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Efficacy of Organo-Modified Nano Montmorillonite to Protect against the Cumulative Health Risk of Aflatoxin B_1 and Ochratoxin A in Rats

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Abstract

The aim of the current study was to prepare organo-modified nano montmorillonite (OMNM) and to evaluate its chemopreventive effects against the hapatonephrotoxicity induced by aflatoxin B_1 (AFB_1) and ochratoxin A (OA) singly or in combination in rats. OMNM was prepared using Cetyltrimethylammoniumbromide (CTAB) as organic modifier. Eighty male Sprague Dawley were divided into 8 groups and treated for 8 weeks as follow: the control group; the group treated orally with AFB_1 (80 µg/kg b.w.); the group treated with OA (100 µg/kg b.w.); the group treated with AFB_1 plus OA, the group treated with OMNM (5 g/kg diet) and the groups treated with AFB_1 and/or OA plus OMNM. At the end of treatment period, blood and tissue samples were collected from all animals for biochemical and histological analysis. The results revealed that the expansion in the basal spacing of the montmorillonite due to the intercalation of CTAB was 7.20 Å and the average particle size of OMNM was 120 nm. The *in vivo* results indicated that treatment with both AFB_1 and OA singly or in combination resulted in a significant increase in liver and kidney function parameters, oxidative stress and tumor markers accompanied with a significant decrease in antioxidant enzyme activities and significant histological changes in liver and kidney tissues. These changes

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were severe in the group received the combined treatment of AFB_1 and OA. OMNM alone did not show any toxic effect and it succeeded to prevent or at least diminish the toxic effects and the histological changes in liver and kidney. It can be concluded that treatment with AFB_1 and OA has a synergistic toxic effects and OMNM is safe and it is a promise candidate as an additive to protect against the exposure to multi-mycotoxins in high risk population.

Keywords

Modified Montmorillonite Nanoparticles, Aflatoxin, Ochratoxin, Mycotoxins, Oxidative Stress

1. Introduction

Mycotoxins are an extremely diverse group of environmentally persistent compounds produced by fungi, which when ingested, inhaled or absorbed from environmental sources, can cause adverse health effects or even death in humans and animals [1]. They can contaminate various agricultural commodities, especially maize and wheat, either before harvest or under post-harvest conditions [2] [3] and considered as the most important chronic dietary risk factor, higher than synthetic contaminants, food additives or pesticide residues [4]. Aflatoxin B_1 (AFB_1) and other naturally occurring aflatoxins (AFs) have been classified as group 1 human carcinogen because of their role in aetiology of liver cancer [5], notably among subjects who are carriers of hepatitis B virus surface antigen (HBsAg) [6]. AFs are considered to be genotoxic carcinogens [7]. The FAO/WHO Joint Expert Committee on Food Additives (JECFA) concluded that the exposure of even <1 ng/kg body weight could contribute to the risk of liver cancer [8]. Moreover, ochratoxin A (OA), another mycotoxin, is classified in group 2B as possible human carcinogens [5]. Consequently, mycotoxin contamination is becoming one of the most insidious challenges to food safety.

Previous studies have demonstrated that multiple mycotoxins can co-contaminate crops and foods intended for both animal and human consumption [9]. Individual mycotoxicosis occurs seasonally on certain areas that hinder an implementation of an effective prophylactic measure [10]. However, interactions between given mycotoxins are still unclear [11]. The presence of a mixture of these toxins may present a problem in terms of determining clinical symptoms of an individual mycotoxicosis. Concomitant exposure to mycotoxins, such as aflatoxin and fumonisin, has been associated with various teratogenic, mutagenic, estrogenic, neurogenic and immunotoxic effects, as well as growth faltering, cancer, and even death in acute incidences [5].

A new practical and effective strategy for reducing food-borne exposure to mycotoxins is the inclusion of various binding agents or sorbents in the diet to adsorb mycotoxins in the gastrointestinal tract of animals and reduce bioavailability, and toxicity. Montmorillonite, bentonite, and hydrated sodium calcium aluminosilicate (HSCAS), as anticaking agents for animal feed, have been reported to prevent disease associated with aflatoxicosis in farm animals, including chicks, turkey poults, and pigs [12] and laboratory animals [11]. Montmorillonite belong to the structural family called 2:1 phyllosilicates, which present a structure composed by two tetrahedral layers formed by Si and O atoms, fused with an octahedral layer with aluminum and magnesium atoms bonded to oxygen and hydroxyl groups [13]. Organically modified nanoclay (OMNC) is the clay modified with organic surfactants. These hydrophobic materials have attracted much interest because they have found wide applications. Organoclays have also been tested for treating ground and surface water and for other toxic organic chemicals from pharmaceuticals and pesticides industries. Organoclays can offer dramatic performance improvements in many other adsorption applications, including removing oil; grease; heavy metals; and polychlorinated biphenyl; organic matter; such as humic and fulvic acids; poly-nuclear and polycyclic aromatics; and sparingly soluble hydrophobic; chlorinated organics. Removing radio-nuclides, including pertechnetate, from water is another application with tremendous potential [14]. The aim of the current study was to develop organo-modified montmorillonite for the protection against the combined toxicity of AFB_1 and OA in rats.

2. Materials and Methods

2.1. Chemical and Kits

AFB_1 and OA standards were purchased from Sigma Chemical Co. (St. Louis MO, USA). Kits of Transaminase

(ALT, AST) were purchased from Quimica Clinica Aplicada (Amposta, Spain). Kits of alkaline phosphatase (ALP), Gamma-Glutamyl Transpeptidase (G-GTP), urea, uric acid, createnine, nitric oxide (NO), Malondialdehyde (MDA), Total antioxidant capacity (TAC), Alpha feto protein (AFP), catalase and reduced glutathione (GSH) were obtained from Biodiagnostic (Giza, Egypt).

2.2. Clay Sample

Montmorillonite was supplied by Egypt Bentonite and Derivatives Co. (Alexandria, Egypt). The chemical composition of the montmorillonite was found to be as follows: 43.731% SiO_2, 2.5% MgO, 15.38% Al_2O_3, 0.98% K_2O, 1.31% CaO, 1.41% TiO_2, 4.17% Na_2O, 0.13% P_2O_5, 10.86% (FeO + Fe_2O_3), 18.70 % loss on ignition. It is observed that the major constituents in the raw clay are SiO_2, Al_2O_3 and Fe_2O_3 in a descending order. The higher SiO_2 and lower Al_2O_3 content are mainly due to the predominance of montmorillonite clay mineral. Cetyltrimethylammoniumbromide (CTAB) as chemically pure surfactants was purchased from Sigma Aldrich Co. (Irvine, Scotland) and used to modify montmorillonite.

