Int.J.Curr.Microbiol.App.Sci (2016) *5*(12): 795-818

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 5 Number 12 (2016) pp. 795-818**
Journal homepage: http://www.ijcmas.com

Original Research Article http://dx.doi.org/10.20546/ijcmas.2016.512.090

Efficacy of Zinc Oxide Nanoparticles and Curcumin in Amelioration the Toxic Effects in Aflatoxicated Rabbits

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Keywords

Aflatoxicosis, Rabbits, ZnO-Nps, curcumin, GSH, Hepatoprotective effect, anti-hepatocellular carcinoma, Antioxidant, Fibrosis, Apoptosis.

Article Info

Accepted:
28 November 2016
Available Online:
10 December 2016

A B S T R A C T

As the first step in this direction; biosynthesis and characterization of zinc oxide nanoparticles (ZnO-NPs) and their use to ameliorate the toxic effects of aflatoxicosis in rabbit in comparison with curcumin were studied. Thirty-five healthy New Zealand rabbits were divided into 5 groups, each including 7 rabbits. Group 1 was kept as negative control without any treatment. Rabbits in the groups 2, 3, 4 and 5 were given AFB_1 orally at a dose of 50 ug dissolved in 0.5 ml of olive oil/ animal daily, for 4 weeks. On day one following the administration of AFB_1, curcumin was orally administered at a dose of 15 mg/kg b.w. for group 3. While, animals of Group 4, were orally administered AFB1 + ZnO-NPs (25 ug / kg of B.W. of animal /0.2 mL of buffer/day)(low dose). Animals of Group 5, were orally administered aflatoxins + ZnO- NPs (50 ug / kg of B.W. of animal /0.2 mL of buffer/day) (high dose). The biochemical evaluation of rabbit sera after experimental study showed that AFB_1 increased the concentration of NO and MDA, while decreased the level of GSH and activities of SOD, CAT and GSH-P_X. Significant decreased values in plasma total protein, albumin, alpha globulin, beta globulin, and gamma globulin. Decrease in serum globulin in toxin fed group might be due to the adverse effect of aflatoxin B_1 on synthesis of total proteins and globulin. The AFB_1 had a drastic effect on liver and kidneys with marked genotoxic effect and inhibition of DNA, RNA and protein synthesis. These changes were improved by administration of ZnO-NPs or curcumin to aflatoxicated animal through the immune strengthening effect and protection of lipid and protein from oxidative damage. It is concluded that the addition of ZnO-NPs and curcumin to animal feed possesses hepato-protective effect through scavenging of free radicals, or by enhancing the activity of antioxidant, which then detoxify the free radicals. These factors protect hepatic cells from the damaging effect by AFB_1 that could be progress to hepatocarcinmoa.

Introduction

The most potent mycotoxin to human and animal health is aflatoxin B_1 (AFB_1); the secondary metabolite of *Aspergillus flavus and A.parasiticus* that grow on a variety of food and feed commodities at any stage during growth, harvest, storage, and transportation (Henry *et al.*,2000). Aflatoxin B_1 (AFB_1) has a great concern because of its detrimental effects on the health of humans and animals, including carcinogenic, mutagenic, teratogenic, and immunosuppressive effects (Fernandez *et al*., 2001).

In developing countries; it appears that there is a direct correlation between dietary aflatoxins intake and the incidence of liver cancer (FDA, 2000, Bahtnager *et al.,* 2002 and Refai and Hassan, 2013). In addition, it has been well documented that AFB_1 leads to liver-specific carcinogenicity through induction of a guanine (a purine) to thiamine (a pyrimidine) substitution at codon 249 on the p53 gene (Verma *et al.,* 2004).

Also, AFB_1 is able to induce reactive oxygen species (ROS) generation during the metabolic processing of AFB_1 by liver enzymes (Kanbur *et al.,* 2001), which causes oxidative stress, leading to oxidation of proteins, lipids and DNA (Mary *et al.*, (2012). Therefore, numerous physical, chemical and biological methods had been proposed to detoxify or inactivate aflatoxins in contaminated feedstuffs.

Recently, the nano minerals are widely used in diversified sectors including agriculture, animal and food systems. The nano-sized particles having higher potential than their conventional sources and thus reduce the quantity required (Patil *et al*., 2012 and Sri Sindhura *et al.,* 2014). They have more significant growth promoting, immuno-modulatory, antibacterial effects than the conventional counterparts. They also alter the rumen fermentation pattern on supplementation in the animal feeds, nano minerals are reported to enhance the reproduction in the livestock and poultry (Partha *et al.,* 2015) and also Zn-NPs were found to enhance growth and improve the feed efficiency in piglets (Yang and Sun, 2006) and poultry (Lina *et al.,* 2009 and Mishra *et al.,* 2014).

Moreover, ZnO-Nps improve the immunity of the animals as the reduction in somatic cell count in subclinical mastitic cow and an increase in milk production were observed due to supplementation of ZnO-Nps (Rajendran, 2013).

On the other hand, Curcumin is a phytochemical material and considered as an active component of the spice turmeric obtained from the rhizome of the Curcuma longa plant. It has been widely used throughout history for the treatment of diverse ailments that include inflammatory conditions, gastrointestinal disorders, and cancer (Goel *et al.,* 2008). Moreover, curcumin is known for its antioxidant properties and acts as a free radical scavenger by inhibiting lipid peroxidation and DNA oxidative damage (Jayaprakasha *et al.,* 2006).

Therefore, the present study was undertaken to evaluate human food and animal feed for aflatoxins contamination and production of aflatoxins by *A.flavus* that isolated from animal and poultry feeds.

The biosynthesis, characterization of ZnO-Nps by scanning electron microscope (SEM) and evaluation of their efficacy in ameliorating the toxic effects in aflatoxicated rabbits in comparison with curcumin were investigated.

Materials and Methods

Feed and Food Samples

One hundred and twenty samples of human food (frozen meat and raw milk) and animal feed (poultry feed, yellow corn, hay and tibn) (20 of each), were collected from different markets and animal farms in Egypt. Samples were aseptically transferred into sterile polyethylene bags without delay and transported to the laboratory for detection of their aflatoxins content.

Laboratory animals: Thirty-five healthy New Zealand male rabbits, aged 6-8 weeks and weighing between 1 and 1.5 kg were used. The rabbits were housed individually in wire cages and fed with commercial pellet feed and the water was supplied *ad libitum*.

Production of aflatoxins on yellow corn and their estimation (Smith, 1997):

The aflatoxigenic *A. flavus* strains that were recovered from feed and food samples were screened for aflatoxin production on yellow corn. In a flask containing 100 gm of finely ground yellow corn and 40-50 ml of distilled water were mixed and autoclaved at 121°C for one hour. The flask was shaken to prevent cooking of yellow corn. It was inoculated with spore suspension of 2 slants of *A. flavus* and incubated for 4 weeks at 25-28°C. After end of incubation period, the corn was removed from flasks, dried; finely ground and 50 g of each were subjected for estimation of aflatoxins. The estimation of prepared aflatoxins was measured qualitatively by TLC according to the recommended method of AOAC (1990) and Refai and Hassan, (2013).

Biosynthesis, identification and characterization of zinc nanoparticles

Preparation of *C. albicans* cells culture (Hartsel and Bolard, 1996) : The spore suspension of *Candida albicans* (10^5/ml) of 2-5 days age cultures was inoculated into 50 mL of semi defined medium (SDM) and incubated at 30°C under shaking condition (200 rpm) for 96 hrs.

Mycelia were separated from the culture broth by centrifugation at 4500 rpm, 10°C, for 15 min. The settled cells were washed with deionized water. 1% of the washed *C. albicans* cells were inoculated into flasks containing 100 ml of Sabouraud broth medium incubated for 24 hours at 30^o C and treated with 1.0% NaCl.

