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Protective Effect of *Hypophae Rhamnoides* Oil Against Ochratoxicosis in Chickens

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Abstract. The aim of this study was evaluate the prophylactic of oil *Hypophae rhamnoides*, when included in a diet containing ochratoxins and fed to broiler chicks. The criteria of the evaluation included body weight gain, haematological profile and biochemistry, in addition to associated lesions in chicks. The biochemical analysis showed a considerable decrease in the serum alanine aminotransferase (ALT), increase of aspartate aminotransferase (AST), uric acid, cholesterol levels, a reduction in the serum total proteins, albumin and globulins. The addition of *H. rhamnoides oil*, diminished the adverse effects of ochratoxins. Chickens who received *H. rhamnoides* oil had a better body weight gain. Finally, it was concluded that effective in the amelioration of the toxic effects of aflatoxins that may be present in poultry rations. OTA residues from liver and kidney were significantly reduced in chickens treated with *H. rhamnoides* oil.

Keywords: Ochratoxine A, chickens, *Hypophae rhamnoides,* antioxidants

INTRODUCTION

Ochratoxin A (OTA) is a nephrotoxic, hepatotoxic and teratogenic mycotoxin produced by storage molds (mainly by species of *Aspergillus* and *Penicillium*) on a variety of commodities. Ochratoxins affect many species including humans, dogs, feeder pigs, dairy cattle, and chickens. The molecular mechanisms involved in OTA-induced nephrotoxicity (16), carcinogenesis (3, 15), teratogenic effects (25), immunosupression (5, 8) were studied. Data regarding OTA metabolism are controversial. Several metabolites have been characterized in vitro and/or in vivo, whereas other metabolites remain to be characterized. Several major mechanisms have been shown as involved in the toxicity of OTA: inhibition of protein synthesis, promotion of membrane peroxidation, disruption of calcium homeostasis, inhibition of mitochondrial respiration and DNA damage. The genotoxic status of OTA is still controversial because contradictory results were obtained in various microbial and mammalian tests, notably regarding the formation of DNA adducts. More recent studies are focused on the OTA ability to disturb cellular signalling and regulation, to modulate physiological signals and thereby to influence cells viability and proliferation (8, 9). Oxidative damage may be one of the manifestations of cellular damage in the toxicity of ochratoxin A. Reactive oxygen species (ROS) have a major role in the mediation of cell damage (12). Several defence mechanisms attempt to minimize the production and the action of harmful oxidants. Free radical damage initially induced by mycotoxins can be propagated and magnified by lipid peroxidation chain reactions (18).

Antioxidants are substances that can inhibit reactions of free radicals, such as reactive oxygen species (ROS) by neutralizing oxidants and thus reducing oxidative damage. Thus antioxidants aid in the overall detoxification process in the liver and in cells and thus, may aid

in alleviation of mycotoxicosis (19, 21). Reported that selenium which is an antioxidant enhances the formation of water soluble conjugated forms of ochratoxin A which promotes the clearance of the toxin and enhanced chick growth (20). *H. rhamnoides* contains different natural antioxidants such as: vitamin C, mineral elements, monosaccharides sugars, organic acids, free amino acids, carotenoids, vitamin E, volatile compounds, different flavonoids, quercetin, myricetin, kaempferol, fatty acids, triacylglrcerol (25).

The aim of the study was evaluate the antitoxic potential of *Hypophae rhamnoides* oil, when included in a diet containing ochratoxin A and fed to broiler chicks.