2.3. Preparation of Organo-Modified Nano Montmorillonite (OMNM)

OMNM was prepared according to the method described by Zawrah *et al.* [15]. In brief, 5 g of milled montmorillonite was dispersed in 300 ml distilled water for 24 h at room temperature using a magnetic stirrer at 600 rpm and then a desired amount of surfactant (CTAB) was slowly added. The reaction mixtures were stirred for 5 h at 80˚C. Consequently, the cation exchange reaction occurs rapidly. The resulting organoclay suspension was mixed further for 12 h. The product was washed until free from bromide anions and dried at 90˚C. Finally, the resulting material was ground using SFM-1 Desk Top Planetary Ball Miller (MTI) for 3 h, in order to obtain a nanoscale powder. The phase composition and d-spacing of OMNM were identified by X-ray using a Philips 1730 diffractometer with Ni filter, Cu Ka radiation at a scan speed of 0.5/ min. The microstructure of OMNM with different surfactants was examined using scanning electron microscope (Philips XL 30) after coating with gold thin films.

2.4. Experimental Animals

Three-month old male Sprague Dawley rats weighing 150 - 160 g were purchased from Animal House Colony, National Research Centre Dokki, Giza, Egypt. Animals were maintained on the specified diet and housed in filter-top polycarbonate cages in a room free from any source of chemical contamination, artificially illuminated (12h dark/light cycle) and thermally controlled (25˚C ± 1˚C) and humidity (50% ± 5%) at the Animal House Lab., National Research Centre Dokki, Giza, Egypt. All animals received human care in compliance with the guidelines of the Animal Care and Use Committee of the National Research Centre**.**

2.5. Experimental Design

Animals were divided randomly into eight groups and treated for 8 weeks as follow: group (1); control animals, group (2); animals treated orally with AFB_1 alone (80 µg/ kg b.w.) in corn oil, group (3); animals treated orally with OA alone (100 µg/kg b.w.) in corn oil, group (4); animals treated orally with AFB_1 plus OA, group (5); animals received OMNM in the diet (5 g/kg diet), group (6); animals received the OMNM plus AFB_1, group (7); animals received OMNM plus OA and group (8); animals received OMNM plus AFB_1 and OA. The animals were observed daily for any signs of toxicity. At the end of the treatment period (*i.e.* day 56) all animals were fasted for 12 h, then blood samples were collected from the retro-orbital venous plexus by means of capillary tubes under diethyl ether anesthesia. Sera were separated using cooling centrifugation at 3000 rpm for 15 minutes and stored at −20˚C until analysis. ALT, AST, ALP, G-GTP, urea, createnine, uric acid and AFP were determined in serum samples of all groups according to the kits instructions.

After the collection of blood samples, all rats were sacrificed by cervical dislocation and samples of the liver and kidneys of each rat were dissected, weighed and homogenized using glass homogenizer (Universal Lab. Aid MPW-309, Mechanika Precyzyjna, Poland) with ice-cooled phosphate buffer (pH 7.4) to give 20% w/v homogenate [16]. This homogenate was centrifuged at 1000 rpm for 10 min; the resultant supernatants were stored at −80˚C until analysis for the assessment of lipid peroxidation (MDA), GSH, TAC, Catalase and NO. Another portion of the liver and kidney tissue of each animal was dissected and fixed in natural formalin (10%) then hy-

drated in ascending grades of ethanol, cleaned in xylene and embedded in paraffin. Sections (5 mm thick) were cut and stained with hematoxylin and eosin (H & E) for histopathological investigation according to the method described previously [17].

2.6. Statistical Analysis

All data were statistically analyzed using the General Linear Models Procedure of the Statistical Analysis System. The significance of the differences among treatment groups was determined by Waller-Duncan k-ratio. All statements of significance were based on probability of $P \leq 0.05$

3. Results

XRD of OMNM is presented in **Figure 1**. The XRD pattern of OMNM was recorded and basal spacings of 19.80 Å was observed. The expansion in the basal spacing of the montmorillonite due to the intercalation of CTAB was calculated as $\Delta d = d - 12.60$ Å (where d is the basal spacing of the CTAB-treated clay and 12.60 Å is the thickness of a clay layer) and it was found to be 7.20 Å.

The morphology of OMNM was carried out using SEM (**Figure 2**). SEM photomicrographs of nano mont-

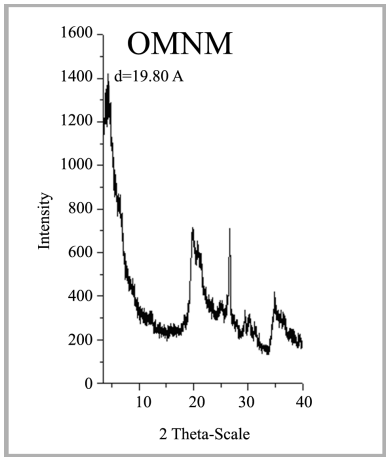

Figure 1. XRD pattern OMNM.

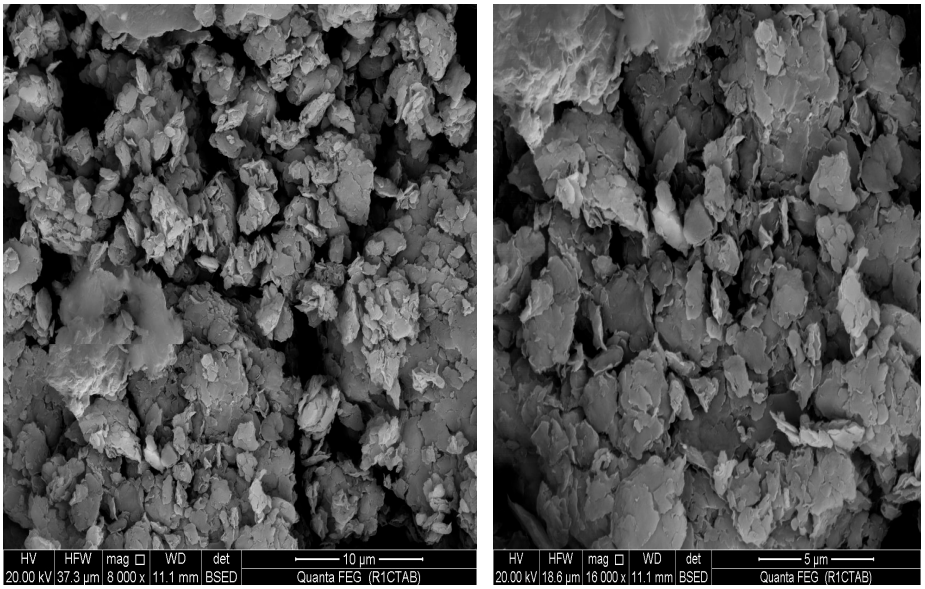

Figure 2. SEM images of OMNM.

