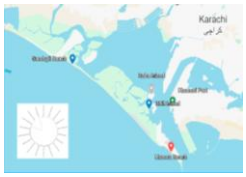

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Protective Effect of Thymoquinone Coated Zinc Oxide Nanoparticles Against Aflatoxin B_1 Induced Hepatotoxicity in Albino Rats

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ABSTRACT: *Herbal Aflatoxins are a group of mycotoxins which can cause hepatotoxicity and can eventually lead to liver cancer. The current study was designed to check the hepatoprotective effect of thymoquinone coated zinc oxide nanoparticles against aflatoxin induced hepatotoxicity in albino rats. Aflatoxin B1 (AFB1) was produced by solid state fermentation on rice using Aspergillus flavus and quantified by HPLC. TQ-coated ZnONPs were prepared by the conjugation of thymoquinone with ZnONPs and characterized by UV-Visible spectrophotometer, and Zeta potential analysis. The albino rats were fed with different diets by dividing them into five groups i.e., group A (normal diet), group B (20 ppb AFB1), group C (20 ppb AFB1 and 25 ppb ZnONPs), group D (20 ppb AFB1 and 10 ppb TQ) and group E (20 ppb AFB1 and 25 ppb TQ-ZnONPs). Blood sample and liver tissues were taken for histopathological study. Histopathological examination of liver revealed that AFB1 causes swelling, focal necrosis and decreased sinusoidal spacing. Biochemical tests like ALT (116 U/L) and AST (109 U/L) were significantly raised while there was decline in level of glucose, cholesterol, HDL and TG by AFB1. Liver function enzymes ALT (28 U/L) and AST (35 U/L) were significantly improved by treatment of TQ-ZnONPs. These changes caused by AFB1 were ameliorated by TQ-ZnONPs. It was concluded that the combined effect of TQ and ZnONPs is an effective approach towards the lethal hepatotoxic effects caused by aflatoxins present in animal feed.*

Keywords: Aflatoxin B1, Zinc oxide nanoparticles, Thymoquinone, Hepatotoxicity, Liver

INTRODUCTION

Mycotoxins are considered toxic secondary metabolites produced by fungi. There are five types of mycotoxins, i.e., aflatoxins, ochratoxins, zearalenone, deoxynivalenol and fumonisins. These toxins are present in a variety of food and feed items which badly affect human and animal body as they are readily absorbed, affecting the liver and cause metabolic disorders (Khan et al., 2024).

Aflatoxins are produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus* (Tola and Kebede, 2016; Sun et al., 2022). Different food commodities like spices, millet, cocoa, sesame seeds, wheat, maize, rice, fig, peanut and peanut butter are contaminated with aflatoxins (Mahato et al., 2019). Aflatoxins are difuranocoumarin derivatives in nature and have mutagenic, hepatotoxic, tetrogenic, cytotoxic, immunosuppressive and estrogenic effects in mammals (Benkerroum et al., 2020; Klvana and Bren, 2019). Aflatoxin exposure is not only linked with liver cancer but also leads to malignancies of different organs like kidney, bladder and bone (Dabuo et al., 2022).

Liver is a vital organ as it is involved in metabolism, secrete harmful materials and store useful products in the body. It is the main site of body which remove injurious chemicals, detoxify different drugs and eliminate xenobiotics. Any malfunctioning in the liver leads to liver damage which results in the disturbance of these processes. Liver disease is regarded as a serious public health concern in several parts of the world (Rishi and Subramaniam, 2017). Aflatoxin B_1 is the primary reason of liver cancer in humans and much evidence support that aflatoxins are primarily involved in hepatocellular carcinoma (Rushing and Selim, 2019).

In the light of previous observations, it is very important to develop different physical, biological and chemical approaches to detoxify various mycotoxins. The use of nanotechnology to reduce the toxic effects of aflatoxins on human and animal health is the most promising approach (Ajayi et al., 2015; Hassan et al., 2021). Nanoparticles are the smallest particles having size range from 1 to 100 nm (Irshad et al., 2020). From the past few decades, nanoparticles are extensively used in a variety of fields i.e., medicine, environment, drug discovery, therapy and biotechnology (Patra et al., 2018). Nanoparticles have gained special importance because of their antitoxin, antitumor, antimicrobial and antifungal activity (Kiruthika and Somanathan, 2014). They increase the safety and efficacy of drugs, enhance their bioavailability and