MATERIALS AND METHODS

The experiment used 100 Ross "307" broiler chickens, selected when 6 days old, with an average weight of 79.03± 0.73g, which after a period of one week of acclimatisation to living conditions provided, were randomly divided in 3 groups: experimental (E) and control (C). Chicks were reared on sawdust litter, were provided specific microclimate conditions for their age, with room temperature gradually decreasing. Commercial-type food, free of OTA was administered *ad libitum*. The experimental group received daily by gavage, ochratoxin A diluted in sterilized sunflower oil at a dose of 50 µg/kg b.w (E1), OTA and *Hypophae rhamnoides* oil (Hofigal, Ro.) as caps. x240 mg (E2) and *Hypophae rhamnoides* oil (E3). The control group received only diluent (sterilized sunflower oil). At the end of each week during the experiment, chicks were individually weighed. Five chickens were selected by random from each group and were killed on the 7th, 14th or 21st and 35 days of the experiment. Blood samples from each group were collected and centrifuged at 3000 rpm for 10 min. Separated sera were collected in Eppendorff tubes and kept at –20ºC until biochemical analysis. After chickens were killed the liver and kidney were removed and divided into two parts; the first part was kept at –20oC for biochemical measurements. The second part was kept at – 20ºC for extraction and quantificatation of ochratoxin residues and the last one was collected in 10% neutral buffered formalin solution (BFS) for histopathology. Serum samples were analized for serum total proteins and albumin, serum globulins, ALT and AST, acid uric.

Sample extraction and cleanup of organs. Residues of aflatoxin in kidney and liver samples of all groups before and after withdrawal of ochratoxin were determined by the method of ELISA and HPLC. Samples were performed at 7 and 21 days after onset of acute OTA intoxication; in 5 chicks from each experimental group the OTA levels were determinated. Organ samples were prepared for ELISA and HPLC analysis Aliquot was mixed and weighed in a PP-plastic tube with screw cap. It was added 0.5 mL phosphoric acid, then two times 3 mL ethyl acetate, shake for 30 seconds and centrifuge 1min/2000g at 20^{0}C. Transfer the supernatant (ethyl acetate) layer into a new plastic tube by decanting, after the second extraction add the supernatant on top of the first layer of ethyl acetate. To the combined layers of ethyl acetate is added 3 mL NaHCO (0.65 M), mix vigorously (vortex) and continue agitation on a horizontal shaker for 15 min. Centrifuge 5min/2000g. For samples analyzed by ELISA 1mL of aqueous phase is transferred into a glass tube and heated on a water bath at 100 ^{0} C for 3 min. Add 4 mL distilled water and diluted 1:1 with NaHCO (0.13 M). Use a 50 µl extract per well in the assay.

To measure ochratoxin levels by HPLC method, sample are prepared by the same mixing with an extraction solution, followed by blending and filtering. One mL from the same aqueous extract was loaded onto an immuno affinity column (LC Tech, 3 mL, polypropylene) which contains specific monoclonal antibodies for ochratoxin A in buffer solution. Using OACLEAN immuno affinity column, accuracy of results is ≥ 90% (minimum recovery), the

maximum capacity of the column being 150ng ochratoxin A. For removal of the impurities OACLEAN column is wash with 10 mL phosphate buffer solution (PBS) and 10 mL ultra pure water. At this stage OA binds to antibody on the column. Mycotoxins was eluted with 1 mL of methanol.

. *Toxin determination by ELISA method.* To determine levels of the residues of ochratoxin A (OA) was used a commercial kit RIDASCREEN OA (R-Biopharm, Darmstadt, Germany), which is based on competitive ELISA method for quantitative determination of OA (4). Insert the required number of wells for the standards and evidence in support. Is added to each well in 50 ml standard or sample (extracts). Add 50 ml enzyme conjugate with ochratoxin A in each well, mix by hand and incubated 30 minutes at room temperature (20-25º C). Discard liquid and add 250 m l buffer wash. Repeat washing step two times. Add 100 ml substrate / chromogen to each well. Mix by turning the plate and incubated 15min at room temperature. Add 100 ml stop solution to each well. Homogenize gently rotating the plate and measure its fluorescence intensity UV light at 450nm against the blank air.

Mycotoxins levels determination by high-performance chromatography (HPLC)

For the detection of residues of ochratoxin A (OA) of the liver and kidney samples by HPLC - FL improved method was used to detect OA in tissue samples, proposed by Bozzo et all. (2008), with a limit of detection 0.10-0,30 mg / kg and a recovery of 87 ± 13%. For analysis we used Shimadzu HPLC system equipped with modular type, quaternary pump solvent delivery model LC-2AD, semi Sample injection system Reodzne 7725 model fluorescence detector Shimadzu, model RF-AXL. We used a 120 Acclaine ® C18 column (15 mm, 150 mm x4, 6) with the mobile phase consisted of water: acetonitrile: acetic acid (49,5:49,5:1), degassed at flow rate of 0, 9mL/ min. For mycotoxins detection were used wavelengths of 333nm and 477nm, excitation and emission. After completion of the mandatory steps, involving chromatographic column packing was done to test the samples. External standards, pure mycotoxins solutions with known concentrations are initially injected to determine the standard curve.