morillonite modified with CTAB indicated that the physical appearance of the clay particles changed significantly. Gathered agglomerations with severely curled or crumpled structures were formed much more easer in OMNM. The grain boundaries were steadily disappeared and the flakes of clay minerals are dispersed in the matrices. The laser particle size distribution of OMNM was carried out using SFM-1 Desk Top Planetary Ball Miller (MTI) for 3 hours. The results revealed that the average size of OMNM was 120 nm (**Figure 3**).

The biological evaluation of OMNM to protect against mycotoxins toxicity revealed that ALT, AST, ALP and G-GTP were significantly increased in the groups treated with AFB_1 and/or OA and this increase was more pronounced in the group received the combined treatment (**Table 1**). Treatment with OMNM alone has no significant effect on ALT and AST however; it caused a significant decrease in ALP and a significant increase in G-GTP compared to the control levels. Treatment with OMNM plus AFB_1 and/or OA resulted in significant improvements in all the tested parameters toward the control levels and it succeeded to normalize AST and G-GTP in the group received OA.

The effects of different treatments on kidney function parameters (**Table 2**) revealed that both the mycotoxins increased uric acid, urea and createnine although OA has severe toxicological effect on kidney function compared to AFB_1. The combined treatment with AFB_1 and OA showed synergistic toxicological effect than the single treatment. Treatment with OMNM did not affect uric acid or createnine however; it induced a significant decrease in urea. Treatment with OMNM succeeded to normalize uric acid and createnine in the groups treated with AFB_1 or OA and createnine in the group treated with AFB_1 plus OA and significantly decreased the level of uric acid and urea in the group treated with the combined mycotoxins (**Table 2**).

The data presented in **Table 3** indicated that treatment with AFB_1 and/or OA resulted in a significant decrease in serum TAC and hepatic GSH and CAT. This decrease was pronounced in the AFB_1 alone-treated group and was more pronounced in the group treated with AFB_1 plus OA compared to OA-treated group. Treatment with OMNM alone did not induce any significant effect on serum TAC and hepatic CAT however; it induced a significant increase in hepatic GSH. Animals treated with AFB_1 and/or OA plus OMNM showed a significant im-

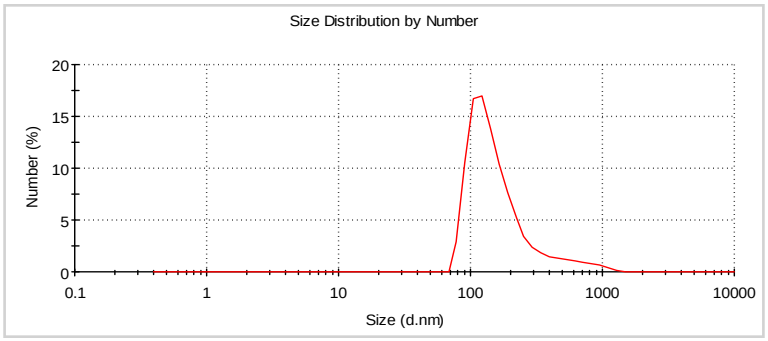

Figure 3. Laser particle size distribution of OMNC.

Table 1. Effect of OMNM on liver function tests in rats treated with AFB_1 and/or OA.

Groups	ALT (U/L)	AST (U/L)	ALP (U/L)	G-GTP (U/L)
Control	9.47 ± 0.20^{a}	7.39 ± 0.32^{a}	6.71 ± 1.08^{a}	888.17 ± 15.74^{a}
AFB_1	16.03 ± 1.31^{b}	15.22 ± 0.79^{b}	15.70 ± 0.75^{b}	1048.84 ± 79.49^{b}
OA	10.37 ± 0.48^{c}	14.48 ± 1.36^{b}	12.86 ± 0.96^{c}	950.79 ± 50.74^{c}
AFB_1 + OA	19.50 ± 0.51^{d}	15.88 ± 1.93^{b}	16.81 ± 1.30^{b}	1376.79 ± 46.78^{d}
OMNM	9.31 ± 0.21^{a}	7.87 ± 0.32^{a}	5.01 ± 1.03^{d}	961.60 ± 17.53^{c}
AFB_1 + OMNM	11.10 ± 0.61^{c}	8.95 ± 0.47^{a}	8.36 ± 0.73^{e}	924.45 ± 11.69^{c}
OA + OMNM	7.56 ± 0.93^{e}	7.99 ± 0.59^{a}	7.59 ± 0.74^{e}	897.62 ± 20.47^{a}
AFB_1 + OA+ OMNM	12.76 ± 0.92^{f}	11.82 ± 1.30^{c}	7.36 ± 0.81^{e}	1016.04 ± 12.10^{b}

Within each column, means superscript with different letters are significantly different ($P < 0.05$).

provement in the antioxidant enzymes activities in serum and hepatic tissue although these parameters were still significantly lower than the control group.

The effect of different treatments on oxidative stress markers (NO and MDA) in liver tissue and tumor marker (AFP) in serum are presented in **Table 4**. These results showed that both the mycotoxins induced a significant

Table 2. Effect of OMNM on kidney function tests in rats treated with AFB1 and/ or OA.