stability, allow targeted delivery and improve their effectiveness in the target tissue. A wide range of drugs can be delivered via nanoparticles to various organs like liver, brain, spleen, lungs and lymphatic system (Zahin et al., 2020). They have positive effects on the reproduction of livestock and poultry. They alter the fermentation process in rumen of animals (Swain et al., 2015). ZnONPs have been regarded as effective agents in both vivo and in vitro studies. They can easily absorb through body barriers, efficiently targeting cells and molecules in various diseases (Tang et al., 2016). ZnONPs were reported to reduce the toxic effects of AFB_1 in rabbits by scavenging free radicals. This mechanism guard liver cells from toxic effects of AFB_1 (Atef et al., 2016).

Thymoquinone is a biologically active chemical present in the seeds of *Nigella sativa*. It is commonly named kalonji, black cumin or black seed. It exhibits a variety of properties like antioxidants, anti-cancerous, antibacterial, anti-hypersensitive, immunomodulatory and anti-inflammatory (Torabi et al., 2017). It has been reported that it possesses several pharmacological characteristics including antioxidant activity and protective effects against hepatotoxins (Meydan et al., 2019). It could dock with different cancerous and apoptotic targets which make it a hepatoprotective agent. It enhances the anti-cancerous effect of different chemotherapeutics (Khan et al., 2019). According to previous research, ZnONPs when coupled with TQ show more cytotoxicity (Banupriya et al., 2020; Banupriya et al., 2022).

Thymoquinone coated zinc oxide nanoparticles can lead to enhanced anti-cancerous and hepatoprotective effects. It will be a novel approach towards reliable therapeutical intervention against aflatoxins induced hepatotoxicity. So, this study is designed to determine the hepatoprotective effect of TQ coated ZnONPs against aflatoxin B_1 in terms of biochemical and histopathological analysis.

MATERIALS AND METHODS

Synthesized and characterized ZnONPs

Synthesized and characterized ZnONPs were procured from the Biotechnology Department, Kinnaird College for Women University.

Preparation and characterization of TQ coated ZnONPs

The thymoquinone stock solution was prepared by dissolving 2 mg/mL in acetone. 10 mg ZnONPs were dissolved in 1 mL of acetone. The ZnONPs solution was supplemented with the thymoquinone solution. After 24 hours of stirring, the thymoquinone was completely

adsorbed to the surface of the zinc oxide, creating the TQ-ZnONPs. The resulting suspension was centrifuged at 6,000 rpm before being washed three times with distilled water and dried in a vacuum (Perera et al., 2020). Synthesized ZnONPs were characterized by Ultraviolet-visible spectroscopy at 280 nm and zeta potential analysis.

Production of AFB_1

AFB1 was produced using rice by solid-state fermentation. 20 g of rice was taken in flask and 5 mL of distilled water was added and autoclaving was done for 15 min at 121 °C and 15 psi. The rice was inoculated by *A. flavus* and shaken daily. The flask was incubated at 28 °C in dark for 21 days (Lai et al., 2015). 3 g fermented rice was mixed with 20 mL of acetonitrile water (70:30) and was placed in a shaking incubator for 2 hours. The mixture was filtered using Whatman filter paper (0.45µm). The filtrate was then purified by liquid-liquid extraction using chloroform. The chloroform was evaporated by placing the filtrate in a water bath at 70 ^{o}C and the remaining crust was dissolved in 1 mL of methanol. Further analysis of toxin was done by using HPLC (Bayman et al., 2002). The level of AFB1 in 3g rice sample was calculated using formula given below:

$$\text{Concentration of sample (µg/mL)} = \frac{\text{Area of sample}}{\text{Area of standard}} \times \text{Concentration of standard}$$

Study Design

Two months old, 20 albino rats were randomly selected and divided into 5 groups (A-E) each including 4 rats. Five types of diets were designed for five different groups as A (control group with normal feed), B (20 ppb AFB_1 contaminated diet), C (20 ppb AFB_1 contaminated diet and 25 ppb ZnONPs/kg feed), D (20 ppb AFB_1 contaminated diet and 10 ppb TQ/kg feed) and E (20 ppb AFB_1 contaminated diet and 25 ppb TQ-ZnONPs/kg feed). The experiment was conducted according to the Ethical Review Committee, University of Veterinary and Animal Sciences, Lahore.

