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Residual Aflatoxin and Histopathological Changes in Broiler Chickens Amended with Rhino-Hepato ® Forte During Aflatoxicosis

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Abstract

Four groups of broiler chickens have been treated as follows: Group 1 (control group, without AF or RHF; Group 2: (Feed contaminated with 500ppb AF without RHF), G3 (Feed contaminated with 500ppb AF+ 1 ml of RHF in drinking water), G4 (Feed contaminated with 500ppb AF+ 2 ml of RHF in drinking water). ELISA assay was conducted to estimate the AFB1 residual in the liver and the histopathological changes in livers as a result of AFB1 consumption per se or with antidote RHF.

No AFB1 residues were observed in the control group livers of. Residues of 15.18 µg/kg were found in the liver tissues of the group that was fed with 500 ppb of AFB1. Significantly lower residues of 1.14 and 1.012 µg/kg were detected in livers of G3 and G4 respectively compared to AFB1fed group (G2).

feeding broilers with AFB1 at levels of 500 showed measurable AFB1 residues in the tissues of the liver and enhancing mild-moderate to severe histopathological changes. Amending broiler chickens fed AFB1 with RHF significantly ameliorated the AFB1 negative effect on AFB1 residual in the liver content and was able to counteract the histopathological changes of the toxin on liver parenchyma. Therefore, the researcher conducted the study to estimate aflatoxin residues of B1in the liver (AFB1), and histopathological changes in liver via the chronic aflatoxicosis in chicks that are fed with AFB1and Rhino-Hepato ® Forte (RHF).

Keywords: Broilers, AFB, RHF, ELISA, liver histopathology.

Introduction

Poultry meat is an excellent, accessible and somewhat cheap source of protein so one might notice a growing inclination to consume poultry meat globally in the recent years (1). In spite of this, Poultry sector has different challenges, of these the contamination of numerous crops utilized as basic components of poultry feed, like peanut meal, corn, cotton seed and sorghum are subjected to the contamination with mycotoxin and this stands for a high risk for the presence of mycotoxins in poultry diets with different mycotoxins [2,3,4,5]. The poor hygienic conditions, under which broilers are brought up are regarded as poor biosecurity precautions that exacerbate the negative impact of these secondary molds metabolites. They introduce, with special reference to aflatoxins, in the poultry production process through feed harvesting, storage and processing, in addition to its possible field contamination(6).

Aflatoxins are considered the group most intensively researched concerning the impact of mycotoxins and aflatoxins on broilers productivity.

Former studies demonstrated that aflatoxins have a wide range of passive effects, such as slower growth (8), carcinogenic effects, immune-suppression and high sensitivity to diseases (9). Among the known aflatoxins, aflatoxin B_1 (AFB_1) is the most potent hepatotoxin (10) and is classified as a Group I carcinogen by the International Agency for Research on Cancer (2012) (11).

Feed that are contaminated by mycotoxins in general and aflatoxins in particular, is considered a severe hazard on the health and productivity of poultry. The importance of