Biosynthesis, identification and characterization of zinc oxide nanoparticles : (Awodugba and Ilyas (2013) and Shamsuzzaman *et al.(*2013): Twenty-five ml of the above prepared culture of *C.albicans* were taken in a separate sterilized flask and 20 ml aqueous solution of 1 mM zinc oxide were added to the culture broth and the flask was kept at 30^o C for 24 h until white deposition started to appear at the bottom of the flask, indicating the initiation of transformation of zinc oxide to zinc nanoparticles.

The culture solution was cooled and incubated at room temperature in the laboratory ambience. After 12-15 hours, white clusters deposited at the bottom of the flask. The reaction mixture was subjected to centrifugation for 15 min. The sediment was collected, washed by deionized water and filtrated through Whatman filter paper No. 1 and the filtrate was discarded.

The obtained powder in the filter paper was dried in hot oven at 50-60 ^{O}C. The prepared ZnO-NPs sizes and morphology were observed and measured under transmission electron micrograph (TEM) HITACHI H-800 (Hitachi) and scanning electron microscope (SEM) (Joe, JSM-5600LV,

Japan).

Experimental Design (Neeta and Ramaje, 2007; Sahoo *et al.*, 2014 a&b and Partha *et al*., 2015)

The induction of hepatotoxicity by aflatoxicosis and ameliorated their effects by ZnO-NPs and Curcumin in rabbits were investigated.

Thirty-five healthy New Zealand male rabbits were divided into 5 groups, each including 7 rabbits. Group 1 was kept as negative control without any treatment. Rabbits in the groups 2, 3, 4 and 5 were orally given AFB_1 at a dose of 50 ug dissolved in 0.5 ml of olive oil/ animal/ day, using stomach tube, for 4 weeks. On day 1 following the administration of AFB_1, curcumin was orally administered at a dose of 15 mg/kg b.w. for group 3. While, animals of Group 4, were orally administered aflatoxin B_1 + ZnO-NPs (25 ug/kg of B.W./0.2 mL of buffer/day)(low dose). While, animals of Group 5, were orally administered aflatoxins + ZnO-NPs (50 ug /kg of B.W. /0.2 mL of buffer/day) (high dose). All the treatments were given orally using a feeding tube attached to a hypodermic syringe (Çam *et al*., 2008). The experimental study was extended for further 4 weeks.

Biochemical Analysis

Serum and plasma parameters

At the end of the experiment, two blood samples were collected from each group into small-labeled dry and clean vials with or without anticoagulant. Blood samples were centrifuged (3000 rpm, 10 min) and the plasma and serum were separated for biochemical parameters. The levels of aspartate amino-transferase (AST), alanine aminotransferase (ALT) were measured according to (Reitman and Frankel, 1957). Plasma Nitric oxide (NO) levels were measured by enzymatic Greiss reaction method described by Green *et al.* (1982). Triglyceride according to Wahlefeld (1974) and cholesterol according to Watson (1960), Whereas, the estimation of plasma total protein and electrophoretic pattern were carried out according to Davis (1964) and SonnenWirth and Jaret (1980*)*, respectively and calculated according to Syn Gene S. No. 17292*14518 sme*mpcs

Lysate preparation and assays of antioxidant parameters

RBCs were separated from plasma by centrifugation, washed three times with saline and lysed (Tietz, 1996). The lysate was mixed with an equal volume of Drabkin`s reagent to determine hemoglobin levels (Van Kampen and Zijlstra, 1965). Catalase activity; lipid peroxidation as malonaldehyde (MDA) and reduced glutathione (GSH) in lysated RBCs were determined according to Aebi (1974), Okhawa *et al.*, (1979) and Ellman (1959*)*, respectively. Also SOD according to Nishikimi *et al*. (1972), GSH-px according to Paglia and Valentine (1967) and GSH-R according to Goldberg and Spooner (1983). The obtained results were calculated as U/gHb, U/gHb, U/mgHb, U/gHb, U/gHb and nmol/gHb, respectively, for SOD, GSH-px, GSH-R and CAT activities, and GSH and MDA levels.

Pathological Examination

Pathological examination; tissue specimens from livers and kidneys were taken, from different groups, after ethical scarifying and careful post mortum examination; and divided into 2 halves (formallin fixed and frozen). One half was preserved in 10% formalin. Formalin-fixed tissues were

dehydrated in alcohol, cleared in xylene and embedded in paraffin wax. Sections of 4-5 um were stained routinely with hematoxyline and eosin (H&E) according to Suvarna *et al.* (2012).

Set of sections was cut on positive charged slides for IHC technique, using Poly (ADP-ribose) polymerase (PARP) -Ab. IHC/DAB kit (Acu-Stain™ Mouse+ Rabbit HRP kits) was purchased from Genemed (Biotechnologies, Inc.). Procedures were applied according to kits' instructions.

Frozen half of the tissues were used to estimate the genotoxicity using alkaline Comet assay according to Singh *et al.* (1988).

Statistical Analysis

Results were expressed as means ± standard errors. Data obtained were analyzed using one way analysis of variance (ANOVA) according to SPSS 14 (2006) values with $P \leq 0.05$ were considered as significant.

Results and Discussion

In the present work, one hundred and twenty samples of frozen meat, raw milk, poultry feed, yellow corn, hay and tibn (20 of each), were collected from different markets and animal farms in Egypt. The samples were examined for detection of aflatoxin residues and the obtained results tabulated in (Table 1).

The current results revealed that the incidence of aflatoxins (Afs) residues in animal feeds was comparatively higher than human food. Where, the Afs residues were detected in 80% of poultry feeds and tibn samples, all samples of the yellow corn and hay were contaminated by Afs (100%). Whereas, Afs residues were detected in 50% and 30% of frozen meat and raw milk samples, respectively. However, the highest mean values of aflatoxins residues were detected in animal and poultry feeds as in hay samples the detected mean value was (230±1.63 µg/kg), followed by tibn (120 ± 1.89 µg/kg), poultry feed (97.5± 1.63 µg/kg) and yellow corn (31±1.36 µg/kg), respectively. Whereas, the comparatively lower levels were detected in human food of animal origin as frozen meat (9.2±0.71 µg/kg) and raw milk (3.0±0.1 µg/kg), respectively.

The mycotoxins are natural contaminants of foods and feeds even when the most efficient condition of culture, harvest, storage and handling are used. The prevalence of these toxins in feed samples varies depending on geographical location and season of the year and the specific qualities of climate, vegetation and land are the important factors in connection with certain geographical location the air with the wind or in combination of wind and rain (Mikulec *et al*., 2005).

Nearly every food or feed commodity can be contaminated by fungal organisms and many of these fungi are capable of producing one or more mycotoxins, which are toxic metabolites of concern to human and animal health. It is estimated that 25 to 50% of the crops harvested worldwide are contaminated with mycotoxins. The percentage is highest in tropical regions, where, up to 80% of the crops are reported to contain significant amounts of mycotoxins (Refai and Hassan, 2013 and El-Hamaky *et al*., 2016).

There are many studies about whether or not the ingestion of meat, milk, and eggs originating from mycotoxin exposed food production animals is a significant pathway for mycotoxins among humans (Wafia and Hassan, 2000). In other study, Hassan *et al*.

(2010) detected aflatoxins in 30% of feed samples with the mean value of (3.4 ± 0.1 ppm). While, Hassan *et al*. (2012), reported that 60% of cattle blood had the mean levels of aflatoxins (15.20±0.01 ppb) and the used feed samples in breeding of these animals had the amounts of AFB_1 detected in (60%) of feed samples, with the mean levels of (55.00±1.50 ppb).Whereas, El-Hamaky *et al. (*2016) screened one hundred feed samples for aflatoxigenic fungi and recovered 106 fungal isolates comprising, *Aspergillus flavus, A. ochraceus* and *A. niger*. Thirty three isolates of 47 *A. flavus* produced aflatoxin B_1 and B_2 at average levels of (170-750 ppb).