Histopathological examination

Liver tissues were collected and fixed in 10% of formalin solution for 24 hours. In the next step, dehydrating agent, ethyl alcohol is used for dehydration of tissues. Then, clearing of tissues was done by xylene. Following this, tissues were kept in a jar of molten paraffin wax, embedded in paraffin blocks, and cut into sections of 4µm thickness. Tissue sections were stained with hematoxylin and eosin solutions and viewed through light microscope for histopathological examination (Bancroft et al., 1984).

Estimation of Serum Biochemical Parameters

At the end of biological trial, 5 mL of blood was collected through

cardiac puncture at random from each group of rats. Serum was separated and stored at -20 ºC. Alanine transaminase (ALT), Aspartate transaminase (AST), Alkaline Phosphate (ALP), cholesterol, triglycerides, blood glucose, LDL, HDL and bilirubin were analyzed from serum samples by using kits on a chemistry analyzer (Micro-lab 300; Merck).

STATISTICAL ANALYSIS

All the results were analyzed statistically by one-way ANOVA and means will be compared by Duncan's Multiple Range test using SPSS software. The P-value < 0.05 will be considered as significant.

RESULTS AND DISCUSSION

Estimation of AFB_1

The quantity of aflatoxin extracted from rice was estimated by HPLC against the standard. The calculated value of AFB_1 was 0.008 µg/mL per 3 g of rice.

Characterization of TQ Coated ZnONPs

UV-Visible Spectroscopy was done to confirm the synthesis of ZnONPs. Reduction of Zn ions to ZnONPs was observed by mixing the plant extract and zinc acetate stock solution at 1:50 ratio (pH 12) and stirring for 2 hours. Appearance of colloidal solution was the first indication for the initiation of ZnONPs synthesis. Free electrons in metal NPs exhibit an absorption band of surface plasmon resonance (SPR) due to the resonance of electron vibrations with light waves. The emergence of peaks during UV–Visible characterization reveals the specific SPR which could be a

Fig. 1: UV- Visible Spectrophotometric Analysis of TQ-ZnONPs, TQ and ZnONPs

confirmation for each type of produced NPs. The UV-Vis absorption spectrum of ZnONPs, TQ and TQ-ZnONPs is shown in (Fig 1).

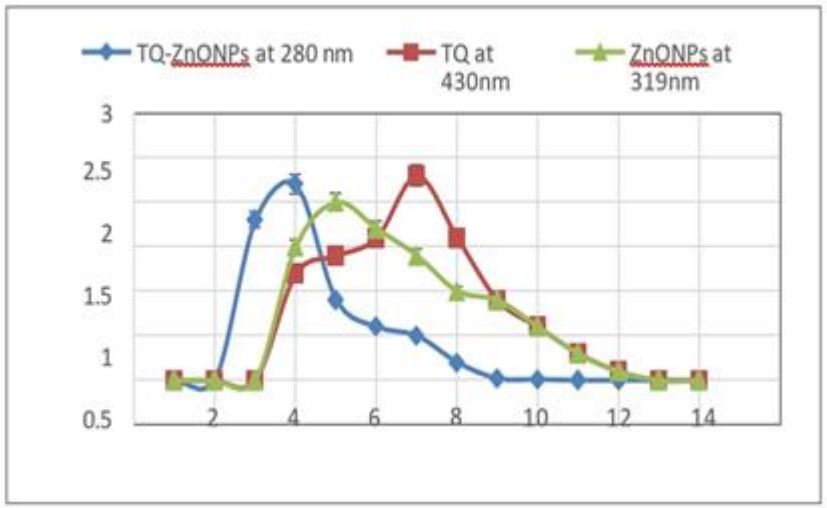

Fig. 1: UV- Visible Spectrophotometric Analysis of TQ-ZnONPs, TQ and ZnONPs

UV-Visible Spectroscopy showed the absorbance of TQ, ZnONPs and TQ-ZnONPs at 430 nm, 319 nm and 280 nm respectively. A prominent peak at 319 nm using UV-Visible Spectrophotometer shows the characteristic band of ZnONPs which is comparable with 320 nm, band of ZnONPs synthesized from Cayratia pedata leaf extract (Jayachandran et al., 2021) whereas TQ give the absorbance at 430 nm as reported in several studies (Mohammed et al., 2019; Laskar et al., 2016).