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Aflatoxins are related to their hepatotoxicity, hepatocarcinogenicity, mutagenicity and teratogenicity which affects many animal species including poultry (IARC, 1987). In addition to that, the residues of mycotoxin in the products of poultry is likely to stand for a danger to human due to their carcinogenic, mutagenic, teratogenic, immunosuppressive and other negative impacts [2,12,13,14]. additionally, several crops that are mentioned as well as about 25% of the food supplies around the globe are contaminated with mycotoxins [3,5,12,13,14,15]. Four essential groups of aflatoxins: B1, B2, G1 and G2 have been emphasized [16,17]. As for aflatoxin B1 (AFB1), which is considered the most toxic and it is recognized as carcinogen [16,17,18], it was included in the Group number one, that involves the carcinogenic agents listed by the International Agency for Research on Cancer (IARC) [2,5,14,19]. Although Aflatoxins are generated by some Aspergillus flavus species and in spite that almost all the Aspergillus parasiticus are growing in number, however, Aflatoxin B1, are considered the most important and have been identified as natural contaminant of feedstuff that is used for the formulation of poultry feeds. Liver is the targeted organ affected by Aflatoxins, which is manifested by pale, hemorrhagic gross. The effect of afalatoxin on biochemical profile of broiler chicken was studied by the author (20). Aflatoxin residues, AFB1 and its metabolites in particular, might exist in tissues, in eggs of the poultry fed with diets that are contaminated with AFB1 to become a potential hazard on man health [2,5,13,16,21,22]. It was shown that the intake of AFB1 is accompanied by a high rates of human liver cancer infection [3,14,18], and also with cancers of prostate breast, gastro-intestine; malnutrition of protein-energy for children. It is also associated with HIV infection progression, particularly in states with low-standard of living [3,14,23,24]. As it is regarded as a significant hazard to public health, the exposure of people to AFB1 by the food, which is source is animals was mentioned by several researchers [3,14,15,25,26] and it is permanently superintended in the developed states via various methods of chromatography and immune-enzymes [5,13, 27, 28, 29,30, 31, 32, 33]. The monitoring does not take place in a big number of the developing countries. In the evaluations of histopathology, the livers of chicks, which ate feed that contains AFB1 demonstrated multifocal and various cytoplasmic vacuolization, degenerating foci, severe fatty changes, fibrosis of the portal regions and hyp**erplasia of bile duct (34)**. Immunosuppresion and growth retardation are almost always accompanied Aflatoxicosis in broilers. Different physical and chemical methods were used for amelioration the adverse effects of Aflatoxin in different avian species (35) Milk thistle as one of the plant extract entered the race track for remedy of Aflatoxicosis in poultry (36). Rhino-hepato ®, a product containing Milk thistle (*silymarin marianum*), was effective in ameliorating Aflatoxin negative effect on the blood profile of broiler chickens (37). The main effect of *silymarin marianum* extracts is in liver protection (38), through their anti-inflammatory, cytotoxicity and Anti-carcinogenicity effects (39). Milk thistle as an antioxidant extract contains many flavonolignans having well known anti-inflammatory and hepatoprotective activity (40). **This research has been performed to assess the utilization of Rhino-hepato ®, as** protective product on the residual Aflatoxin and on the histological architecture of the liver tissue.

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Materials and methods

The trail was accomplished at the college of veterinary medicine, department of veterinary public health, from 1/10/2020 to 5/11/2021.

Aflatoxigenic strain of *Aspergillus flavus* was used to produce AF using rice culture (41). Aflatoxin B1 was estimated to be 500 ppb using Neogen Enzyme linked immunosorbent assay (competitive ELISA) (Neogen company, USA).

Rheno-Hepato Forte (Germany)

Liquid complementary feed for poultry to prevent and treat liver and kidney disorders

Analytical constituents and levels:

1% potassium, 2% methionine

Additives per L:

Vitamin B12, 15.000 µg; L-carnitine, 20.000 mg; Betaine, 40.000 mg; Choline chloride, 80.000 mg; Sorbitol, 200.000 mg; Flavour, 2.000 mg; 1,2-propanediol, 50.000 mg; Magnesium sulphate, 10.000 mg; Total seasoning flavorings, 40.000 mg; Milk thistle extract, 32.000 mg; Artichoke extract, 8.000 mg.

One hundred twenty (120) **male broilers** with an age of **one day (Ross 308) were procured from obtained from private-sector hatchery** (Mosul governorate) with approximately equal weight. Were used in completely randomize block design was applied in this experiment (CRBD) with two factors: (i): the level of Rhino-hepato ®forte (RHF), (ii): the level of AF. **The Chicks were categorized into 4 groups**. Group 1 (control group, without AF or Rhino-hepato ® forte) (RHF); Group 2: (Feed contaminated with 500ppb AF without RHF); G3 (Feed contaminated with AF+ 1 ml of RHF in drinking water) G4 (Feed contaminated with AF+ 2 ml of RHF in drinking water). Three replicates with 6 broilers per replicate were involved in this experiment. Birds were housed in an isolated room, **which was supplied with electrical heating system, a negative pressure, a unit of forced ventilation in addition to a feeder and a trough water per a cage. The temperature for the purpose of brooding was set to 33~35Cº for day 0 and it was reduced step by step be 23~21 Cº till the twenty first day and was kept in this range until the end of the experiment. The relative humidity and the temperature were examined each three hours. Efforts were made to have and resume the optimal conditions of relative humidity and temperature according to the Broiler Management Handbook of Ross** (2014)(42). Birds were raised on deep litter in wooden pens at the animal house of the college of veterinary medicine. Broilers were offered feed and water *ad libitum*.The birds were vaccinated with Newcastle vaccine at 8 and 28 days of age.