Food and Drug Administration (FDA,1999) established regulatory working guidelines on the acceptable levels of aflatoxins in human foods and feeds set at 20 ppb for total aflatoxins, with the exception of milk which has an action level of 0.5 ppb of aflatoxins (Bullerman, 1979). The detected levels of aflatoxin residues in the present samples were compared with the international permissible limits of WHO (1979), FAO (1995) and FDA (1999). The main route of human exposure to mycotoxins has been identified as the direct ingestion of contaminated cereals, grains and food of animal origin (Morris *et al.*, 1997). In the present study, human food of animal origin (some of frozen meat and milk samples) showed residues of aflatoxins more than the permissible limits of 15 ppb (WHO) and 20 ppb (FAO). While, all animal feeds were contaminated with aflatoxins residues more than the permissible limits of (WHO) and (FAO), thus its consumption can be resulted a health hazard for human and animal.

The effects of mycotoxins in human and animals varied from carcinogenic; nephrotoxic and immunosuppressive health effects (Hassan *et al*., 2009). In developing countries, it appears that there is a direct correlation between dietary aflatoxins intake and the incidence of liver cancer (FDA, 2000 and Bahtnager and Ehrlich, 2002). The exposure to AFB_1 can result in suppressed immune response, malnutrition, proliferation of the bile duct, centrilobular necrosis, fatty infiltration of the liver, hepatic lesions and even hepatomas. It is one of the most commonly found metabolites and has a highest toxigenic effect (Richard, 2007).
Because aflatoxins contamination of food cannot be avoided, numerous detoxification strategies have been proposed to alleviate its impact. Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidizing chain reactions (Velioglu *et al.*, 1998).

However, not only the emergence of chemical drugs and herbs resistance among different pathogenic strains and their toxins but also their high toxicity had prompted research on new antifungal and antimycotoxins agents (Mukherjee *et al.*, 2003). Till up date, the metal of zinc was used as antioxidant feed additives for animals and human. Bio-nanotechnology has emerged for developing biosynthesis and environmental-friendly technology for synthesis of nano-materials. Among them, the metallic nanoparticles are most promising as they contain remarkable antimicrobial properties due to their large surface area to volume ratio, which is of interest to researchers due to the growing microbial resistance against metal ions, antibiotics and the development of resistant strains (Gong *et al*., 2007; Rai *et al.*, 2009; Hassan *et al*., 2013 and Nabawy, 2015). Among nano- material Zn-NPs have gained more attention due to their special properties and less hazard to environment, however, like most of nano-particles, they are toxic to micro-organisms, which can be used as

antibacterial, antifungal and antiviral agents (Hosseini *et al*., 2011). In addition, Nano Zn improves the immunity of the animals, for an instance, a reduction in somatic cell count in subclinical mastitic cow and an increase in the milk production was observed due to supplementation of Zn-NPs (Rajendran, 2013).

In the present study, the biological synthesis of ZnO-Nps by fungal strains of *Candida albicans* was investigated. The appearance of white clusters deposited at the bottom of the flask indicated the reduction of metal ion and the formation of nano-particles has been taken place. Bio-reduction indicates the presences of reducing agent which served as electron shuttle in this reduction reaction and it was also reported that, fungus reduction was most probably either by reductase action or by electron shuttle quinones or both (Nelson *et al.*, 2005). Moreover, this process can be easily scaled up, economically viable with the possibility of easily covering large surface areas by suitable growth of mycelia (Nelson *et al.*, 2005).

The prepared ZnO-Nps were identified and characterized by visual inspection; in a UV-visible spectrophotometer and Scanned by Transmission electron microscope (TEM) and Scanning electron microscope (SEM) for detection of their particle size and the purity of the prepared powder. The particle size of prepared ZnO-NPs was 60 nm with spherical and granular morphology with uniform distribution (Fig., 1).

It should be noted that small-sized particles have a higher proportion of atoms on their surfaces than do bulk-sized particles, so the former is more reactive and responsive than the latter. However, this raises concern about the possible biological counterparts, which may have an adverse effect by interfering with biological functions, presumably due to their large surface area and enhanced reactivity (Sawai and Yoshikawa, 2004). Various nano-particles as zinc oxide (ZnO-NPs), gold NPs, carbon nano-tubes, and iron oxide nano-particles had been explored for improving bacteria and biofilm adhesion, penetration, generating reactive oxygen species and killing bacteria, potentially providing a novel method for fighting infections that is drug resistance (Taylor *et al*., 2011). Also, Zhang *et al*. (2007) detected that the adhesion of ZnO-NPs with fungal and bacterial cells causing membrane disruption through direct interactions or through free radical production.

Recently, they have significant growth promoting, immuno-modulatory, antibacterial effects than the conventional counterparts. They also alter the rumen fermentation pattern on supplementation in the animal feeds. A part from these nano-minerals are reported to enhance the reproduction in the livestock and poultry (Partha *et al.*,2015). Also, ZnO-Nps was found to enhance growth and improve the feed efficiency in piglets (Yang and Sun, 2006) and poultry (Mishra *et al.*, 2009 and Lina *et al.*, 2009).

Furthermore, Hassan *et al*. (2013) detected that the growth of aflatoxigenic moulds and aflatoxins production were inhibited by addition of 8µg/ml of ZnO-NPs; while that of ochratoxin A and fumonisin B_1 producing mould and mycotoxins production were inhibited by addition of 10µg/ml of ZnO-NPs to tested medium. On the other hand, Nabawy (2015) illustrated that ZnO-NPs was effective in the prevention of aflatoxin production by *A.flavus* in feed when nano-particles were added to it before contamination with *A.flavus* at the concentrations ranged from 50-250 ug/kg of feed.

In addition, it appears that nano-materials hold excessive potential to pass some of the barriers to efficient targeting of cells and molecules in many diseases Singh Suri *et al.* (2007)| and Said *et al.* (2012). Nano-structural of ZnO has been used in the various biomedical applications in the modern world. Metal nano-particles are believed to be safe for applications because they are more stable and with salient properties (Zhao and Castranova, 2011).

As the first step in this direction, the ZnO nano-particles were used in our study to ameliorate the toxic effects of AFB_1 in rabbits in comparison with traditional antimycotoxin (curcumin).

In the present study, the biochemical evaluation of rabbit sera after experimental aflatoxicosis and treatment with ZnO-NPs or curcumin were undertaken. The redox reaction in the animal body followed by imbalance and oxidative damage often leads to sub-clinical hepatitis, inflammatory necrotic hepatitis, liver cirrhosis and even cancer (Zhu *et al.,* 2012). Our results showed that AFB_1 increased the concentration of NO and MDA while, decreased the level of GSH and activities of SOD, CAT and GSH-P_X in the serum rabbits (Table: 2).

The nitric oxide synthase is present in the liver parenchymal cells and Kupffer cells (Curran *et al.,* 1989). While, nitrogen oxides can form nitro-cool acid, as reactive intermediates which causes necrosis of liver cells, inhibition of mitochondrial function and consumption of cell pyridine nucleotides, leading to breakdown of DNA. Also, nitric oxide and peroxynitrite ion radicals can combine to form hydrogen peroxide compound, and leading to cell damage (Diesen and Kuo, 2011). Increase in the MDA is an indicator of oxidative stress in both serum and liver. The activity of superoxide dismutases (SOD), glutathione peroxidase (GSH-PX) in chronic liver cirrhosis and hepatitis is significantly lower than control (Osman *et al.,* 2007).

