Comparative Study between the Use of Bulk and Nanoparticles of Zinc Oxide in Amelioration the Toxic Effects of Aflatoxins in rats

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A B S T R A C T

The ability of zinc oxide nanoparticles (ZnO-NPs) to ameliorate the toxic effect of aflatoxin B_1 (AFB_1) in rats in comparison with traditional bulk zinc oxide was evaluated in this study. Two doses of bulk zinc oxide and ZnO-Nps (25 and 50 µg/kg body weight rat of each) were given orally to rats experimentally treated with 50µg/kg b.wt rat AFB_1 produced by aflatoxigenic *Aspergillus flavus* strains. The experimental study extended for further 30 days. The obtainedresults revealed that the low doses of zinc oxide nanoparticles were more effective than low or high doses of bulk ZnO in amelioration the toxic effects of aflatoxicosis which reflected as enhancement in antioxidant activity and histopathological picture of aflatoxicated rats.

Keywords: ZnO-NPs- *Aspergillus flavus*- aflatoxin B_1- aflatoxicosis -amelioration.

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1. INTRODUCTION

Breast cancer is still today one of the leading causes of cancer mortality despite the development of improved diagnostic tools and novel therapeutic modalities. At present, combination therapy (i.e., combinations of different chemotherapeutic drugs in a chemotherapy regime) is becoming a more popular attractive strategy for effective anticancer treatment because it generates synergistic anticancer effects, reduces individual drug related toxicity, and suppresses multi-drug resistance through different mechanisms of action (Parhi, 2012) .

Over the past two to three decades, gallium (Ga) compounds have gained a steady interest in the field of clinical medicine due to the proven ability of Gacations to inhibit tumor growth both in vitro and in vivo, on the one hand, and enhanced bioavailability and efficacy provided by the conversion of Ga into chelate complexes. (Collery, 2012). In recent years, nanotechnology-based combination drug delivery systems to the cancer tissue have emerged as an effective strategy by overcoming many biological, biophysical, and biomedical barriers, largely due to the physical and chemical properties of these nanomaterials. Therefore, administration of different chemotherapeutic drugs in combination, with a suitable nanocarrier platform could be considered as an emerging approach for the treatment of cancer in near the future by offering smart drug delivery systems (Parhi, 2012).

Zinc oxide (ZnO) NPs belonging to a group of metal oxides are characterized by their

photocatalytic and photo-oxidizing ability against chemical and biological species. In recent times, ZnO NPs have received much attention for their implications in cancer therapy (Zhang et al., 2011) . Several studies have shown that ZnO NPs induce cytotoxicity in a cell specific and proliferation-dependent manner, with rapidly dividing cancer cells being the most susceptible, and quiescent cells being the least sensitive (Premanathan et al., 2011) . Surfactantsare wetting agents that lower the surface tension of a liquid, allowing easier spreading, and lower the interfacial tension between two liquids. CTAB is a quaternary ammonium compound belonging to a group of small molecules known as delocalized lipophilic cations (DLCs). Because of their lipophilic nature and delocalized positive charge, DLCs can penetrate the hydrophobic barriers of plasma and mitochondrial membranes and accumulate in the mitochondria in response to the negative transmembrane potential, resulting in mitochondriotoxicity (Chen, 1988).

PH plays an important role in almost all steps of metastasis (Hashim et al., 2011) Lysine is α-amino acid that is used in the biosynthesis of proteins. It contains α-amino, α-carboxylic acid group, and a side chain lysyl ($(CH_2)_4NH_2$), classifying it as a charged (at physiological pH), aliphatic amino acid. It is essential in humans, meaning the body cannot synthesize it and thus it must be obtained from the diet. Free-base lysine (pKa = 10) have all been shown to be effective in reducing metastases in vivo (Robey et al., 2009) Reduction of metastasis is dependent upon buffering (Ribeiro et al., 2012).buffer therapy was initiated before inoculation to prevent progression to metastatic disease. Previous studies show buffer therapy has little effect on reducing primary tumor growth, but significantly reduces spontaneous metastasis formation (Robey et al., 2009)

Sodium ascorbate is considered a powerful hydro-soluble antioxidant capable of deoxidizing the reaction of oxygen and nitrogen free radical species. Therefore sodium ascorbate is able to prevent important deleterious oxidative effects on biological macromolecules, such as DNA, lipid and protein (Soheili et al., 2003).