Surface charges and stability of the synthesized ZnONPs and THQ-loaded ZnONPs were revealed through zeta potential analysis. For ZnONPs and THQ-loaded ZnONPs, strong peaks at negative potentials of -12.3 mV and -14.3 mV were detected, respectively as shown in (Fig 2). Metal oxide NPs possessing negative potential are particularly common while employing plant extract. Furthermore, the emergence of single peaks with negative potential demonstrates not only the constant distribution of surface charge but also the stability of the material in aqueous media (Jebril et al., 2020).

Histopathological examination

Liver tissue of control group (A) demonstrated normal architecture of the liver hepatocytes in cord as well as normal sinusoidal spaces between hepatocytes (Fig 2). Severe swelling represented with a black arrow appeared in the hepatocytes of rats fed with 20 ppb AFB_1 (group B). Focal necrosis was observed in some areas in this group and illustrated using a red arrow. There was a

decrease in sinusoidal spacing as shown by the blue arrow. Histopathological examination of liver tissue of rats fed with AFB_1 and ZnONPs (group C) showed mild swelling of hepatocytes with distorted hepatic cords as compared to the toxin group. Normal architecture was retained with less distortion of hepatic cords in the liver of group D treated with AFB_1 and TQ as compared to groups B and C. Group E which was given TQ-ZnONPs also retained its normal appearance. According to the findings of the histopathology examination, the hepatocytes of the toxicated group B were noticeably larger than those of the control group. A disorganized pattern of hepatic fibers was seen in group B as well. Qing et al. (2022) reported some histopathological abnormalities like hepatocyte cellular damage, oedema, inflammatory cell in the portal triad, and localized hepatic necrosis followed by hepatocellular apoptosis that occurred in the livers of aflatoxicated rats. Another change observed in this study was mild swelling of hepatocytes in ZnONPs treated group which was also documented by Naqvi et al. (2023). In another study, thymoquinone showed preserved hepatocytes arrangement, sinusoids, central veins and portal areas (Abduh et al. 2023). The liver sections in TQ treated group displayed partial improvement in liver architecture. A mild degree of inflammation with moderate congestion in the portal vein and little infilteration in inflammatory cells were seen in TQ treated group (Mohamed et al., 2021). Usual central vein, normal hepatocytes, blood sinusoids and Kupffer cells are indicated in the livers of thymoquinone nanoparticles and thymoquinone groups (Nassar et al., 2023).