It could be concluded from our findings that the treatment with ZnO-NPs and curcumin possesses anti-hepatotoxic effect as evidenced by the significant and dose dependent decrease in lipid peroxidation (LPO) and increase in the level of antioxidant enzymes (GSH, CAT, SOD, GPx and GPr) through scavenging of free radicals, or by enhancing the activity of antioxidant, which then detoxify free radicals. These factors protect cells from ROS damaging effect in aflatoxicosis.

The mechanism could be attributed to dissociation of ZnO-NPs with subsequent increase in the tissue Zn concentration.

It is well known that Zn is a powerful antioxidant metal; it is the core constituent of antioxidant enzymes such as SOD and a recognized protector of sulfhydryl groups; it is also thought to impair lipid peroxidation by displacing transition metals such as iron and copper from catalytic sites (Bray and Bettger , 1990). ZnO-NPs are able to protect cell membrane integrity against oxidative stress damage, increase antioxidant enzyme levels, and decrease MDA level. It can improve antioxidant activity through enhancing the activities of antioxidases, and decrease the levels of free radicals (Dawei *et al.,* 2009). Our results are in agreement with Sharma *et al.* (2012) who stated that ZnO-NPs can improve antioxidant activity, enhance the activities of antioxidases and decrease the levels of free radicals.

Currently, the biochemical changes in the sera of rabbit due to aflatoxicosis presented in table (3), showed a significant increase in

the serum activities (AST and ALT), cholesterol and triglyceride concentration. Similar findings were obtained by Hassan and Mogda (2003) in quails, Mogda *et al.* (2014) and Hassan *et al.* (2016) in rabbits. This may be due to necrosis of hepatic cells, as indicated by histopathological results, and resulted in the release of these enzymes into circulation (Lyuch *et al.,* 1971). Such hepatic toxic effect of aflatoxin attributed to its active metabolite in liver as epoxide (Netke *et al.,* 1997), which covalently bind to DNA and may affect structural and enzymatic protein function (Culler and Newbern, 1994).

The results presented here suggest the protective effects of curcumin against the disorders induced by AFB_1 administration, which affected serum level of liver enzymes (AST, ALT), and cholesterol and triglyceride concentration (Table: 3).

It is suggested that the administration of Curcumin reversed the changes induced by AFB_1 supporting the hypotheses that plant products are effective anti-oxidative agents. Possible mechanism behind this is that the Curcumin scavenges or neutralizes the free radicals, interacts with oxidative cascade, quenches oxygen, inhibits oxidative enzymes like cytochrome P450, and chelates metal ions like Fe+2, inhibits peroxidation of membrane lipids and maintains cell membrane integrity and their function in the liver, lungs and kidneys (Sharma *et al.*,2011).

Moreover, our results detected that the AFB_1 induce significantly decreased values in plasma total protein, albumin, alpha globulin, beta globulin, fibrinogen and gamma globulin (Table 4). Decrease in serum globulin in toxin fed group might be due to the adverse effect of AFB_1 on synthesis of total proteins and globulin. These results in agreement with Osuna and Edds (1982) in pigs, Hassan and Mogda (2003*)* in quails and Mogda *et al.*(2014) and Hassan *et al.*(2016) in rabbits. Various studies illustrated that aflatoxins cause an inhibition of DNA and protein synthesis as well as immunosuppression due to the inflammation, cirrhosis of liver and renal-pathy (Harvey *et al.,* 1991 and Salem *et al*., 2007). Also, aflatoxicosis decreased factors V, VII and VIII activity, fibrinogen concentration, platelet number and plasma fibrin monomers (Baker and Green, 1987).

Additionally, ZnO-NPs increase the level of zinc which is an important element for all aspects of the immunity (Sherman, 1992) and is critical for the integrity of the cells involved in the immune response (Dardenne *et al*., 1985); also Zn can improve humeral immune responses Zhenya (2013). In addition, ZnO-NPs improves the immunity of the animals due to supplementation of nano ZnO (Rajendran, 2013).

Currently, our study yielded that the globulin components showed drop in $\alpha 1$, $\alpha 2$, $\beta 1$ and $\gamma 1$ globulin in all of the treated groups, while, there was increase in $\beta 2$ globulin as compared with healthy animals. The results coincided with the tune of total proteins and albumin. This may be attributed to that AFB_1 causes hepatotoxic, nephrosis, hemorrhages (liver and kidneys), which was explored in the histopathological results and in agreement with Tietz (1996). In addition, AFB_1 has immunosuppressive effect through inhibition of nearly all cellular and humeral immunologic factors Tietz (1996) and Hassan and Mogda (2003). Decrease in serum globulin in toxin fed group might be due to the adverse effect of AFB_1 on synthesis of total proteins and globulin (Mogda *et al.,* 2014 and Hassan *et al.,* 2016) in rabbits.

The hazardous effect of AFB_1 was suspected to its ability to induce oxidative stress and distortion of antioxidants enzymes (Alm-Eldeen *et al.,* 2015 and Hassan *et al.,* 2016), with subsequent lipid peroxidation and DNA damage (El-Agamy, 2010), so that hazardous effect could be mitigated by antioxidants (Souza *et al.,* 1999). In the present study we evaluate Curcumin as an herbal medicine having a bi-functional effect in alleviating the aflatoxicosis effect; depending on its ability to react directly with reactive species and indirectly through up-regulation of various cytoprotective and antioxidant proteins (El-Bahr, 2015*)* in comparison with ZnO-NPs.

Clinical investigation revealed noticeable decrease in the animal body weights of aflatoxicated group. Careful post mortem examination of liver and kidneys revealed slight enlargement with paleness in of the liver and kidneys in the aflatoxicated group which directed to normal appearance in the treated groups.

The histopathological investigation, of the current study, detected high incidence of hepatocytes degeneration, in aflatoxicated groups, ranged from severe vacuolar degeneration to cell death with features of both apoptosis and necrosis (Fig.2). These cytopathic features could be attributed to the pathological status generated by AFB_1 through production of high level of ROS which are capable of damaging cell compounds as well as membranes (El-Agamy, 2010), eventually leading to the impairment of cell functioning and cytolysis (El-Nekeetya *et al.,* 2014). While, rising in the mitochondrial matrix free Ca^{2+} concentration lead to matrix swelling with inner membrane unfolding and eventually outer membrane rupture with release of apoptogenic proteins and cell death through convey both apoptotic and necrotic death signals (Rasola and Bernardi, 2011).

In curcumin treated group, there was a noticeable improvement of the hepatocytes status with moderate stage of vacuolar degeneration. Some degenerated foci exhibited features of regeneration in form of bi-nucleated hepatocytes (Fig.3). Fu *et al*. (2008) explained the protective effect of curcumin on the hepatocytes through suppressing hepatic inflammation and attenuating hepatic oxidative stress.

In the present investigation, ZnO-NPs at two level doses showed diverse biological effects. Where, zinc considered as an essential trace element had a critical biological role at the level of enzymes, protein and molecules including RNA and DNA with an additional regulatory role of apoptosis (Hambidge and Krebs, 2007*)*. The beneficial role of ZnO-NPs appeared in our study, at low dose level, in form of mitigation the degree of hepatocytes vacuolar degeneration, associated with increase in the incidence of hepatocytes bi-nucleation (Fig.4). While, high dose of ZnO-NPs exhibited marked cytopapthic effect on hepatocytes in from of vacuolar degeneration and increase in the incidence of necrotic foci (Fig.5), which could be attributed to the persistence of cell membrane lipid peroxidation status with cell membrane disruption (Guan, *et. al.,* 2012); and continuous cytosolic Zn^{2+} elevation lead to its sequestration by the mitochondria and collapse of the mitochondrial membrane potential and dysfunction followed by caspase activation and cell apoptosis (Kao *et al.,* 2012); on the other side DNA oxidative damage lead to necrotic end result, these are in accordance with the findings of Sharma *et al.* (2012) and similar to the results of our study.