The present study wasdesigned tointroduce a newly synthesized metal-based compound, namely Lysine-Cetrimonium gallium Complex with zinc oxide nanopatricle and sodium ascorbate (NNC), as achemotherapeutic agent withspeculatedreduced toxicity risk accompanied with higher potential in cancer treatment

2. Materials and methods

2.1. Aflatoxigenic strains

Aflatoxigenic strains that isolated from feeds and their ability to produce aflatoxins was evaluated at laboratory of Mycology and Mycotoxins, Animal Health Research Institute, Agriculture Research Centre.

2.2 Production and estimation of aflatoxins by *Aspergillus flavus* on liquid medium according to Gabal*et al.*, 1994.

2.3. Production of aflatoxins on yellow corn and their estimation according to Smith, 1997.

2.4. Biosynthesis, identification and characterization of zinc nanoparticles

2.4.1. Preparation of C. albicans cells culture (Hartsel and Bolard, 1996) **:**The spore suspension of *Candida albicans* (105/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.

*2.4.2. Biosynthesis, identification and characterization of zinc oxide nanoparticles: (Awodugba and Ilyas (2013) and Shamsuzzaman et al.(2013)***:** Twenty-five mlof the above prepared culture 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).

*2.5. Experimental design***:**

Sixty young inbred, male albino rats (*Musmusculus*),weighing approximately 100-120 g were divided into six groups each of 10 animals and caged separately. The experimental study was extended for further 30 days. The groups were divided as following:

-Group1 (vehicle group) received 0.2 ml olive oil/rat/day.

-Group2 (aflatoxigenated group) was treated with AFB_1 50µg/kg body weight (b.wt).

-Group3 was treated with AFB_1 50µg/kg b.wt with bulk zinc oxide 25µg/kg b.wt

-Group4 was treated with AFB_1 50µg/kg b.wt with ZN NPs 25µg/kg b.wt .

Group5 was treated with AFB_1 50µg/kg b.wt with bulk zinc oxide 50µg/kg b.wt.

Group6 was treated with $AFB_1$50µg/kg b.wt with ZN NPs 50µg/kg b.wt **.**

All the treatments were given orally using a feeding tube attached to a hypodermic syringe (Çam*et al*., 2008) .The used toxic doses of aflatoxins in this study was prepared by dissolving 50 µg / kg of b.w. of rats in 0.2 ml of olive oil/day and given orally (Neeta and Ramaje, 2007).The identified zinc oxide nanoparticles powder were dissolved in buffer to the required doses (25 and 50 µg/ml) for further study in experimental animal (Sahoo*et al.,* 2014 a&b).

2.6. Blood sampling and assays of antioxidant parameters:

At the end of the experiment, blood samples were collected for determination of lipid peroxidation as malonaldehyde (MDA) and reduced glutathione (GSH) were determined according to Okhawa*et al.,* (1979) and El-lman (1959*),* respectively. Also SOD according to Nishikimi*et al*. (1972)

2.7. Histopathological Examination:

Tissue specimens from liver were takenfrom different groups at the end of the experiment and preserved in 10% formalin according to Suvarna *et al.* (2012).

2.8. Statistical analysis:

Collected data were statistically analyzed using one wayanalysis of variance (ANOVA) with $P \leq 0.05$ (the level of significance) using Graph Pad InStat Software (version 3.0, IFF-Italy), (SPSS, 2006).

3. RESULTS

3.1. Results of aflatoxin B_1 production by aflatoxigenic*Aspergillusflavus* on liquid synthetic (YES) medium and natural medium (Yellow corn) were illustrated intable 1 & figure 1.