Aflatoxigenic strain of *Aspergillus flavus* already isolated on Sabouraud agar from contaminated poultry feed sample was used for the production of aflatoxins, using a rice culture as described by Shotwell et al., 1966 (41). Aflatoxin B1 was estimated to be 50ppm using Neogen Enzyme linked immunosorbent assay (ELISA) kit (Neogen company,USA).

Fourty eight liver samples were collected and **taken to the lab of the of Veterinary Public Health department, College of Veterinary Medicine, Mosul University. All the samples were stored at -20 Cº until analysis was conducted.**

Livers were collected (n=24) for estimation of residual Aflatoxin, and another (n=24) for histopathological examination.

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During collection, liver samples were **examined in depending on color, and they were categorized as "normal", "pale or yellowish" and "moderate", in accordance with the procedures that were described in the study of Dos Anjos et al. [43] and USDA [44]. The "normal" livers were defined as those with the color from tan to deep mahogany red, but the livers that are 'moderate' included the pale or yellowish in color in the area that constitutes two-thirds of their total area.**

AFB1 analysis of content was conducted by the competitive ELISA method by means of using the AFB1 MaxSignal® commercial kit (1055-04, MaxSignal®, Bioo Scientific Corporation, Austin, TX, USA) that involves 96-well micro-titer plates that were sensitized by monoclonal antibody, specified to AFB1. Fifty (50) µl of each standard solution and each sample. After that, 100 µL of aflatoxin B1-horseradish peroxidase conjugate was added to every well of the plate. The plate is shaken manually for one minute and then incubated at the room temperature for 30 minutes. After the incubation, a micro-titer plate wells were emptied fully and washed for 3 times with 250 µL of the 1× wash solution in each wash and then dried up by tapping several times on a paper towel layer. The unbound conjugate was removed during washing. After washing, 100 µL of tetramethylbenzidine (TMB) substrate was added to each well of the plate. Then, the plate was manually shaken again for 1 minute and then incubated at the room temperature for 15 minutes (counted from the first addition of the substrate). The reaction was halted through adding 100 µL of the enzyme reaction inhibition buffer. The absorbance was measured immediately at 450 nm in a BioTek® ELISA plate reader (EL-800, BioTek®, Winooski, VT, USA). It was discovered that the intensity of absorption inversely related to the concentration of AFB1 in the sample. The concentrations of AFB1 and the determination of the standard curve were processed on a specific aflatoxin MaxSignal® Excel analysis program (Bioo Scientific Corporation, Austin, TX, USA) setting the dilution factor to be 20, as it is recommended in the procedures of the kit.

Specimens (n=6 per group), from the liver were collected and fixed at once in 10% buffered neutral formalin solution followed by routine processing. Paraffin with sections thickness of five microns were prepared and were cut into 5-µm-thick sections. After being deparaffinized, sections were, stained by Hemotoxylin&Eosin (H&E) then examined microscopically for histopathology, using a ProgRes C5 digital camera (Olympus DP72) attached to a light microscope (Olympus BX53/U-LH 100HG, Olympus Corp., Tokyo, Japan)

The concentrations of AFB1 were analyzed by through the use of **the analysis of variance (ANOVA). The significance level was set to $p < 0.05$** for all statistical analyses tests.

Data are presented as means±SEM. Data were subjected to one-way analysis of variance (ANOVA). In all cases, *p* values <0.05 were used to indicate statistical significances.