Groups	Uric Acid (mg/dl)	Creatinine (mg/dl)	Urea (g/dl)
Control	2.64 ± 0.22^{a}	4.90 ± 0.23^{a}	48.06 ± 2.56^{a}
AFB_1	7.74 ± 0.54^{b}	15.39 ± 0.40^{b}	73.9 ± 0.40^{b}
OA	9.76 ± 0.38^{c}	13.63 ± 0.34^{c}	81.52 ± 2.36^{c}
AFB_1 + OA	12.36 ± 0.73^{d}	17.98 ± 0.45^{d}	91.96 ± 1.55^{d}
OMNM	2.69 ± 0.49^{a}	5.30 ± 0.71^{a}	39.64 ± 2.03^{e}
AFB_1 + OMNM	2.16 ± 0.48^{a}	6.67 ± 0.35^{e}	53.47 ± 2.83^{f}
OA + OMNM	2.08 ± 0.20^{a}	5.50 ± 0.44^{f}	44.52 ± 6.78^{g}
AFB_1 + OA + OMNM	4.19 ± 0.33^{e}	4.55 ± 0.47^{a}	45.43 ± 3.12^{g}

Within each column, means superscript with different letters are significantly different ($P < 0.05$).

Table 3. Effect of OMNM on serum total antioxidant capacity and liver reduced glutathione and catalase activity in rats treated with AFB1 and/ or OA.

Groups	TAC (mM/L)	GSH (mg/g. Tissue)	CAT (nmol/g Tissue)
Control	0.88 ± 0.03^{a}	13.78 ± 1.66^{a}	11.92 ± 3.42^{a}
AFB_1	0.15 ± 0.003^{b}	5.27 ± 1.34^{b}	5.00 ± 1.00^{b}
OA	0.16 ± 0.003^{b}	6.26 ± 1.34^{b}	6.00 ± 0.50^{c}
AFB_1 + OA	0.053 ± 0.009^{c}	3.13 ± 0.44^{c}	3.50 ± 0.50^{d}
OMNM	0.78 ± 0.01^{a}	15.95 ± 0.76^{d}	10.06 ± 0.12^{e}
AFB_1 + OMNM	0.59 ± 0.073^{d}	9.04 ± 0.53^{e}	8.38 ± 1.38^{f}
OA + OMNM	0.51 ± 0.034^{e}	9.62 ± 1.43^{e}	9.17 ± 3.50^{g}
AFB_1 + OA + OMNM	0.25 ± 0.03^{f}	8.11 ± 0.48^{f}	7.13 ± 0.53^{c}

Within each column, means superscript with different letters are significantly different ($P \leq 0.05$).

Table 4. Effect of OMNM on oxidative stress and tumor markers in rats treated with AFB1 and/ or OA.

Groups	Nitric Oxide (µmol/L)	MDA (nmol/g Tissue)	AFP (ng/ml)
Control	162.47 ± 13.02^{a}	0.67 ± 0.12^{a}	2.69 ± 0.15^{a}
AFB_1	240.36 ± 13.00^{b}	4.4 ± 0.17^{b}	4.91 ± 0.28^{b}
OA	197.75 ± 35.51^{c}	3.83 ± 0.23^{b}	3.89 ± 0.27^{c}
AFB_1 + OA	322.29 ± 25.99^{d}	5.03 ± 0.38^{c}	6.28 ± 0.05^{d}
OMNM	162.49 ± 12.29^{a}	0.62 ± 0.08^{a}	2.16 ± 0.04^{a}
AFB_1 + OMNM	166.62 ± 16.40^{a}	1.43 ± 0.15^{d}	2.22 ± 0.05^{a}
OA + OMNM	167.75 ± 12.61^{a}	1.30 ± 0.06^{d}	2.41 ± 0.12^{a}
AFB_1 + OA + OMNM	182.84 ± 4.11^{e}	1.57 ± 0.14^{d}	2.64 ± 0.06^{a}

Within each column, means superscript with different letters are significantly different ($P \leq 0.05$).

increase in MDA, NO and AFP levels compared to the control group. The increase in these parameters was pronounced in the AFB_1-treated group and more pronounced in the group received the combined treatment of AFB_1 and OA. Treatment with OMNM did not induce any significant changes in these parameters. However, OMNM succeeded to normalize AFP in the groups treated with AFB_1 and OA and the groups treated with AFB_1 or OA accompanied with a significant improvement in NO in the group treated with AFB_1 plus OA and hepatic MDA in the groups treated with the single or combined mycotoxins (**Table 4**).

The aforementioned biochemical results were confirmed by the histological examination for the liver and kidney tissues. The microscopic examination of the liver of the control animals revealed normal architecture of hepatic lobule (**Figure 4(a)**). The liver of AFB_1-treated rats showed thickening in the wall of the portal tract with necrosis, fibrosis and bile duct proliferation with fatty droplets in the hepatocytes around the central vein (**Figure 4(b)**). Animals treated with OA showed pronounced hepatic histological changes around the enlarged portal tracts than the central vein in the form of necrosis obliteration or proliferation in the bile ducts surrounded by aggregation of fibrous tissues (**Figure 4(c)**). However, animals treated with AFB_1 plus OA showed disorganization of hepatic cords and vascuolar destruction, bile duct proliferation and aggregation of inflammatory cells (**Figure 4(d)**).

The microscopic examination of liver sections from rats treated with OMNM showed normal radiating hepatocytes and central veins (**Figure 5(a)**). However those treated with OMNM with AFB_1 showed maintenance of cellular integrity in the central area around the central vein without any inflammation and foci of regenerating nodule (**Figure 5(b)**). The liver of animals treated with OMNM plus OA showed regeneration in hepatocytes with normal acidophilic cytoplasm and central vein although local foci of the inflammatory cells are seen (**Figure 5(c)**). However, the liver of animals treated with AFB_1 and OA plus OMNM showed same picture of normal hepatocytes and prominent decrease in the different abnormal archetictuer (**Figure 5(d)**).