3.2. Identification and Characterization of ZnO-NPs were demonstrated in figure 2& 3.

3.3. Results of antioxidant changes in aflatoxicated rats' blood and their amelioration by treatment with bulk or zinc oxide nanoparticles were reveled in table 2.

3.4. Histopathological picture of aflatoxicated rat liver with or without treatments by bulk zinc oxide and zinc oxide nanoparticles were revealed in figures (4:19)

Table 1: Levels of aflatoxins produced by *A. flavus* isolated from feed samples on synthetic liquid medium (YES) and natural medium (Yellow corn) (PPm):

Medium used for production of AFs by aflatoxigenic*A. flavus*	**No. of used isolates**	**Levels of aflatoxins produced (ppm)**			
		Maximum	**Minimum**	**Mean**	**Types of produced aflatoxins**
Synthetic medium(YES)	**10**	**42.0**	**15.0**	**38±8**	**B_1**
Natural medium (Yellow corn)	**10**	**55.0**	**23.0**	**46±10**	**B_1**

Table (2): Results of antioxidant changes in aflatoxicated rats' blood and their amelioration by treatment with bulk or zinc oxide nanoparticles:

Groups	SOD (u/mgHb)	GPX (u/mgHb)	MDA (nmole/mg Hb)	GSH U/ML (nmole/mg Hb)
G1: Vehicle control	5.85 ^{a} ± **0.84**	6.61 ^{a} ± **0.41**	1.13 ^{c} ± **0.086**	16.3 ^{b} ± 1.11
G2: +ve control aflatoxicated	2.51 ^{C} ± **0.42**	1.38 ^{c} ± **0.15**	4.30 ^{a} ± **0.34**	10.2 ^{c} ± 0.20
G3: AF+ bulk Zn 25 ug/kg b.w	4.54 ^{B} ± **0.98**	2.09 ^{bc} ± **0.63**	1.96 ^{b} ± **0.18**	15.4 ^{b} ± **1.0**
G4: Nano Zn 25 ug/kg of b.w	5.87 ^{a} ± **0.20**	5.27 ^{a} ± **1.44**	1.073 ^{c} ± **0.014**	20.34 ^{a} ± **1.53**
G5: AF+ bulk zn 50 ug/kg b.w	3.84 ^{Bc} ± **0.52**	3.65 ^{b} ± **0.86**	1.56 ^{bc} ± **0.17**	11.52 ^{c} ± **3.51**
G6: Nano Zn 50ug/kg bw	3.45 ^{C} ± **1.94**	2.69 ^{bc} ± **1.10**	1.99 ^{b} ± **0.41**	16.11 ^{b} ± **1.68**

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.

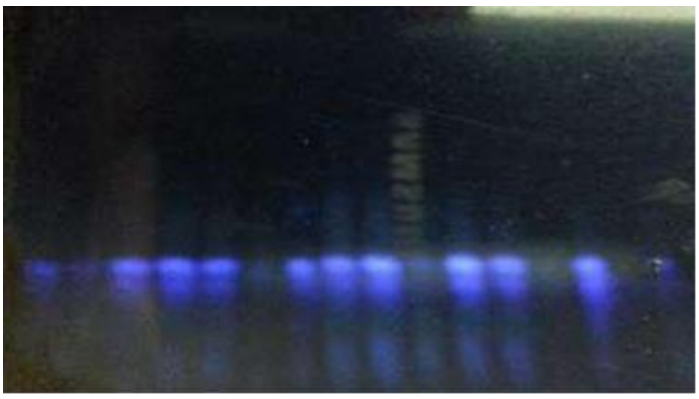

Figure (1): TLC for determining aflatoxin B_1 of used aflatoxigenic*A. flavus* strain.