Results

From table (1), there is an obvious significant (<0.05) residual AFB1 content in livers of (G2) birds that consumed AFB1contaminated feed at a rate of 500 (µ/kg) and reached 15.18 µ/kg, when compared with control group (G1) or the treated groups (G3&G4), in which birds were amended with 1 and 2 ml of RHF/ liter of drinking water. It also found that there was no significant differences of the residual AFB1 in RHF between the treated groups, being

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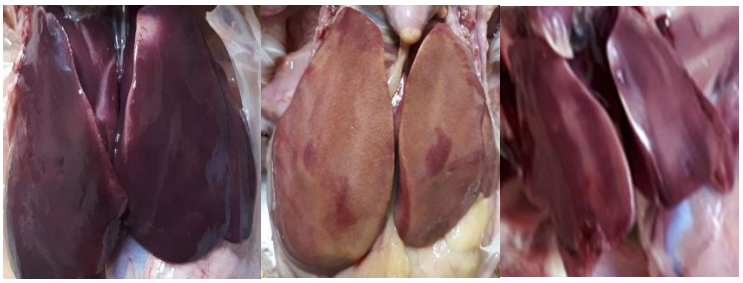
1.14 µ/kg in livers of G3 and 1.012 µ/kg in G4, since the addition of RHF to the drinking water was responsible for a significant (<0.05) reduction (13-14) folds in AFB1 livers content when compared with group (G2) which fed AFB1 alone.

Table 1: Concentration of AFB1 in broilers liver (µ/kg)

Diet	Residual AFB1 (µ/kg) in liver
G1: Control	not detected
G2: 500ppb AF	15.18*
G3: 500ppb AF+1 ml of RHF in drinking water	1. 14
G4: 500ppb AF+2 ml of RHF in drinking water	1.012

* Significant at a level of *p* values (<0.05).

Liver samples were collected from four groups of broiler chickens show different colors categorized as deep mahogany in both control group fed no AFB1 or RHF (A), while those fed 500ppb AFB1 were pale to yellow in color(B). Restoring to the normal color was obtained by amending group 3 and 4 fed 500ppb with RHF at a rate of 1 and 2 ml in drinking water (C).



A (G1) B (G2) C (G3) (G4)

Figure 1: Control (Fig. A); Pale or yellow (Fig. B); Normal (Fig. C) livers of broiler chickens with or without AFB1or RHF.

Histopathological changes in livers of chickens did not receive Aflatoxin (500ppb) or RHF (G1= control group), show the normal architecture of hepatocytes, portal area and the central vein (Fig.2).

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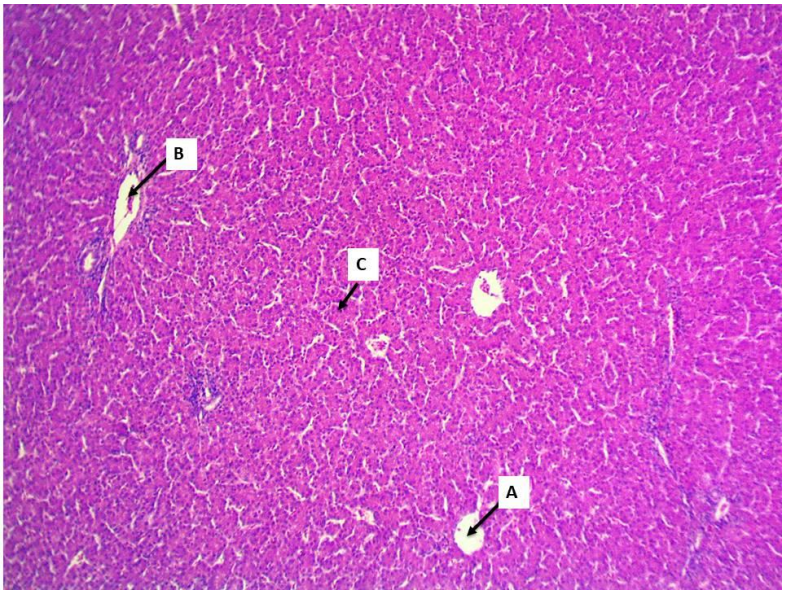

Figure 2: A photo of the liver of one of the the control group chicks, which shows normal architecture represented by the central vein (A), portal area (B) and hepatocytes (C). H&E stain, 100X.

Livers of the birds that consume diets that contain AFB_1 at a rate of 500 ppb, showed significant histopathological changes, such as loss of architecture, congestion of central vein, mild to severe aggregation of inflammatory cells, lymphoid cell and heterophils in periportal and parenchymatous areas, fatty degeneration, massive multifocal coagulative necrosis of hepatocytes in portal area, ecchymotic hemorrhages, dilatation of sinusoids and proliferation of bile ducts (Fig.3).