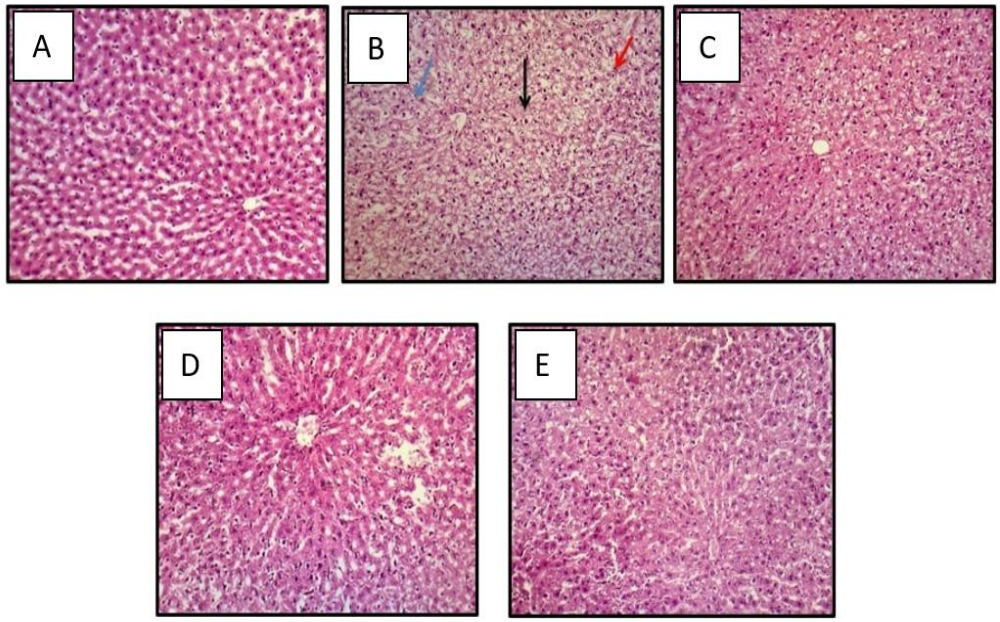

Fig. 2: Histopathological analysis of the liver intoxicated with AFB1 and treated with ZnONPs, TQ and TQ-ZnONPs. (A) Liver of control group, (B) Liver of rats fed with 20 ppb AFB1 revealing distorted pattern of hepatic cords, severe swelling of hepatocytes (black arrow), focal necrosis (red arrow), decreased sinusoidal spacing (blue arrow), (C) Mild swelling of hepatocytes with distorted hepatic cords in the liver of rats fed with 20 ppb AFB1 and 25 ppb ZnONPs, (D) Normal architecture is maintained with less distortion of hepatic cords treated with AFB1 and TQ, (E) Photomicrograph showing improvement in hepatocytes fed with TQ-ZnONPs. All photomicrographs were stained with H and E staining and viewed under 40X objective lens.

Estimation of serum biochemical parameters

The results of liver function tests are given in Table 1. ALT was observed significantly highest for the toxin group while significantly lowest for the group treated with ZnONPs and thymoquinone group. The ALP values were marked significantly highest for the positive control group while there was a significant decline for the ZnONPs and thymoquinone treated group. A significant elevated level of AST was observed in groups treated with contaminated feed while there was a decrease in AST for group treated with ZnONPs and thymoquinone group. According to the findings, liver biomarkers improved in the groups that were treated with TQ-ZnONPs. This shows the combined treatment is

most effective in lowering values of ALT, ALP and AST. There was an increase in bilirubin value in groups fed with AFB_1 and the second highest value was observed for the ZnONPs treated group. The group treated with thymoquinone alone gave almost similar values of bilirubin like nanoparticles group. The most effective treatment was the combined treatment of ZnONPs and thymoquinone as the group showed the lowest bilirubin value. According to Shrestha et al. (2021), an increase in enzyme activity occurs when liver cells are injured, which also results in an increase in the permeability of the cell membranes of the liver cells releasing ALT and AST into the blood. The administration of ZnONPs significantly decreased the levels of ALT and AST in rats (Mirzaei et al., 2024). The treatment of aflatoxins with thymoquinone led to considerable changes in liver enzymes, as well as the histological appearance of liver tissue (Asgharzadeh et al., 2017). Elevated activity of bilirubin shows the liver damage as literature showed that liver's ability to process bilirubin is impaired, leading to an increase in its level in blood. Long term liver damage and biliary obstruction which is blockage in the bile ducts that carry bile from liver to small intestine can prevent the elimination of bilirubin leading to buildup in blood (Guerra Ruiz et al., 2021).

Table 1 Effects of AFB1, ZnONPs, TQ and TQ-ZnONPs on Liver function tests of albino rats (Means±SD)

Group	**Level of Liver parameters**			
	ALT (U/L)	**AST (U/L)**	**ALP (U/L)**	**TB (mg/ dL)**
A (Normal diet)	75 ± 1.00^{a}	59 ± 4.00^{b}	111 ± 1.00^{b}	0.6 ± 0.01^{a}
B (20 ppb AFB_1)	116 ± 1.00^{b}	109 ± 4.00^{a}	136 ± 3.00^{b}	0.8 ± 0.02^{a}
C (20 ppb AFB_1+ 25 ppb ZnONPs)	45 ± 1.00^{c}	59 ± 4.00^{a}	94 ± 3.00^{c}	0.7 ± 0.03^{a}
D (20 ppb AFB_1+ 10ppb TQ)	39 ± 1.00^{d}	53 ± 3.00^{b}	116 ± 1.00^{b}	0.6 ± 0.01^{a}
E (20 ppb AFB_1+ 25 ppb TQ-ZnONPs)	28 ± 1.00^{e}	35 ± 4.00^{a}	56 ± 1.00^{a}	0.5 ± 0.02^{a}

Different superscripts written on means in a column show significant differences among groups ($p<0.05$)