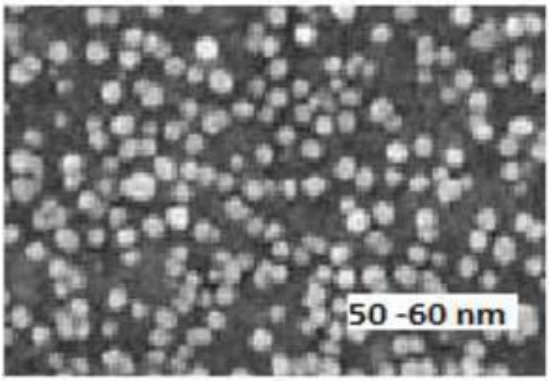

Fig. (2): The micrograph of the particles size of ZnO-NPs (50-60nm) (black dots) under SEM. (×20000)

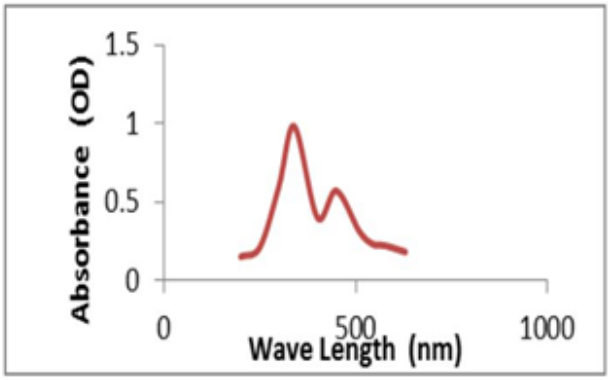

Fig. (3): The UV-VIS absorbance spectra of ZnO-NPS (the optimal W.L was 340 nm)