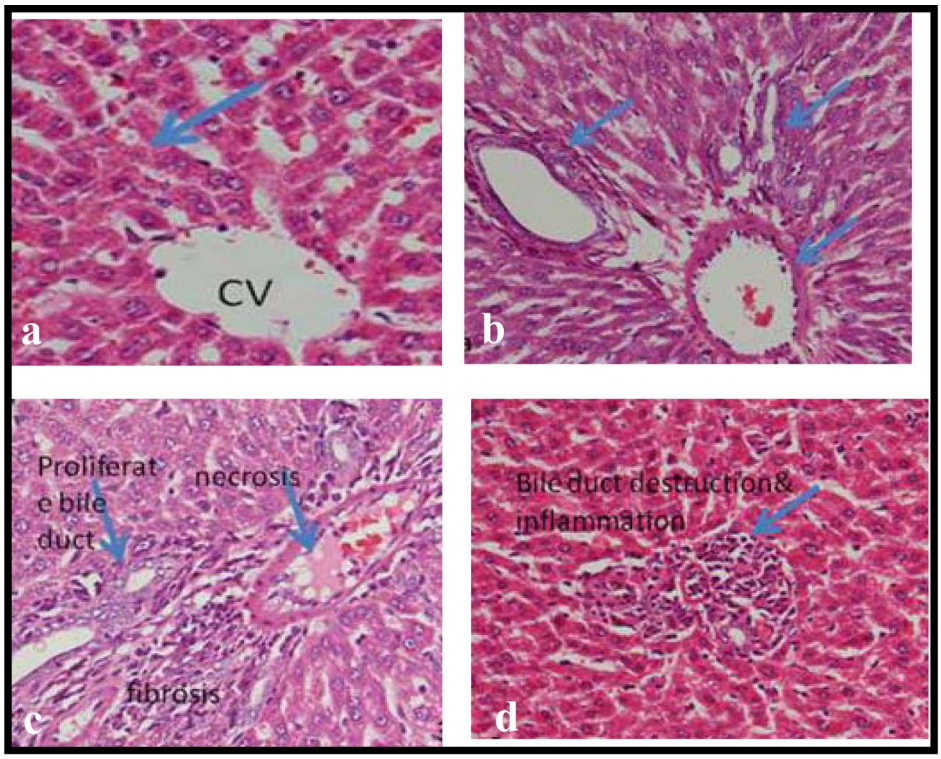

Figure 4. Photomicrographs of the liver section of (a) the control rats showing normal architecture of hepatic lobule in which hepatocytes are radiating from central vein to the periphery of lobule; (b) AFB_1-treated rats showing thickening in the wall of the portal tract with necrosis, fibrosis and bile duct proliferation. The hepatocytes around the central vein revealed fatty droplets; (c) OA-treated rats showing more pronounced hepatic histopathological changes around the enlarged portal tracts than the central vein in the form of necrosis obliteration or proliferation in the bile ducts surrounded by aggregation of fibrous tissues and (d) OA plus AFB_1-treated rats showing disorganization of hepatic cords and vascuolar destruction, bile duct proliferation and aggregation of inflammatory cells. (H & E × 400).

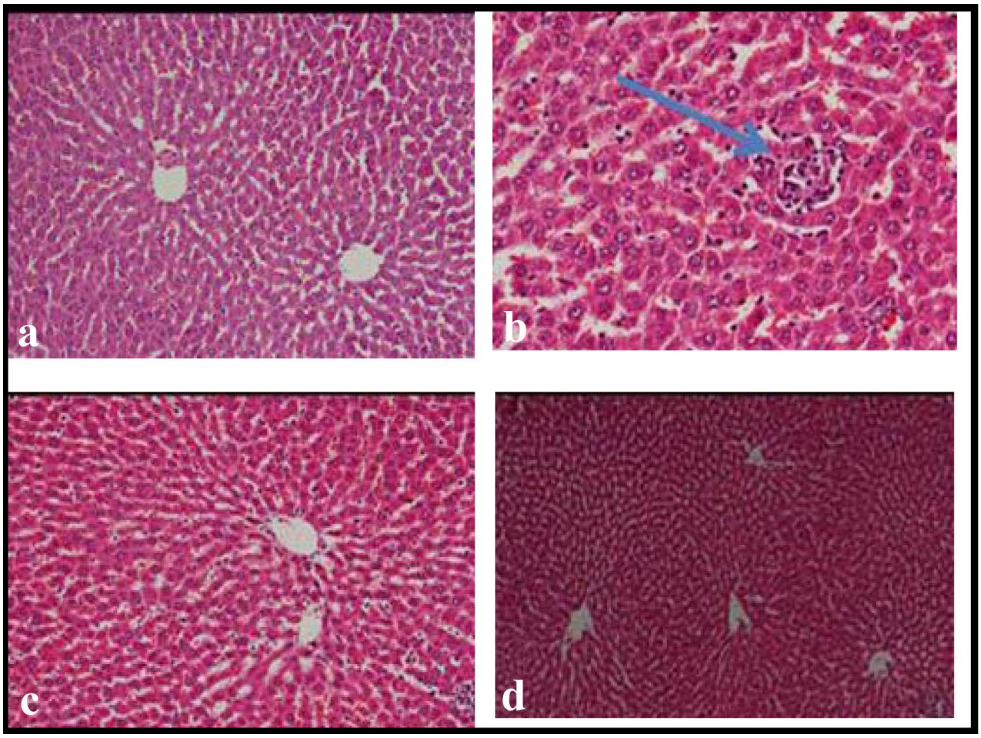

Figure 5. Photomicrographs of the liver section of (a) rats treated with OMNM alone showing normal radiating hepatocytes and central veins; (b) rats treated with AFB_1 plus OMNM showing maintenance of cellular integrity in the central area around the central vein without any inflammation and foci of regenerating nodule; (c) rats treated with OA plus OMNM showing regeneration in hepatocytes with normal acidophilic cytoplasm and central vein, local foci of the inflammatory cells are seen and (d) rats treated with AFB_1 and OA plus OMNM showing same picture of normal hepatocytes and prominent decrease in the different abnormal archetictuer. (H & E × 400).

The histological examination of the kidney cortex of the control animals showed normal renal corpuscle with parietal layer of Bowman's capsule, glomerulus, preserved renal space and normal proximal and distal convoluted tubules were also seen (**Figure 6(a)**). The examination of the sections in the kidney cortex of AFB_1-treated rat showed shrunken or atresia in some renal corpuscle and tubular necrosis with pyknosis of their epithelial cells were formed. The same group showed tubular necrosis and obliteration, the epithelial cells in some tubules were disintegrated or pyknotic. The glomerular capillaries were expanded, less cellularity and vacuolated (**Figure 6(b)**). The kidney cortex of the rats treated with OA showed an increase in number of shrinking or damaged renal corpuscle and foci of tubular necrosis and dilatation with epithelial cells pyknosis (**Figure 6(c)**). However, the kidney section of the rats treated with AFB_1 plus OA showed marked increase in tubular necrosis, vacuolation and obliteration with more cleared pyknotic epithelial cells. The same sections showed that the necrotic tubules were scattered others had vacuolar degeneration with pyknotic nuclei, interstitial haemorrhage and fibrous tissues were also seen with hyperplastic changes glomeruli (**Figure 6(d)**).