Statistical analysis

Data analysis covered by this study was made on account of some basic statistical indicators such as the arithmetic mean ($\bar{X}$), standard deviation (σ_X) and coefficient of variation (V%). It also was intended to test the hypothesis that there are significant differences between the individuals of control and experimental lots for the values of body, various organs weight (liver and kidney) and biochemical blood parameters (alanine aminotransferase, aspartate aminotransferase total protein, albumin uric acid and cholesterol).

As checking this hypothesis was used Student t-statistics, calculated as follows:

$$t = \frac{\bar{X}_M - \bar{X}_E}{\sqrt{\frac{\sigma_M^2}{n1} + \frac{\sigma_E^2}{n2}}}$$

Where,

$\bar{X}_M, \bar{X}_E$ = average of the control sample, respectively experimental;

σ_M^2, σ_E^2 = undistorted estimator of the two samples variance;

n = sample size (number of individuals).

In the Student table, the theoretical value of this indicator has been identified for a probability of 95% ($p = 0.05$) and v degrees of freedom ($v = n1 + n2 - 2$). Given that the t - Student calculated was greater than the theoretical one, was accepted the hypothesis through which was guaranteed, with a probability of 95%, that between the values determined in the two groups of animals were significant differences.

RESULTS AND DISCUSSIONS

Evolution of chickens body weight during the experiment is presented in Table 1.

Relative weight of livers (reported to body weight) was significantly higher in E1group (table 2) than in all other groups and progressively increased, when in control group it decreased. In E2 group (treated with OTA and *H. Rhamnoides* oil), relative weight of liver progressively decreased, differences from E1 group being significant. The same tendency were observed when comparing relative weight of kidney between groups.

Tab. 1.

Chickens weight (g)

Age of chickens	Nr	Control group	E1 (OTA)	E2 (OTA and *H. rhamnoides* oil)	E3 (*H. rhamnoides* oil)
6	5	79,8±0,75	78,9±0,89	79,4±0,91	78,1±0,76
13	5	211,5±1,11	129,5±1,89	162,4±1,13	209,4±0,89
21	5	422,1±1,12	276,3±2,11	376,7±1,33	418,3±1,13
28	5	783,9±1,21	429,2±2,21	667,5±2,15	793,1±1,11
35	5	1 643,1±1,14	888,3±1,88	1 200,4±1,96	1632,9±1,21

Tab. 2.

Relative weight of liver and kidney.

Days of experiment	Control group	E1	E2	E3
Relative liver wheight				
7	3,02±0,33	3,80±0,27	3,30 ±0,21	3,04±0,20
14	3, 0±0,15	3,68±0,13	3,26 ±0,12	3,09±0,11
21	2,97±0,13	3,40±0,33	3,12±0,23	2,96±0,39
35	2,92±0,03	4,17±0,33	3,41±0,26	2,94±0,43
Relative kidney wheight				
7	0,71±0,01	0,79±0,12	0,77±0,01	0,75±0,02
14	0,69±0,11	0,84±0,05	0,76±0,04	0,72±0,12
21	0,65±0,01	0,88±0,04	0,74±0,01	0,68±0,02
35	0,63±0,02	0,97±0,11	0,80±0,03	0,65±0,01

The activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST) were increased, total protein and albumin concentrations decreased significantly together with the stage of liver steatosis. 7days after experimental poisoning significant decrease of total

blood proteins, albumins and increase of blood uric acid and cholesterol was noticed only in E3 group (Table 3)

Tab. 3.