Another suspected mode of ZnO-NPs induced cytotoxicity is indirectly, through

saturation of the oxidative defense compounds, which are unavailable to bind other transition metal ions (Watson, *et al*., 2015).

Where, AFB_1 control group had a well-developed portal- portal bridging in between hepatic lobules (Fig.6) and portal traid's fibrosis which is a hallmark of aflatoxicosis (Fig. 7). In this study we used PARP, as an indicator, to investigate the association between ROS production and induction of genotoxicity on the variety of hepatic cells with the fibrosis as an outcome. Our result referred to the oxidative genotoxic effect of AFB_1 on both of hepatocytes and non-parenchymatous cells (Fig.8).

Neither portal- portal bridging formations (Fig.9) nor portal traid's fibrosis (Fig.10) could be detected in curcumin treated group. This beneficial effect of the curcumin, in agreement with the findings of Pinlaor *et al.* (2010), their study revealed that curcumin alleviated periductal fibrosis due to its anti-inflammatory property (Lin, *et al.*, 2009) and a modulator effect on cytokine TGF- β1 (Mourad, *et al.*, 2006) and EMT-transcription factors (Kong *et al.*, 2015) with end result of tissue restoration (Pinlaor *et al.*, 2010). Lin *et al.* (2009) suggested that curcumin exerted antifibrotic effects, possibly through two different mechanisms depending on its concentrations. At lower concentrations curcumin exerted antifibrogenic effects, whereas at higher concentrations curcumin induce fibrosis through induction of apoptosis in hepatic satellite cells (HSCs). Our results could add that protective effect of curcumin on liver cells through the mitigation of aflatoxicosis ROS induced genotoxic effect mainly on non- parenchymatous cells, as parenchymatous cells react positively with PARP (Fig.11), that in accordance with findings of Sun and Peng (2014), who stated that curcumin had potent anti-fibrosis activity, contributing to the inhibition of HSCs activation as a result of increasing in the level of cellular GSH.

Similar beneficial effect was detected in ZnO-NPs/ low dosed group which showed absence of portal-portal birding (Fig.12) associated with marked reduction in the incidence of genotoxicated non-parenchymatous cells along the portal areas (Fig.13) with a subsequent inhibitory effect kupffer cell activity (Watson *et al.*, 2015) and induction of ito cell myofibroblastic transformation (Xidakis *et al.*, 2005).

Whereas, ZnO-NPs/high dosed group had signs of portal-portal bridging formation with neo-vascularization of sinusoids (Fig.14) in addition to massive increase in the parenchymal fibrocytes which enclosed newly proliferated bile ductules and hepatic arterioles in the portal traid's areas. Also, periductul fibrosis, in high dosed-group, might be reflecting the biliary excretion of ZnO-NP (Paek *et al.*, 2013) with induction of fibro-genic effect, due to persistence the ROS-induced genotoxic effect on non-parencymatous cells, which was detected in our study (Fig.15).

The present study revealed the renal cytopathic effect of AFB_1 in form of marked enlargement of the glomerular tufts and increase in their cellularities, which lead to obliteration of the urinary spaces (Fig.16) (Rubin and Strayer, 2008 and Hassan *et al.*, 2016). Another common feature was cystic dilation of the distal convoluted tubules, while proximal convoluted tubules suffering from severe hydropic degeneration with high incidence of coagulative necrosis (Fig.17). In curcumin, ZnO-NPs/ low and ZnO-NPs/ high doses treated groups renal features revealed moderate to marked (respectively) improvement in the AFB_1 -

toxicated renal structure, with reduction in the incidence of coagulative necrosis of renal tubular lining epithelia.

Desquamated epithelia were detected as cellular cast formation in the renal medulla, these findings correlated with the findings of Al- Ghasham *et al.* (2008). In our study, the severity of desquamation of tubular lining epithelia lead to cellular casts' formation associated with increasing in the dose of ZnO-NPs (Fig.18), which is in accordance with the findings of Esmaeillou *et al.* (2013). Yan *et al.* (2012) , explain that effect of ZnO-NPs/high dose associated nephrotoxicity to the disruption in the energy metabolism with subsequent mitochondrial dysfunction with impairment of the cell membrane, ending by necrosis of tubular epithelial cell lining.

Table.1 Aflatoxin residues (ppb) detected in examined food and feed samples (n=20)

Samples	Total Aflatoxins Residues (ppb)				
	Positive	% of Positive	Min.	Max.	Mean±SE
Frozen meat	10	50	5.5	20	9.2±0.71 ppb
Raw milk	6	30	0.5	19	3.0±0.1 ppb
Poultry feed	16	80	50	250	97.5±1.63 ppb
Yellow Corn	20	100	20	50	31± ppb
Hay	20	100	40	400	230±1.63 ppb
Tibn	16	80	40	200	120±1.89 ppb

The permissible limit of Aflatoxins is > 15 ppb as WHO(1979) and > 20 ppb as FAO (1995) & FDA (1999)

Table.2 The effect of AFB1 and the investigating agents on the antioxidant parameters of the serum and the blood lysate of the rabbits

Group / Parameter	Control	AFB_1	ZnO-NP (25 ug/kg b.w)	ZnO-NP (50ug/kg bw)	Curcumin
NO m mole/l	18.23^{e} ±0.26	28.40^{a} ±0.60	20.80^{a} ±0.73	25.20b ±0.58	22.80^{c} ±0.37
MAD nmole/mg Hb)	1.67^{d} ±0.05	2.90^{a} ±0.04	1.83^{c} ±0.02	2.31^{b} ±0.06	1.94^{c} ±0.02
GSH nmole/mg Hb)	15.03^{a} ±0.19	10.60^{e} ±0.24	13.37^{b} ±0.17	12.26^{d} ±0.11	12.79^{c} ±0.09
GSH-R(u/mgHb)	7.53^{a} ±0.29	4.68^{e} ±0.10	6.87^{b} ±0.03	5.26^{d} ±0.10	6.29^{c} ±0.07
GHSpx (u/mgHb)	7.03^{a} ±0.15	3.13^{d} ±0.16	5.00^{b} ±0.12	3.97^{c} ±0.18	4.76^{b} ±0.08
SOD (u/mgHb)	88.20^{a} ±5.07	54.60^{c} ±0.75	80.20^{b} ±1.11	59.20^{c} ±1.69	75.60^{b} ±1.03
CAT (u/mgHb)	18.76^{a} ±0.85	11.72^{d} ±0.53	15.46^{b} ±0.18	12.82^{cd} ±0.22	14.12^{bc} ±0.09

Values are Mean±SE. Values across the table with similar superscript are not significantly different at 5% based on ANOVAtest and letters mean the significant differences between treatments according to Duncan´s test.