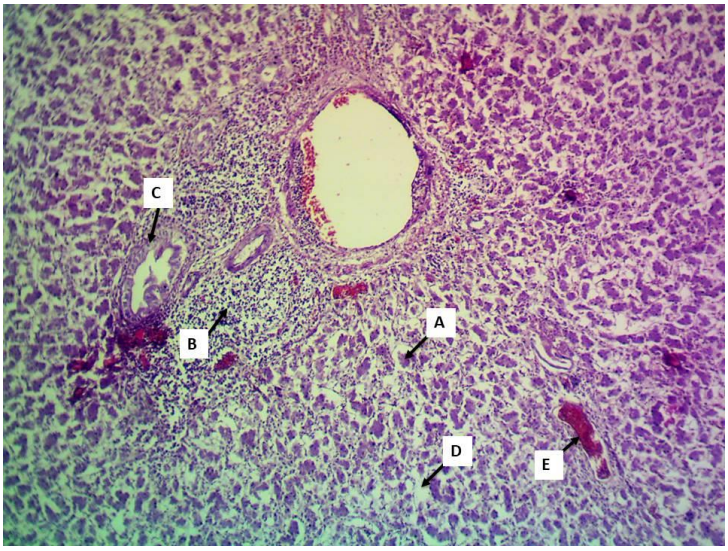

Fig. 3: Histopathological changes in the liver of broilers, which were given 500pb aflatoxin that shows massive coagulative necrosis of hepatocytes in portal area (A), severe inflammatory cells infiltration (B), severproliferation of bile ducts (C), dilatation of sinusoids (D), and congestion of central vein (E). H&E stain, 100X.

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Addition of Aflatoxin (500ppb) and treatment with1 ml of RHF in the birds (G3), or with 2 ml of RHF in drinking water (G4), were effective in amelioration the toxic effect of Aflatoxin in livers and restoring liver architecture to that of the normal one (Fig 4**).**

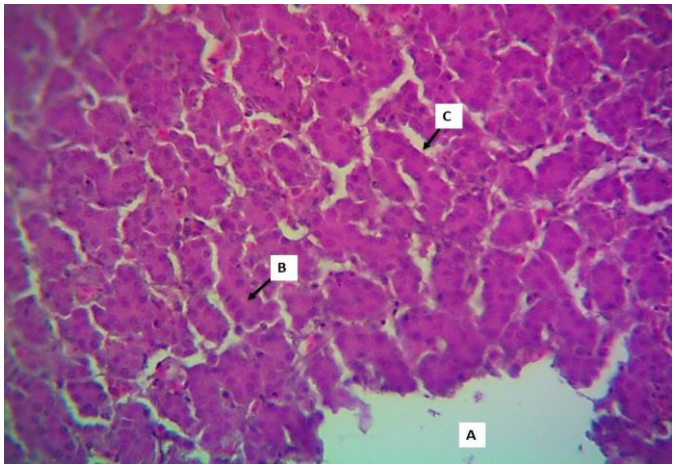

Figure 4: A photomicrograph of a liver of treatment group AF+ 2 ml of RHF in drinking water shows normal architecture representing by central vein (A), hepatocytes (B) and sinusoids (C). H&E stain, 400X.

Summarizing the results of the histopathological analysis in aflatoxin and Rhino-hepato ®, exposed chickens as shown in table 2 indicate that livers of broiler chicks given 500pb aflatoxin (G2) show sever hepatocyte necrosis and proliferation of bile ducts, moderate Hemorrhage and Inflammatory cell infiltration and weak Fatty degeneration. None of these mentioned changes were noticed in control group (G1) and livers in group (4), when the latter group was amended with 2 ml of RHF in drinking water, except of some milder degree of hemorrhage in livers of group (3) that received 1 ml of RHF in drinking water.

Table 2: Results of the histopathological analysis in chickens exposed to aflatoxin and Rhino-hepato ®,.

Group	Parameters				
	Hemorrhage	Hepatocyte necrosis	Infiltration of inflammatory cell	Fatty degeneration	proliferation of bile ducts
Control	-	-	-	-	-
500ppb AF	++	+++	++	+	+++
AF+ 1 ml of RHF in drinking water	+	-	-	-	-
AF+ 2 ml of RHF in drinking water	-	-	-	-	-

Grades are follows: - absent, + weak, ++ moderate, +++ sever.