Biochemical blood parameters

Parameter	ALT	AST	TP	ALB	glob	UA	CHOL
UM	UI/L	UI/L	g/dl	g/dl	g/dl	mg/dl	mg/dl
control	5,7,5±0,17	173,6±2,22	4,15±0.09	1,81±0.02	2,34±0.1	2,73±0.09	108,8±1.09
E1	3,16±0,15	216,6±1,35	2,30±0.11	1,06±0.01	1,24±0.2	5,94±0.06	149,9±0,12
E2	4,41±0,13	204,3±1,73	3,17±0.19	1,10±0.11	2,07±0.09	4,91±0.11	126,5±1,1
E3	5,58±0.09	169,4±2,95	3,46±0.22	1,89±0.19	1,57±0.2	3,82±0.1	94,5±0.09

Distribution of ochratoxin A (OA) in the organs examined by ELISA method was different, depending on the time of administration of the toxin, the dose administered. (Table 4)

Tab. 4.

Concentration of OA residues in kidney and liver ng/g (ppb)

Period of intoxication	E1		E2 p	
	kidney	liver	kidney	liver
7 days	3,531±2,31	2,451±1,12	1,554±1,41	1,45±1,01
21 days	2,805±2,11	1,180±1,81	1,446±11,3	1,156±2,15

The highest concentration of OTA residues was found in the kidneys, in chickens that has received only OTA for seven days after onset of experimental acute poisoning. The statistical interpretation of results, for tissue distribution of the mycotoxins, shows that the differences between the values obtained in this group are statistically insignificant, p> 0.05 in the first 7 days. After 21 days of experimental poisoning, OTA residues from kidney were significantly lower in E2 group than in E1 (1,446 *±11,3as 2,805±2,11*).

High Performance Liquid Chromatography (HPLC) has allowed to obtain information on the concentration of ochratoxin A residual samples from the liver and kidneys of chickens in group E1 who received only mycotoxins, so they were determined precisely and accurately average peak areas obtained by three successive injections. Concentrations were calculated for each sample using the calibration curve equation and the peak areas, taking into account the relative deviation calculated. (Table 5)

In the analysis by HPLC with fluorescent tissue, OTA was detected in both kidney and liver. (table 5). The highest concentration of OTA was found in kidney (0.496 to 1.465 ng / g, with an average of 0.749 ng / g), while the residual concentration of OTA in the liver ranged from 0.430 to 0.937 ng / g, an average of 0.298 ng / kg. In terms of tissue distribution of OA in both noble, the percentage of positive samples was 83.33% higher in the kidney, while only 33.33% of liver samples was detected mycotoxin of interest.

Comparing the results obtained by ELISA and HPLC (Table 6) we can see than HPLC gives higher values.

Tab.5.

OTA residues distribution assessed by HPLC

Sample	Peak area			Area average bit	OTA concentration (ng / g - ppb)
	I	II	III		
kidney					
5R	0.5571	0.6246	0.6387	0.6068	1.465
4R	0.2877	0.1738	0.2188	0.227	0.547
8R	0.2007	0.2319	0.1975	0.210	0.507
11R	0.2062	0.2058	0.204	0.205	0.496
19R	0.4673	0.3022	0.4329	0.401	0.968
22R	0.2789	0.2872	0.3089	0.292	0.704
26R	0.1916	0.2601	0.1667	0.206	0.498
28R	0.2754	0.3467	0.377	0.333	0.804
30R	0.1928	0.2135	0.2628	0.223	0.538
38R	0.445	0.3958	0.3597	0.400	0.966
The average concentration of OTA					0,749 $^{\pm 0,31}$
liver					
5F	0.2144	0.2382	0.2305	0.228	0.550
4R	0	0	0	0	0
8F	0	0	0	0	0
11F	0	0	0	0	0
19F	0.1711	0.1862	0.1765	0.178	0.430
22F	0	0	0	0	0
26F	0	0	0	0	0
28F	0.3707	0.3125	0.4811	0.388	0.937
30F	0	0	0	0	0
38F	0.1892	0.1964	0.2001	0.195	0.471
The average concentration of OTA					0,298 $^{\pm 0,23}$

Tab. 6.