The kidney cortex of the rats treated with OMNM showed less affected tubules, nearly normal (**Figure 7(a)**). However, animals treated with AFB_1 plus OMNM showed disappearance of the intracytoplasmic vacuolation in renal tubules and decrease in foci of necrosis. The glomeruli showed hyperplastic changes with narrowing of their capsular spaces (**Figure 7(b)**). Whereas, kidney cortex of animals treated with OA plus OMNM showed improvement in renal tubules in the form of disappearance of the intracytoplasmic vacuolation in renal tubules or necrosis and increase in size of renal corpuscle with dilated capillaries and narrowing of their capsular spaces (**Figure 7(c)**). Moreover, animals treated with AFB_1 and OA plus OMNM showed improvement in most of renal tubules and renal corpuscle where the capillary tufts were surrounded by capsular spaces (**Figure 7(d)**).

4. Discussion

The expansion in the basal spacing of the montmorillonite due to the intercalation of CTAB was 7.20 Å. This

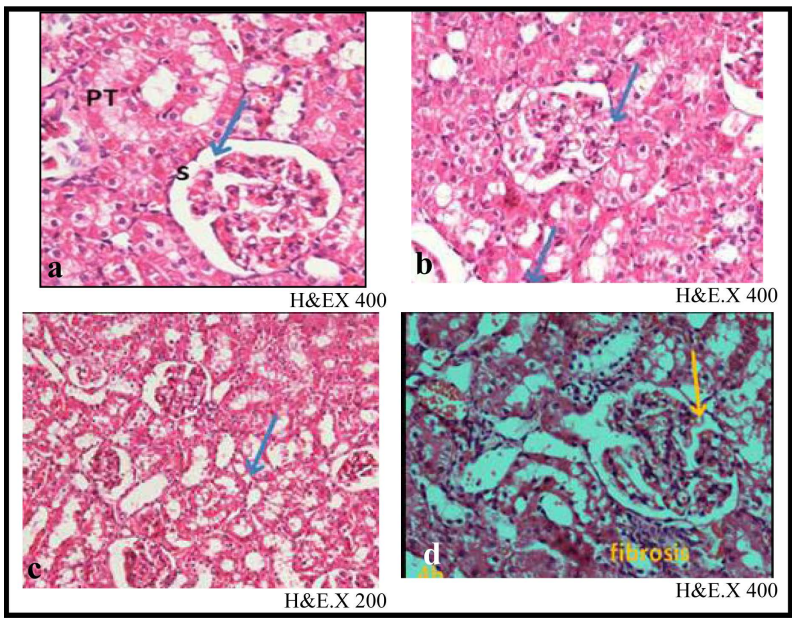

Figure 6. Photomicrographs of the kidney section of (a) control rat showing the renal corpuscle with parietal layer of Bowman's capsule, glomerulus (G), preserved renal space (S), Proximal (PT) and distal (DT) convoluted tubules also seen; (b) kidney cortex of AFB_1-treated rat showing tubular necrosis and obliteration, the epithelial cells in some tubules are disintegrated or pyknotic, the glomerular capillaries are expanded, less cellularity and vacuolated; (c) kidney cortex of OA-treated rat showing increase in number of shrinking or damaged renal corpuscle and foci of tubular necrosis and dilatation, with epithelial cells pyknosis and (d) kidney cortex of AFB_1 plus OA-treated rats showing necrotic tubules are scattered others have vacuolar degeneration with pyknotic nuclei, interstitial haemorrhage and fibrous tissues also seen with hyperplastic changes glomeruli.

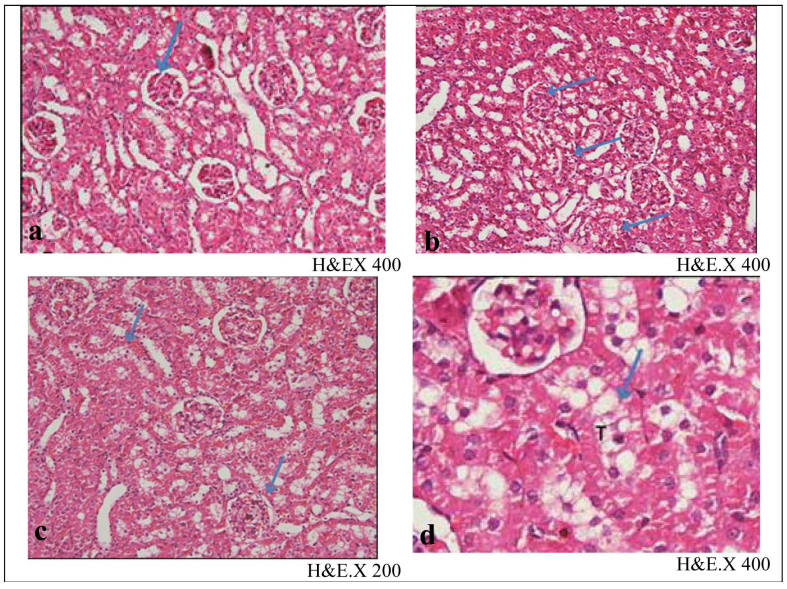

Figure 7. Photomicrographs of the kidney section of (**a**) rat treated with OMNM alone showing less affected tubules, nearly normal. The glomeruli appeared with their capillary tufts surrounded by capsular space and bowman's capsule; (b) rat treated with AFB_1 plus OMNM showing disappearance of the intracytoplasmic vacuolation in renal tubules and decrease in foci of necrosis. The glomeruli showed hyperplastic changes with narrowing of their capsular spaces; (c) rat treated with OA plus OMNM showing improvement in renal tubules in the form of disappearance of the intracytoplasmic vacuolation in renal tubules or necrosis and increase in size of renal corpuscle with dilated capillaries and narrowing of their capsular spaces; (d) rats treated with OMNM plus AFB_1 and OA showing improvement in most of renal tubules and renal corpuscle where is the capillary tufts are surrounded by capsular spaces.

morphological observation of SEM suggested that CTAB ion molecules intercalate in to the interlayers of montmorillonite with a monolayer arrangement. The shifting of these peaks to lower 2θ and increase in d-spacing confirms the increase in gap between clay platelets and diffusion of Cetyl-trimethylammoniumbromide (CTAB) into the layer of silicates [15]. These changes in the morphologies and particle sizes indicate that the intercalation was accompanied by adsorption. In general, the loading of surfactant onto clay is a "self-assembly" process [18].