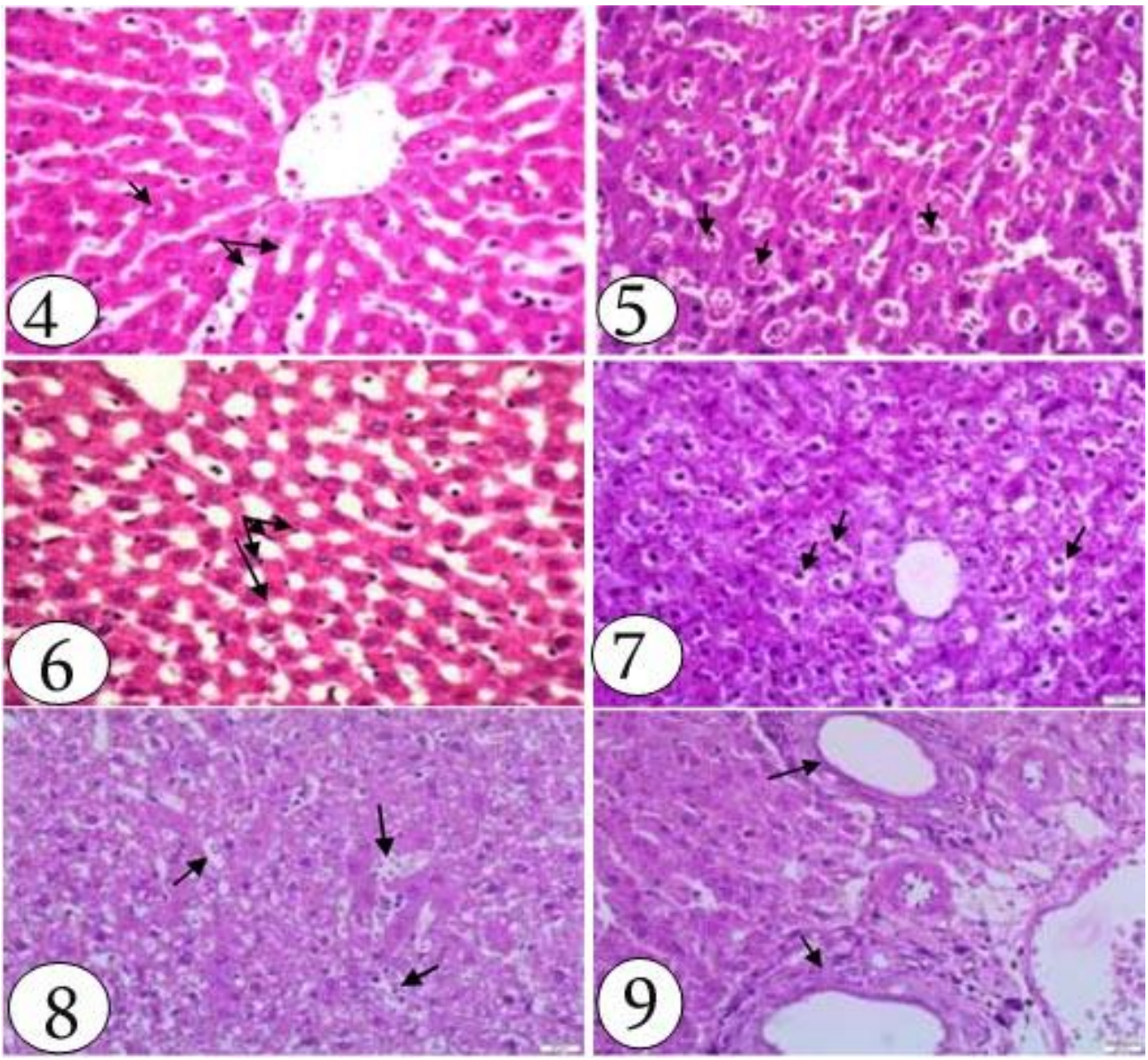

Fig.4: Section of liver of aflatoxicated rats showing widely dilated hepatic sinusoids, Individually cell necrosis of hepatocytes *H&E X 400*. Fig. 5: Section of liver of aflatoxicated rats showing depilating individual hypertrophied hepatocytes, swollen cells with strongly eosinophilic cytoplasm with hyper chromatia of the nuclei with multiple nuclei. *H&E X 400*.Fig.6: Section of liver of aflatoxicated rats showing adenoid pattern of hepatocytes and proliferation of von kupffer cells *H&E X 400*. Fig. 7: Section of liver of aflatoxicated rats treated with bulk zinc (25 ug/kg of b.w.), the hepatocytes showed mild centro-lobular vacoular degeneration H&E X 400 .Fig. 8: Section of liver of aflatoxicated rats treated with bulk zinc (25 ug/kg of b.w.), the hepatic sinusoids were severely dilated and congested in association with minute areas of coagulative necrosis H&E X 400. Fig. 9: Section of liver of aflatoxicated rats treated with bulk zinc (25 ug/kg of b.w.), the Portal traid's area had mild proliferation of endothelial lining its vasculatures. H&E X 400.