Table.3 Showing the serum biochemical parameters in different groups of rabbits

Group / parameter	Control	AFB_1	ZnO-NP (25 ug/kg b.w)	ZnO-NP (50ug/kg bw)	Curcumin
AST u/l	55.0^{d} ±1.82	100.4^{a} ±1.96	66.0^{c} ±1.38	84.8^{b} ±0.86	67.0^{c} ±1.52
ALT u/l	26.0^{e} ±1.22	73.6^{a} ±0.87	44.6^{d} ±1.21	64.6^{b} ±0.93	50.0^{c} ±0.84
Cholesterol mg/dl	80.2^{e} ±1.59	109.2^{a} ±1.39	82.00^{c} ±2.81	87.60^{b} ±1.96	81.80^{c} ±0.66
Triglyceride mg/dl	53.6^{e} ±1.03	95.2^{a} ±1.46	60.6^{d} ±0.93	84.0^{b} ±1.05	67.2^{c} ±0.73

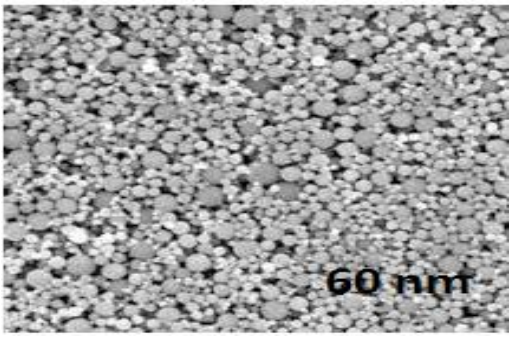
Values are Mean ± SE. Values across the table with similar superscript are not significantly different at 5% based on ANOVA test and letters mean the significant differences between treatments according to Duncan´s test.

Table.4 Total plasma protein (g/dl) and its electrophoresis in control and different groups of rabbits

Group / Parameter	Control	AFB_1	ZnO-NP (25 ug/kg b.w)	ZnO-NP (50ug/kg bw)	Curcumin
T.P	7.12^{a} ±0.07	5.57^{ab}±0.68	6.17^{ab}±0.28	5.99^{ab}±0.30	6.52^{ab}±0.19
Albumin	2.4^{a} ±0.01	1.83^{b} ±0.22	2.12^{ab} ±0.05	2.05^{ab}±0.10	2.24^{a}±0.11
Alpha1a	0.3^{a} ±0.02	0.30^{a} ±0.02	0.28^{a} ±0.04	0.32^{a} ±0.01	0.31^{a} ±0.06
Alpha1b	0.46^{a}±0.06	0.32^{a} ±0.03	0.35^{a} ±0.02	0.34^{a} ±0.05	0.38^{a} ±0.04
Alpha2	0.37^{a} ±0.06	0.28^{a} ±0.04	0.28^{a} ±0.04	0.30^{a} ±0.03	0.32^{a}±0.01
Tt-alpha	1.22^{a}±0.06	0.90^{b} ±0.03	0.93^{b} ±0.02	0.96^{b} ±0.04	1.01^{b}±0.10
Beta1	0.52^{a} ±0.03	0.59^{a} ±0.10	0.62^{a} ±0.06	0.61^{a}±0.06	0.66^{a} ±0.01
Beta2	0.81^{a}±0.06	0.40^{b} ±0.02	0.50^{b} ±0.08	0.43^{b} ±0.03	0.53^{b} ±0.04
T.beta	1.33^{a} ±0.03	0.98^{b} ±0.11	1.12^{ab} ±0.14	1.04^{b} ±0.04	1.19^{ab} ±0.03
Fibrinogen	0.44^{a} ±0.01	0.41^{a} ±0.09	0.47^{a} ±0.02	0.44^{a} ±0.04	0.50^{a} ±0.07
Gamma1	1.37^{a} ±0.12	1.20^{a} ±0.17	1.27^{a} ±0.09	1.27^{a} ±0.09	1.29^{a} ±0.04
Gamma2	0.28^{a} ±0.04	0.24^{a} ±0.07	0.26^{a} ±0.02	0.25^{a} ±0.05	0.29^{a} ±0.04
T.gamm	1.65^{a} ±0.12	1.44^{a} ±0.23	1.54^{a} ±0.08	1.51^{a} ±0.14	1.57^{a} ±0.07
T.globulin	4.20^{a} ±0.07	3.33^{b} ±0.37	3.59^{ab} ±0.21	3.51^{ab} ±0.16	3.77^{ab} ±0.13
A/G	0.57^{a} ±0.01	0.55^{a} ±0.01	0.59^{a} ±0.02	0.58^{a} ±0.00	0.60^{a} ±0.03

Values are Mean±SE. Values across the table with similar superscript are not significantly different at 5% based on ANOVAtest and letters mean the significant differences between treatments according to Duncan´s test.

Fig.1 The micrograph of the particles size of ZnO-NPs (60nm) (black dots) under SEM. (×20000)



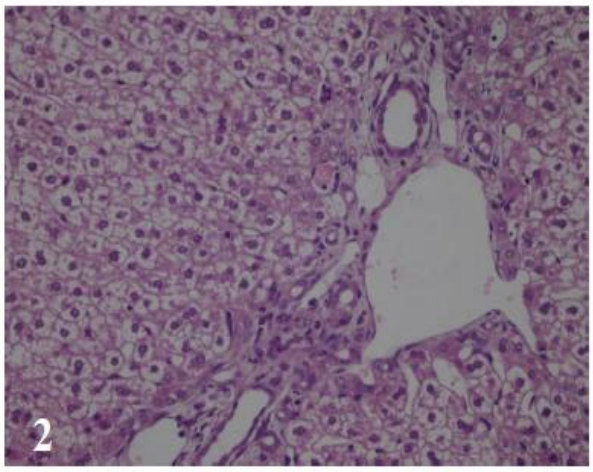


Fig.(2): Aflatoxicated control group showing vacuolar degeneration and marked proliferation of bile ductules with features of apoptosis and necrosis. **H&E X400**

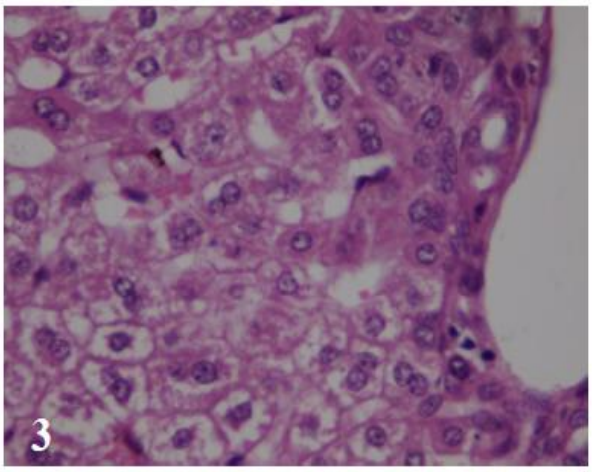


Fig. (3): Curcumin group showed moderate stage of hydropic deg. with evidence of regeneration in form of bi-nucleated hepatocytes.**H&E X1000**

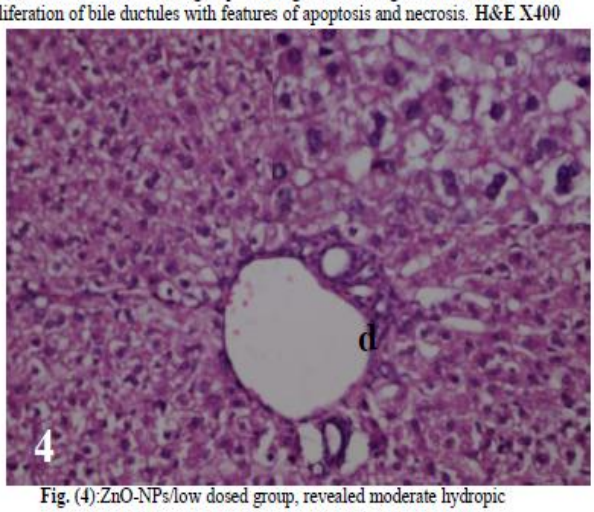


Fig. (4): ZnO-NPs/low dosed group, revealed moderate hydropic deg. Some hepatocytes had bi-nuclei (in set). **H&E X400**