Fig 3 depicts the results of lipid profile and glucose level. Cholesterol levels were observed highest for the negative control as well as for the group treated with ZnONPs and thymoquinone. There was a decline in cholesterol level in positive control. The results showed no positive effect of the combined ZnONPs and thymoquinone treatment in lowering cholesterol levels. An elevated value of high-density lipoproteins was observed for negative control while the lowest value was observed for positive control. The combined treatment of both thymoquinone and ZnONPs gave an HDL value close to positive control. In negative control, low density lipoproteins value was significantly lowest. The highest value was observed for the ZnONPs treated group. The positive control and the group treated with combined treatment of ZnONPs and thymoquinone showed almost similar values for LDL. The lowest triglycerides value was observed for the ZnONPs treated group whereas the highest value was seen in group treated with the combined treatment of thymoquinone and ZnONPs. The negative control also had a higher value of TG as compared to positive control. All these parameters were satisfactorily normalized after treatment with TQ-ZnONPs and toxin. A significantly highest glucose level was observed in the negative control. On the contrary, there was a significant fall in the level of glucose observed in the positive control group. The group treated with ZnONPs alone also showed high values of glucose. Almost similar results were observed for the group treated with thymoquinone and that treated with the combination of ZnONPs and thymoquinone. There is a decrease in blood glucose level in group B treated with aflatoxins as compared to other groups. Gowda et al. (2008) found that AFB1 brought about a reduction in cholesterol levels. Another finding revealed an increased level of LDL and a decrease in HDL level because of liver damage (Abdel-Wahhab et al., 2010). A study showed that AFB_1 feeding results in the lowering of glucose level (Amiridumari et al., 2013).

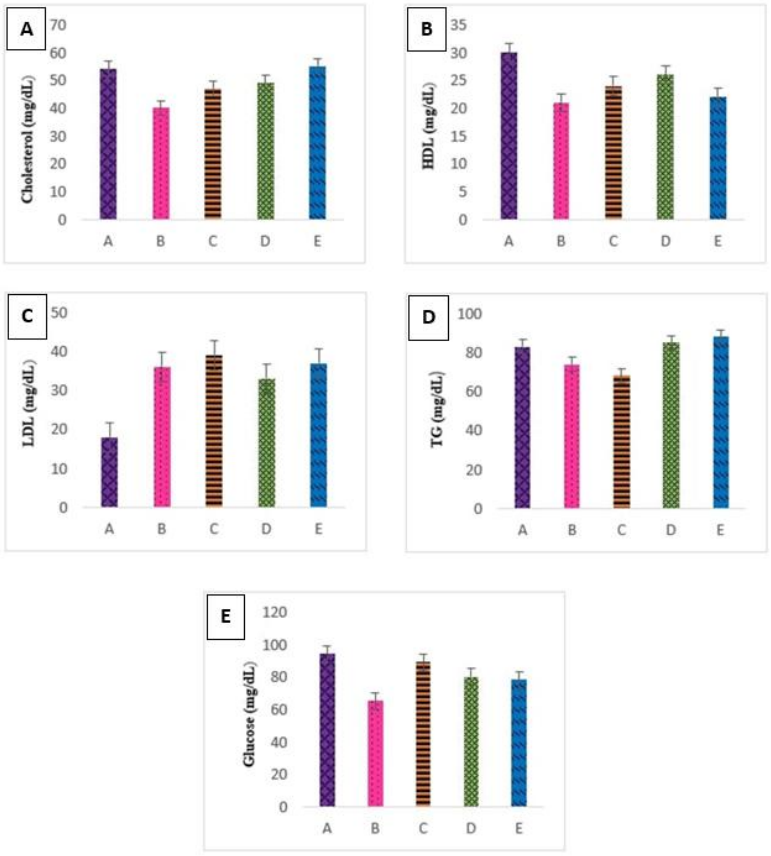

Fig. 3: Effects of AFB1, ZnONPs, TQ and TQ-ZnONPs on (A) cholesterol, (B) HDL, (C) LDL, (D) TG and (E) glucose level. Group A shows control group, group B represents toxin fed group, group C illustrates AFB1 + ZnONPs treated group, group D depicts AFB1 + TQ and group E displays AFB1 + TQ-ZnONPs

CONCLUSION

Based on the findings of current study, it was revealed that AFB_1 had toxic effects on liver in rats. TQ coated ZnONPs have shown promising results in ameliorating the toxic effects of AFB_1 in terms of improvement in histopathological profile and biochemical tests. The combination of TQ and ZnONPs can enhance the hepatoprotective effect, providing an excellent platform for developing safe and efficient therapy against liver damage caused by AFB_1.

ETHICS APPROVAL

All the experimental work related to animal handling and sampling was performed according to the guidelines of the Ethical Review Committee at University of

Veterinary and Animal Sciences, Lahore.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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