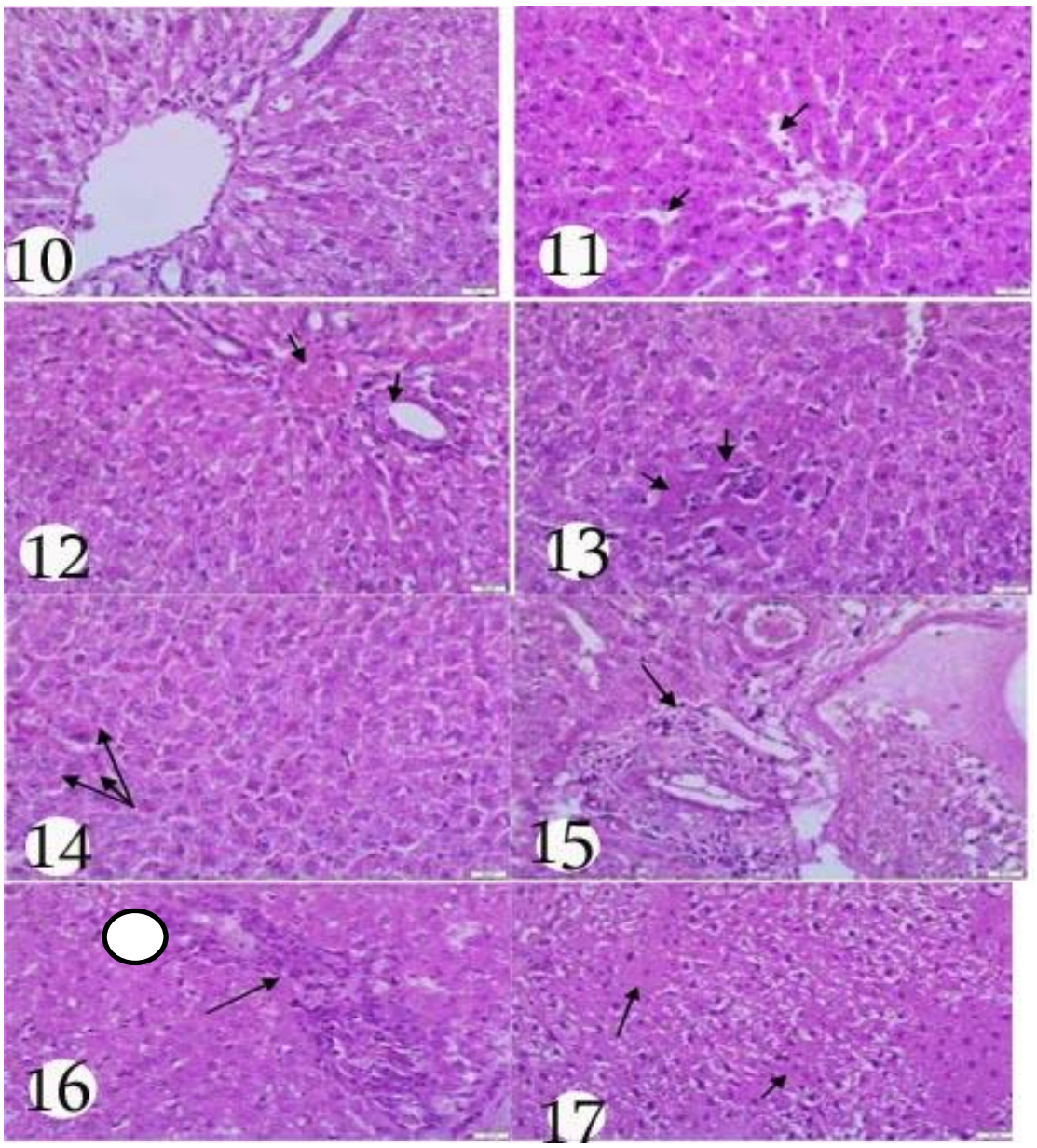

Fig. 10: Section of liver of aflatoxicated rats treated with nano zinc (25 ug/kg of b.w.) The hepatocytes showed almost normal hepatic architectures. *H&E X 400.* Fig.11: Section of liver Liver of aflatoxicated rats that treated with nano zinc (25 ug/kg of b.w.), the hepatic sinusoids revealed mild degree of sinusoidal dilation *H&E X 400.* Fig.12: Section of liver of aflatoxicated rats treated with nano zinc (25 ug/kg of b.w.), the Portal traid's area showed no to lower degree of proliferation of endothelial lining its vasculatures. H&E X 400. Fig.13: Section of liver of aflatoxicated rats that treated with bulk zinc (50 ug/kg of b.w.), the hepatocytes had focal area of coagulative necrosis *H&E X 400.* Fig.14: Section of liver of aflatoxicated rats treated with bulk zinc (50 ug/kg of b.w.), the hepatic sinusoids were engorged by hemolysed blood H&E X 400 .Fig. 15: Section of liver of aflatoxicated rats treated with bulk zinc (50 ug/kg of b.w.), the Portal traid's area showed marked proliferation of endothelial lining its vasculatures. H&E X 400.Fig. 16: Section of liver Liver of aflatoxicated rats treated with nano zinc (50 ug/kg of b.w.) , The hepatocytes had large and more severe focal area of coagulative necrosis. H&E X 400. Fig.17: Section of liver of aflatoxicated rats treated with nano zinc (50 ug/kg of b.w.), the hepatic sinusoids were engorged by hemolysed blood that accompanied with foci of coagulative necrosis H&E X 400.