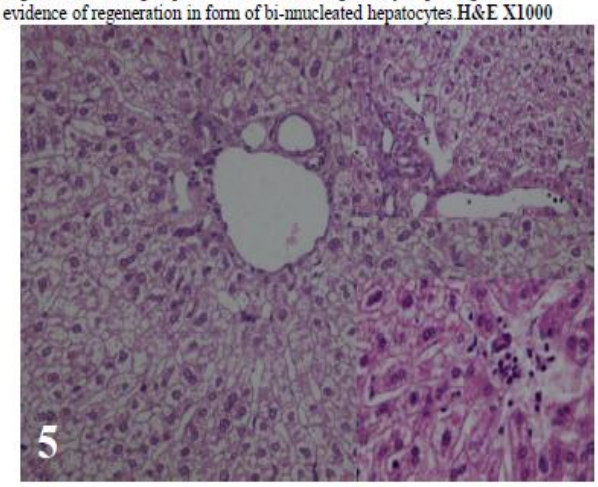


Fig. (5): ZnO-np/ high dosed group, showed hydropic deg. with high incidence of necrotic foci (in set). **H&E X400**

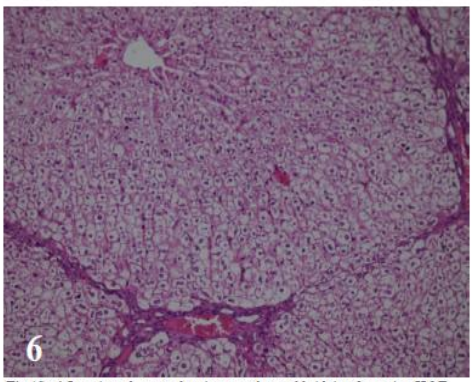


Fig.(6): Aflatoxicated group showing portal-portal bridging formation.H&E. X200

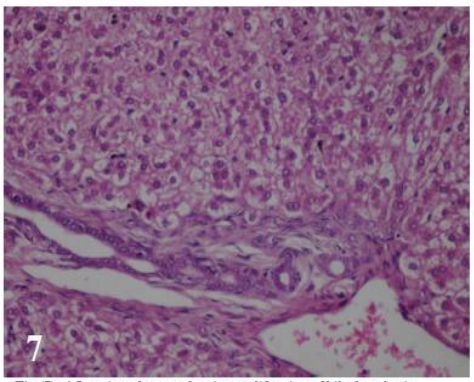


Fig.(7): Aflatoxicated group showing proliferation of bile ductules in the portal traid's area. H&E X400

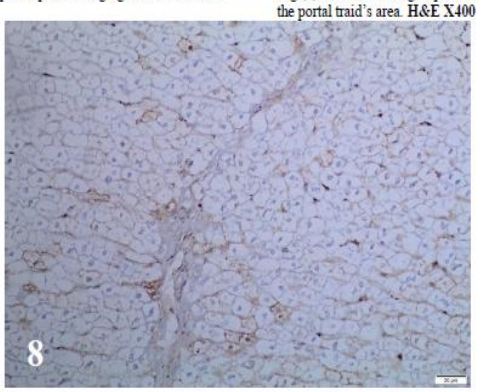


Fig.(8): Aflatoxicated group revealing reactivity of both parenchymatous and non parenchymatous cells to PARP. IHC-peroxidase/DAB X200

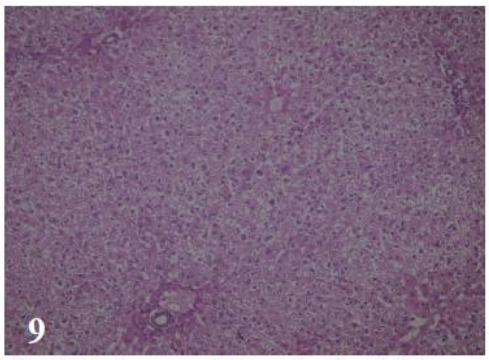


Fig. (9): Curcumin group revealed absence of portal-portal bridging. H&E X 200

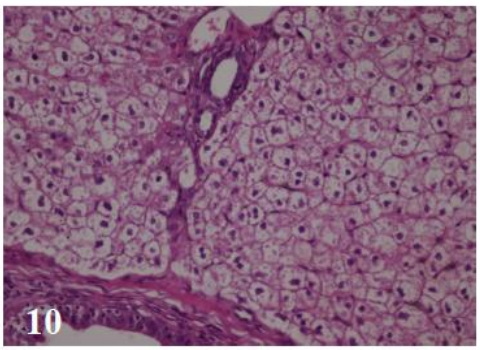


Fig.(10): Curcumin treated group showing normal traid;s area. H&E X400

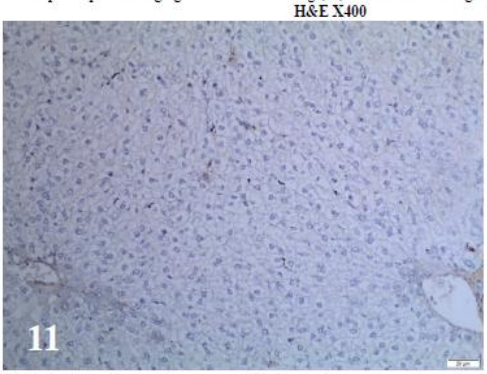


Fig.(11): Curcumin treated group revealing that only parenchymatous cells react with PARA. IHC-peroxidase/DAB X 200

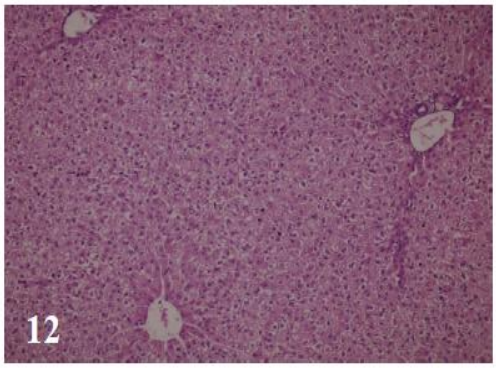


Fig. (12) :ZnO-NPs/low dose group revealed absence of portal- portal bridging. H&E X200

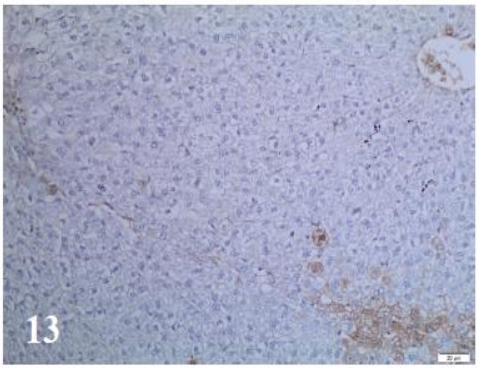


Fig. (13): ZnO-NPs/low dose revealing the reduction in the incidence of the positive reactivity of non-parenchymatous cells to PARP. peroxidase/DAB X200

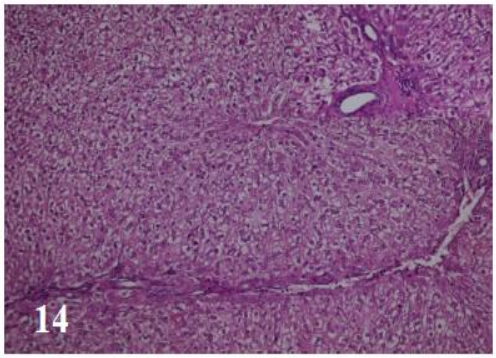


Fig. (14): ZnO-NPs/high dose with marked development of portal-portal bridging. H&E X200