Regarding to the biological evaluation of OMNM, it is documented that the combined toxicity of mycotoxins is very hard to predict because it is influenced by several factors, including chemistry and mechanism of action, toxicodinamics and toxicokinetics, experimental design and endpoints of the study as well as statistical aspects [19]. This means that multiple mycotoxins can affect certain targets and initiate more than one event in the cell machinery leading to extremely complicated cell response [20]. The typical clinical picture of a disease is a result of mixed intoxication and interactions between mycotoxins [11].

The novel technology applied for protecting against mycotoxins toxicity is the utilization of adsorbents mixed with foods which are supposed to bind efficiently mycotoxins in the gastrointestinal tract. Clay eating has been recorded from traditional human societies and is considered 'culturally acceptable' in many African countries and China [21]. The adsorption and ion exchange using natural, synthetic and modified inorganic and organic solids have been explored [22]. In the current study, we evaluated the protective effects of the organic modified nano montmorillonites (OMNM) against the toxicity resulted from the exposure to AFB_1 and/or OA in rat model. The selected doses of AFB_1 and OA were based on our previous work [23] [24] however; the selected dose of OMNM was based on our *in vitro* study (unpublished, data). Animals received the two mycotoxins singly or in combination showed a significant increase in ALT, AST ALP and G-GTP activities. This increase in transaminases in mycotoxins-treated animals is indicative for changes in the hepatic tissues and biliary system [11]. However, the increased activity of ALP may indicate degenerative changes and hypofunction of the liver and the elevated activity of G-GTP in serum is consistent with severe injury of both liver lysosomes and mitochondria [23]. Moreover, there is some evidence that OA induces bone changes which may also be a contributory factor to the increased ALP activity [24] [25]. On the other hand, the significant increase in uric acid, urea and createnine observed in the animals treated with AFB_1 and/or OA may indicate protein catabolism and/or renal dysfunction [23] [24]. These results clearly indicated that both AFB_1 and OA have stressful effects on the hepatic and renal tissues, consistent with those reported in the literature of mycotoxicosis [26].

AFP is considered specific biomarker for liver cancer and it is synthesized mainly in the fetal stage; practically no production of this marker occurs in the normal adult. However, when some adult cells are transformed to cancer cells, the synthesis of AFP commences again. In the current study, the elevated serum level of AFP in the animals treated with AFB_1 and/or OA indicated that both agents are potent hepatocarcinogen, enhance reactive oxygen species (ROS) formation and causes oxidative DNA damage, which may play a role in their carcinogenicity [27] [28]. Therefore, the current study affirmed that AFB_1 and/or OA can induce hepatotoxicity and regeneration in liver cells in rats as indicated by the elevation of AFP level in serum. Similar to the current observations, AFB_1 administration resulted in the elevation of serum AFP level in both ducks [29] and rats [27] and in OA-treated rats [23] [24].

In the current study, NO was found to be increased significantly in the animals treated with AFB_1 and/or OA. Although the role of NO in cell death is complex, Moon and Pyo [30] stated that NO is produced by macrophages and it plays an important role in tumor conditions. The generation of NO by the inducible nitric oxide synthase (iNOS) plays a key role in the cytokine-mediated cell destruction [31]. Consequently, the increased in NO level reported herein in the animals treated with the mycotoxins suggested that these mycotoxins preferentially affect macrophage functions [11].

The current results also revealed a decrease in TAC and GSH in the liver and catalase in serum of AFB_1 and/or OA-treated rats which are might indirectly lead to an increase in oxidative DNA damage [32]. Moreover, the reduced level of TAC may be explained by the association of glutathione peroxidase (GPX) with AFB_1 or its metabolites [32]. Several studies on the mechanisms of mycotoxins-induced liver injury have demonstrated that glutathione and TAC play an important role in the detoxification of the reactive and toxic metabolites of these mycotoxins, and the liver necrosis begins when the glutathione stores are almost exhausted [33]. Similar observations have been reported in weaned piglets received low doses of AFB_1 and OA [10]. Moreover, Gautier *et al.* [34] stated that OA does evoke oxidative stress, which may contribute at least in part to OA renal toxicity and carcinogenicity in rats during long-term exposure. Moreover, OA was found to decrease GSH and TAC which

may be explained by the conjugation of GSH with OA or its metabolites [25].

It is well documented that LP is one of the main manifestations of oxidative damage and it has been found to play an important role in the toxicity and carcinogenicity. However, the antioxidant enzymes represent the major defense system against liver injury and carcinogenesis. Several reports indicated that exposure to either AFB_1 or OA increased LP in liver. In the current study, AFB_1 and/or OA administration enhanced LP as indicated by the significant increase in MDA level which directly results of free radical-mediated toxicity [35]. In a previous works, Abdel-Wahhab *et al.* [23] [25] reported that free radicals are known to attack the highly unsaturated fatty acids of the cell membrane to induce LP which considered a key process in many pathological events and is one of the reactions induced by oxidative stress [36]. Another mechanism for OA-induced injury was suggested by Pfohl-Leszkowicz *et al.*, [37] who reported that the ability of OA to generate free radicals and to enhance lipid peroxidation has been linked to the genotoxicity expressed by DNA adduct formation and to the disturbance of calcium homeostasis due to an impairment of the endoplasmic reticulum membrane. The cellular damage induced by ROS was estimated by monitoring the lipid peroxidation (LP), which is a well-known indicator of cellular damage by oxidative stress. Furthermore, it is well documented that AFB_1 is metabolized by the mixed-function oxidase system to a number of hydroxylated metabolites and to AFB_1 8,9-epoxide, which binds to DNA, forming covalent adducts [38]. Also AFB_1 is known to produce membrane damage through increased lipid peroxidation [33]. However, OA appears to produce many of the effects in the cell such as the increase of the permeability of the cell to Ca^{2+} [39]. Both the enhanced cellular concentration of Ca^{2+} and the presence of the prooxidant OA uncouple oxidative phosphorylation resulting in an increased leakage of electrons from the respiratory chain. This generates O^{2-} and hence H_2O_2 lack of an adequate supply of NAD (P) H and GSH to permit H_2O_2 consumption by the GSH dependent glutathion peroxidase and NAD (P) H dependent glutathion reductase. Furthermore, an increased concentration of free iron within the cell stimulates the production of OH via the Fenton like reaction due to mobilization of Fe^{2+} by Ca^{2+}. This results in further cell damage and may be one of the mechanisms that OA exerts its toxic effects [24]. The biochemical results were further confirmed by the histological examination of the liver tissue. The microscopic examination of the liver tissues revealed severe histological changes typical to those reported in the literature of mycotoxicoses [23].