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So amending group3 and 4 with1 ml of RHF in the birds (G3), or with 2 ml of RHF in drinking water (G4), with (500ppb) Aflatoxin in their feeds were effective in amelioration the toxic effect of Aflatoxin in livers and restoring liver to that of the normal one (Table 2**).**

Discussion

Aflatoxicosis was reported in broilers here in Ninevah governorate (45) and causes economic losses in broiler production even at very low concentration. Several analytical methods could be used for detection of residual mycotoxins in broiler liver, muscles and other poultry organs. Amongst these Immunoassay methods is ELISA, which is always used for the mycotoxin examination in agricultural products and the tissues of organs [46,47,48,49]. ELISA test kits are favored because of its high throughput tests and low requirements for the volume of sample it almost needs less procedures of cleaning the sample extract compared to the traditional methods. In addition to that, the method is characterized with speed, simplicity, sensitivity, specificity and portability that is can be used in the field as well as being quantitative fully [45,46]. Many commercial ELISA aflatoxin tests make the test useful essentially as a test of screening for routine quality control of feed contamination and the residual mycotoxines [45,46].

Significant AFB1 residual was detected in the livers (15.18 µ/kg) of broilers fed with a feeder contaminated with 500ppb (group 2) as a turn-over to liver residues, when boilers were exposed for 35 days to Aflatoxin, explains the fact that feeds is a major source of aflatoxins [7,50,51,52, 53].

Amending drinking water with RHF to birds in both groups 3 and 4 was responsible for the considerable reduction of residual AFB1 in livers of these birds to 1.14 and 1.012 respectively.

The reasons behind this excellent role of this comprehensive water additive could be traced to its components of (potassium, methionine, Vitamin B12, L-carnitine, Betaine, Choline chloride, Sorbitol, Flavour, 1,2-propanediol, Magnesium sulphate, seasoning flavorings, Milk thistle extract, Artichoke extract). These components are reported to improve overall bird's metabolism, through providing antioxidant and hepato -protective action, prevent the penetration of hepatotoxic AFB1 into cells, stimulates the regeneration of hepatocytes and normalizes the functioning of the liver.

The two other Rhino-hepato® important components are betaine and sorbitol, which play by their sides a vital role in counteracting aflatoxicosis in broilers, since betaine as trimethyl glycine derivative, is hepatoprotective and activates the metabolic reactions of methylation and activates fat metabolism in the liver and stimulates digestion, while sorbitol plays an important role in detoxification reproduces blood volume and is involved in energy metabolism.

Artichoke, one of the Rhino-hepato® component is less than silymarin in its beneficial effect on birds expressing as a potent antioxidant thereby protecting liver from oxidative damage and inflammation (54, 55).

In poultry liver is the target organ of aflatoxin effect, characterized by the total hepatic changes in terms of color and the consistency are often the first and the most noticed in poultry species [56,57,58,59].

In this trial, birds ingested aflatoxin B1 caused many different histopathological changes in their livers, since the metabolism of AFB1 takes place in liver via certain cytochrome P450, enzymes to multiple isomers, like aflatoxin-8,9-epoxide which might, after that, bind to the proteins and results in severe toxicity (aflatoxicosis) [60]

The lower residual AFB1 levels, G3 (Feed contaminated with AF+ 1 ml of RHF in drinking water) G4 (Feed contaminated with AF+ 2 ml of RHF in drinking water) of the sampled liver were correlate with their liver morphological findings in restoring the picture to that of the control one [61] (54,55). Restoring liver color by using RHF in both groups of broilers (G3&G4) confirms the above mentioned brilliant properties of milk thistle as hepatoprotective remedy during Aflatoxicosis.

Taking into the consideration the medicinal value the thistle of milk contained in RHF, this research was performed to examine the efficiency of silymarin containing silibinin, silidianin, silichristin, and isosilibinin as hepatoprotective, antiinflammatory, cytoprotective, and anticarcinogenic effects on AFB1 residual level and liver histopathological changes in broiler chickens fed ration contaminated with AFB1.[62]

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