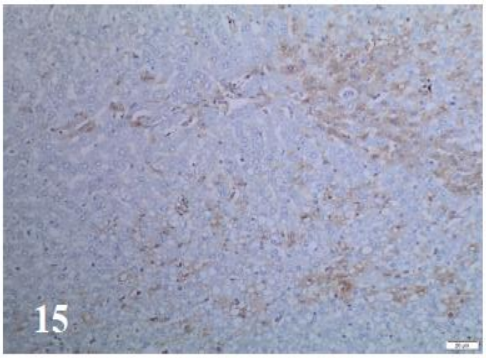


Fig. (15): ZnO-NPs/ high dose showed strong reactivity o non- parenchymatous cells to PARP. peroxidase/DAB X200

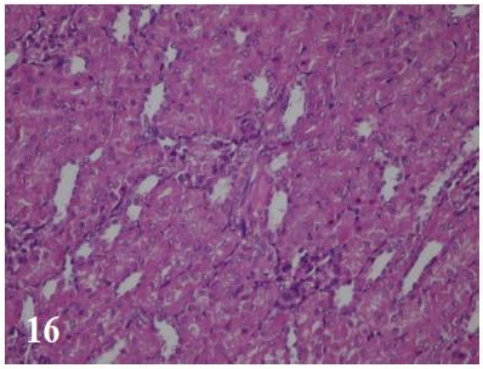


Fig. (16): Aflatoxicated group with signs of gloernular tufts hypercellularity. H&E X400

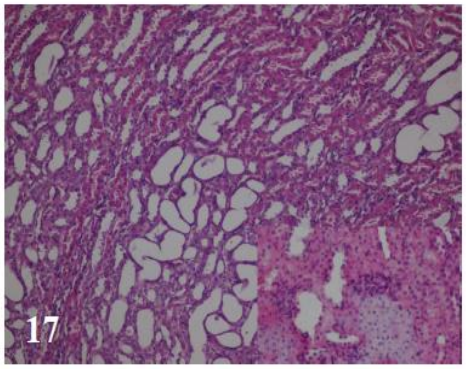


Fig. (17): Aflatoxicated group showing; cystic dilation with coagulative necrosis of proximal convulated tubules. H&E X200

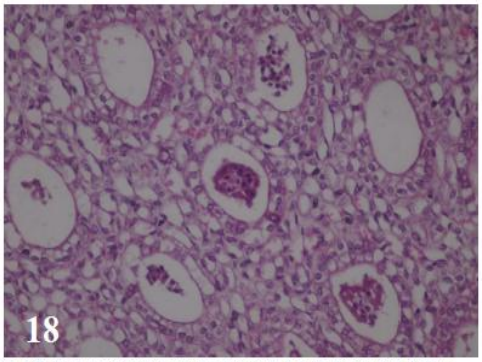
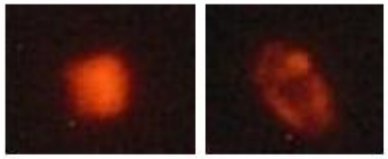
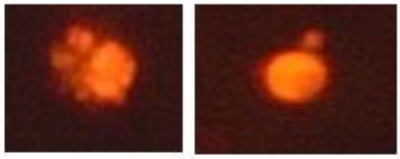
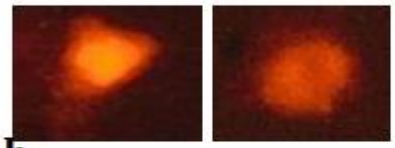
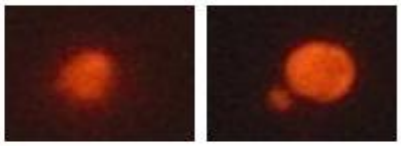
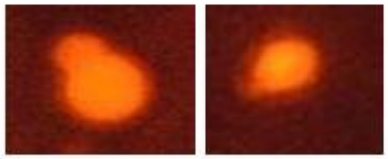
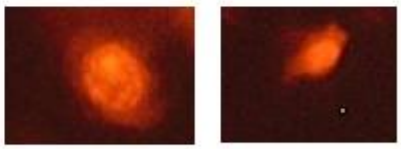
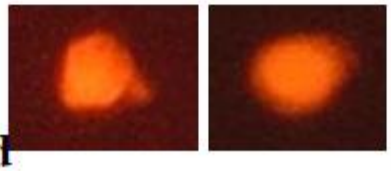
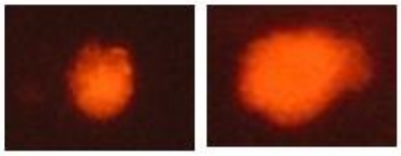


Fig. (18): Cast ormation represent the common feature in all investigated groups. H&E X400

	Fig.(19): *Hepatic cells*	**Fig. (20):** *Renal cells*
AFB1 group	 a	 a
Curcumin group	 b	 b
ZnO-NPs/ ow dose group	 c	 c
ZnO-NPs/ high dose grouop	 d	 d

The genotoxic effect of AFB_1 and the susceptive protective effect of the evaluated therapeutic agents were estimated using Comet-assay in a comparative analysis. Comet assay (Fig.19;a) revealed sever genotoxic effect of AFB_1 which is attributed to its biotransformation and conversion into epoxide with DNA adduction causing DNA strand breaks (Al- Ghasham *et al.,* 2008). Meanwhile, the induction of micronuclei, mainly in renal tissue (Fig. 20;a) could be attributed to the excretion of the formed AFB_1 -N7-guanine adducts in the urine, that in accordance with Wang and Groopman (1999).

Regarding to curcumin, our study revealed significant genotoxic effect, on restricted population of hepatic cellular elements (Fig. 19; b). Cao *et al*. (2007) and Mendonca *et al.* (2009) stated that curcumin shows both genotoxicity and anti-genotoxicity depending on its concentration. The geno-protective role of curcumin, on non-parenchymatoous liver cells, could be attributed to its inhibitory action on bio-transformation of AFB_1 to their active epoxide derivatives (Lee *et al.,* 2001).The same benefit effect was reported in renal tissue (Fig.20; b).

In their study, Wang *et al.* (2006), reported that; the damaging effect on DNA increase reversibly with the dose of ZnO-NPs, which not adapted with our findings that revealed increase in the hepatic (Fig. 19; c &d) and

renal (Fig.20;c&d) protective effect in parallel with increasing in the dose of ZnO-NPs.

In Conclusion, as the first step in this direction, ZnO-NPs at low doses can improve antioxidant activity, enhance the activities of anti-oxidases and decrease the levels of free radicals that resulted from aflatoxicosis in rabbits. The ultimate cyto-protective effect of ZnO-Nps likely reflected by low concentration ZnO-NPs which keep the regulation of enzymes and proteins synthesis that essential for the integrity of the cell membranes. In addition, the biosynthesis preparation of nano-particles by saprophytic fungi is cost-effective and environmental-friendly. Similar findings resulted from use of curcumin plant. Further studies are urgently required to assure more safe doses of nanoparticles before field application in farm animals and poultry.

Acknowledgement

The authors are grateful to Dr. H.H. Mahmoud, Chairman of Laboratory of Elemental and Isotopic Analysis, Nuclear Research Centre, Atomic Energy Authority, Egypt, for his kind assistance and fund in identification and characterization of the prepared and used zinc oxide nano-particles.

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How to cite this article:

Hassan A. Atef, Mogda K. Mansour, Essam M. Ibrahim, Rasha M.H. Sayed El-Ahl, Naglaa M. Al-Kalamawey, Y.A. El Kattan and M.A. Ali. 2016. Efficacy of Zinc Oxide Nanoparticles and Curcumin in Amelioration the Toxic Effects in Aflatoxicated Rabbits. *Int.J.Curr.Microbiol.App.Sci.* 5(12): 795-818. doi: http://dx.doi.org/10.20546/ijcmas.2016.512.090