In the current study, treatment with OMNM plus AFB_1 and/or OA resulted in a significant improvement in all the biochemical parameters tested and the histological picture of the liver. Several reports suggested that the novel technology applied for protecting animals against mycotoxins toxicity is the utilization of adsorbents mixed with foods which are supposed to bind efficiently mycotoxins in the gastrointestinal tract. The adsorption and ion exchange using natural, synthetic and modified inorganic and organic solids have been explored [22]. Clay minerals, which are currently used as an anti-caking agent, may remove mycotoxins from aqueous solutions [40]. The absorbability of different clay minerals differs according to their specific surface area [41]. These clay minerals act as potential ionic exchangers for mycotoxins due to their low cost, high abundance, easy manipulation, and harmlessness to the environment. On the other hand, the addition of different clays to diet did not show any health risk to human or in laboratory and farm animals [42]. In previous works, the dietary inclusion of clay minerals at a level of 0.5% (w/w) did not show any toxic effect regarding biochemical, haematological and immunological parameters in laboratory animals [43]. Furthermore, the addition of calcium montmorillonite did not show any toxic effects in the equilibrium of vitamins and gain minerals in the human blood [44].

The current results clearly indicated that rats treated with montmorillonite nanoclay plus AFB_1 and/or OA showed a significant improvement in all the parameters tested and support the hypothesis that montmorillonite nanoclay bind the AFB_1 and/or OA in the gastrointestinal tract of the animals and the montmorillonite-mycotoxins complex is stable and does not affected by the different metabolizing enzymes. In this concern, Hassan *et al.* [45] suggested that montmorillonite may posse three types of active binding sites: 1) those located at basal planes within interlayer channels, 2) those located on the surface and 3) those located at the edges of clay particles. Moreover, previous reports indicated that montmorillonite has the property of adsorbing organic substances either on its external surfaces or within its inter laminar spaces by the interaction with or substitution for the exchange cations present in their spaces [40].

Although there is a lot of evidence for the good technological performance of nanocomposites, safety issues are also of importance. Available data on clay's toxicity is still scarce, but different authors have already described toxic effects induced by montmorillonite and organoclays [46]. However, these toxic effects were suggested to be due to the modifier used to synthesize the organoclay [46]. Therefore, in the present study, the safety use of montmorillonite-based nanoclays modified with CATB as a commercial surfactant to protect

against AFB_1 and/or OA *in vivo* was studied. The results showed no toxic effects for OMNM itself when used at 0.5% (w/w) in rat diet. In this concern, Houtman *et al.* [47] reported that montmorillonite-based nanoclays had no cytotoxicity on HepG2 cell lines although these authors suggested that the type of modifiers is very importance to improve the compatibility with the polymer matrix. Others studies have evaluated the toxicity of the commercial non modified montmorillonite in the same cell lines [46]. The same authors observed that HepG2 and Caco-2 exposed to organo modified clay did not present higher significant reductions of viability with respect to the controls in the range of concentrations assayed. Moreover, Sharma *et al.* [48] did not obtain any cytotoxic effects in Caco-2 exposed to organo modified clay. In this case, the concentration used of the modified clays showed the same behavior compared to that of the unmodified montmorillonite reported in our previous work [45], indicating that the modifiers employed could not involve changes in the safety profile of the modified clay.

In the case of organoclays, the oral pathway is the most important entrance route for the consumers, and they should know the possible effects of the ingestion of these nanosubstances to the gastrointestinal tract [49]. There are a limited number of toxicological studies in the literature about commercial modified clays. Baek *et al.* [50] evaluated the toxicity effects in human normal intestinal cells (INT-407) in a short and long term exposure, 24, 48, 72 h, and, 10 days to montmorillonite. Thereby, a decrease in cell proliferation showed at all times assayed. On the one hand, significant differences in the short term assays were found above 100 μg/mL concentration levels, on the other hand, a significant inhibition of normal colony formation in the long term was observed at all concentrations tested. Even though, alterations in LDH release were only observed at the highest concentrations at 48 and 72 h. Also, the oligo (styrene-co-acrylonitrile)-modified clay montmorillonite showed an increased LDH release activity and cell viability reduction at a concentration of 1 g/L in mouse embryonic fibroblast (NIH 3T3) cells and human embryonic kidney 293 (HEK 293) cells [51]. The implication of oxidative stress, inflammation or DNA damage, among others, could be related to micro and nanoparticle exposure [52].

For this reason, levels of NO and GSH were assayed in the current study, obtaining insignificant differences with respect to the control group on the GSH levels in the liver. In this concern, Sharma *et al.* [48] reported that modified clays did not induce ROS production in Caco-2. Furthermore, Baek *et al.* [50] reported the evaluation of ROS production in INT-407 cells exposed to montmorillonite at the highest concentration (1000 μg/mL) at all three time points assayed (24, 48 and 72 h). Moreover, it was reported that organo modified montmorillonite did not induce leakage of IL-6, biomarker of an inflammatory response, in any of the cell lines [47]. Montmorillonite, on the other hand, has been reported to rapidly lyse neutrophils and erythrocytes *in vitro*. Furthermore, it can stimulate chemiluminescence, the neutrophil oxidative metabolic burst [53].

5. Conclusion

From the results of the current study, it can be concluded that both AFB_1 and/or OA at the tested doses induce severe hepatic toxicity. Treatment with AFB_1 reveals that this toxin is hepatonephrotoxic however; it is mainly targeted to the liver whereas, treatment with OA revealed that it is also hepatonephrotoxic but it is mainly targeted to the kidney. The co-occurrence of the two mycotoxins suggests the synergistic and adding-up interactions of AFB_1 and OA. OMNM is safe itself and it succeeds to eliminate and even prevent the toxicity of these mycotoxins. Moreover, OMNM is suggested a promise candidate for the protection against multi-mycotoxins in the highly incidence area.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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