Ochratoxin levels measured by ELISA and HPLC

no	Period of intoxication of chicks	OA level (ng/ml) ELISA		OA level (ng/ml) HPLC	
		kidney	liver	kidney	liver
	7 days	3,531±2,31	2,451±1,12	0,796±2,11	0,196±1,8
	21 days	2,805±2,11	1,180±1,81	0,702±1,28	0,281±1,31

A key element in part to be emphasized is that this expression graphically expresses the results clearly highlight the particular tropism for renal tissue of OA, reproduces a dynamic wave phenomenon. The graph is perhaps stronger argument which confirms the reproducibility of the phenomenon itself.

A variety of dietary treatments have been used for eliminating or reducing the toxic effects of OTA in animals, including the use of specific adsorbents to block mycotoxin in the digestive content and the use of antioxidant compounds (12). Hydrated sodium calcium

aluminosilicate (13, 20), activated charcoal, bentonite and cholestyramine, and esterified glucomannan (17) have been used in animal feeds to diminish the adverse effects of OTA.

In our experiment, chickens poisoned with OTA and treated with *H. rhamnoides* oil had a better body weight gain than group who receive only mycotoxin.Transaminases (ALT, AST), cholesterol and uric acid from blood were also higher. OTA residues from kidney and liver were significantly reduced in group treated with *H. rhamnoides* oil, suggesting a protective effect of this. Decreased body weight gain along with increased liver weights and kidney in birds fed OTA are consistent with earlier reports (2, 13, 24). Sea buckthorn has been shown to have a potent antioxidant activity, mainly attributed to its flavonoids and vitamin C content. Sea Buckthorn fruit has a high vitamin C content (695 mg per 100 grams) about 15 times greater than oranges placing sea-buckthorn fruit among the most enriched plant sources of vitamin C as well as vitamin E, carotenoids, lycopene; flavonoids and oils rich in essential fatty acids, Omega-3, 6, 7 and Omega 9 (7, 23, 25).

A clinical trial demonstrated that sea buckthorn extracts helped normalize liver enzymes, serum bile acids and immune system markers involved in liver inflammation and degeneration (10). In addition, sea buckthorn oil protects the liver from damaging effects of toxic chemicals, as revealed in laboratory studies. Recent studies have shown that sea buckthorn contains lots of vitamin A precursors including carotene (14) and unsaturated fatty acids (15). Zhao *et al.*(23) reported that sea buckthorn could protect the liver from damage induced by CCl_4 .

Some other substances, such as antioxidants, have also been evaluated to decrease OTA toxicity in several species. Abdel-Wahhap *et al.* (1) and Özçelik *et al.* (14) found that melatonin exhibits a preventive effect against OTA-induced oxidative stress and structural damage in the kidney through its role in the scavenging of free radicals and/or the prevention of lipid peroxidation. Grosse *et al.* (11) also demonstrated that the incorporation of alpha-tocopherol in the diet decreased by 58% the total DNA adduct provoked in kidney by a single administration of OTA in mouse and rat kidney. In other studies, addition of a plant extract (artichoke extract) or sesame seed to laying hen diets showed protection against the suppressive effect of OTA on egg production and the toxic effect of OTA on various internal organs (22).

The analysis by HPLC with fluorescent tissue, OA was detected in both kidney and liver are the order quantities sites ppb (ng / g). Residual concentration of OA in liver ranged from 0.430 to 0.937 ng / g, with an average of 0.298 ng / kg. The highest concentration of OTA was found in kidneys from 0.496 to 1.465 ng /g, with an average of 0.749 ng /g

These results and the lower content of OTA in the liver of birds fed the OTA + *Hypophae ramnoydes oil* diet appear to support the suggestion that *H. rhamnoides* oil may provide protection against the toxic effects of OTA. The inclusion of new antioxidant products in the diet, such as *Hypophae ramnoydes oil*, can significantly ameliorate many of its adverse effects.

CONCLUSIONS

- *H. rhamnoides* oil express the protective effect in chickens experimentally poisoned with ochratoxin A by significantly higher body weight gain and reduced relative weight of liver and kidney .
- Quantitative evaluation of ochratoxin A residues from liver and kidney shows louwer values in group receiving OTA and *H. rhamnoides* oil than in group receiving only

micotoxin, suggesting that natural antioxidants from *H. rhamnoides* oil play an active role in detoxification of mycotoxin.

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