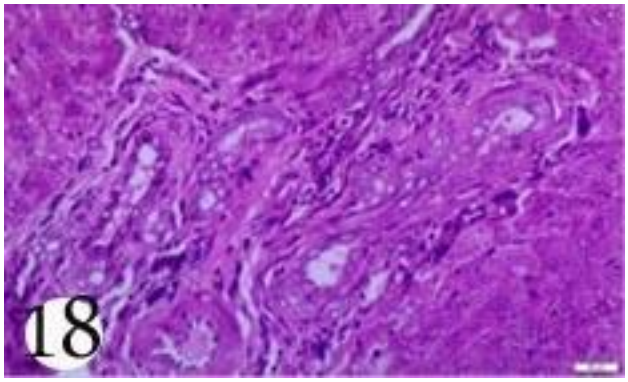

Fig. 18: Section of liver of aflatoxicated rats treated with nano zinc (50 ug/kg of b.w.), the Portal traid's area showed marked proliferation of endothelial lining its vasculatures which also exhibited thrombus formation H&E X 400.

4.DISCUSSION

Most of food or feed commodities 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).

The world organization as 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).

Various studies by several authors as 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).

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 (Nabawy, 2015).

Currently, in the present study, the biosynthesis 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).

As one of the first trials in using zinc oxide nanoparticles as a therapy, the bulk ZnO and ZnO-NP were used in our study to ameliorate the toxic effects of AFB_1 in rats.

The results showed that AFB_1 increased the concentration of MDA while, decreased the level of GSH, SOD and GPX in the serum rats. However, the treatment with bulk zinc oxide or zinc oxide nanoparticles possesses anti-hepatotoxic effect as evidenced by the significant and dose dependent increase in the level of antioxidant enzymes (GSH, SOD and GPx) through scavenging of free radicals, or by enhancing the activity of antioxidant, which then detoxify free radicals. The low doses of ZnO-NPs (25 ug/kg of b.w.) were more effective than low and higher doses of bulk which may be due to the ability to protect cell membrane integrity against oxidative stress damage, increase antioxidant enzyme levels and decrease MDA level. 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).

While, the increase in the MDA is an indicator of oxidative stress in both serum and liver of rats due to Aflatoxicosis and 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).

The mechanism of what 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). Whereas, 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 (Daweie*t al.,* 2009 and Sharma *et al.* (2012).

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 a & b), with subsequent lipid peroxidation and DNA damage (El-Agamy, 2010), so that hazardous effect could be mitigated by antioxidants (Souza *et al.,* 1999).

The small-size particles have a higher proportion of atoms on their surfaces than do bulk-sized particles, so the nanoparticles are more reactive and responsive than the bulk particles. (Sawai and Yoshikawa, 2004)

Concerning histopatholgical results, the foregoing changes in liver tissues 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 and Hassan et al., 2016 a & b), 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 and Hassan et al., 2016 b).

In the present investigation, the treatment of aflatoxicated rats with ZnO-NPs at two level doses showed diverse biological effects particularly the low doses of ZnO-NPs (25 ug/kg b.w) which observed more significant beneficial effects than high doses(50 ug/kg b.w.). 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*)*.

It is suggested that the significant protective effects of ZnO-NPs 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). Similar beneficial effect was detected in ZnO-NPs/ low dosed group by Hassan et al.,(2016 b) which showed absence of portal-portal birding associated with marked reduction in the incidence of genotoxicated non-parenchymatous cells along the portal areas with a subsequent inhibitory effect kupffer cell activity (Watson *et al.*, 2015) and induction of cell myofibroblastic transformation (Xidakis*et al.*, 2005).

5. CONCLUSION

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 rats. In addition, the protective effect of ZnONPs keeps the regulation of enzymes and proteins synthesis that essential for the integrity of the cell membranes. Also, the biosynthesis preparation of nano-particles is cost-effective and environmental-friendly.. Further studies are urgently required to assure more safe doses of nanoparticles before field application in farm animals and poultry

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