RESIDUAL ANALYSIS OF CERTAIN MYCOTOXINS IN COLOURED BROILER CHICKEN TISSUES

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CERTIFICATE

This is to certify that the thesis entitled "***Residual analysis of certain mycotoxins in coloured broiler chicken tissues***" submitted by **Ms. JAYASHREE PATTAR.,** I. D. No. **DVHK 1137** in partial fulfillment of the requirements for the award of **DOCTOR OF PHILOSOPHY** in **VETERINARY PHARMACOLOGY AND TOXICOLOGY** of the Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar is a record of bonafide research work carried out by her during the period of her study in this University under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, associate ship, fellowship or other similar titles.

Bangalore,

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Dedicated To,

My Beloved Brother Shankar

My loving Uncle Virupaxi and

My Parents

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LIST OF ABBREVIATIONS

AFB 1	Aflatoxin B1
ADG	Average daily gain
ADI	Acceptable daily intake
ADFI	Average daily feed intake
@	At the rate of
AFB 2	Aflatoxin B2
AFG 1	Aflatoxin G1
AFG2	Aflatoxin G2
AFR0	Aflatoxicol
AICRP	All India Coordinated Research Project
ALT (SGPT)	Serum alanine transaminase
ALP	Serum alkaline phoshphatase
AST (SGOT)	Serum aspartate transaminase
BW/b.w	Body weight
°C	Degree Celsius
CAST	Council For Agricultural Science and Technology
CE	Collision Energy
CV	Co-efficient of variation
D.E	Diatomaceous earth
dL	Deci liter
DP	De-clustering potential
EU	European Union
EU MRL	European Union maximum residue limit

Fig.	Figure
F/G or FCR	Feed conversion ratio
FD	Fluorescence detector
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
g	Gram
GGT	Gamma glumyltranpeptidase
GC	Gas chromatography
GMP	Good manufacturing practice
h or hr	Hour
HPLC	High-performance liquid chromatography
HPTLC	High Performance Thin Layer Chromatography
HQC	High quality control
IAC	Immunoaffinity column
IARC	International Agency for Research on Cancer
IAEC	Institutional Animal Ethics Committee
ISTD	Internal standard
JECFA	Joint Expert Committee for Food Additives
kg	Kilogram
L	Liter
LC–MS/MS	Liquid chromatography–tandem mass spectrometry
LD_{50}	Lethal dose in 50 per cent of population
LOD	Limit of detection
LOQ	Limit of quantification
LLOQC	Lower limit of quality control

LQC	Lower quality control
MQC	Medium quality control
MS	Mass spectrometry
MRM	Multiple reaction monitoring
µg	Microgram
µm	Micrometers
mm	Millimeter
mg	Milligram
mM	Mill molar
nm	Nanometer
µl	Micro liter
ml	Milliliter
min	Minute
mm	Millimeter
MRL	Maximum Residue Level
MRLs	Minimal Risk Levels
m/z	Mass to charge ratio
ng	Nanogram
N	Normality
OTA	Ochratoxin A
PDA	Photodiode array
PDA	Potato Dextrose Agar
pH	-log[H^+]/ negative log of hydrogen ion concentration
%	Per cent
P	Probability

ppb	Parts per billion
ppm	Parts per million
ppt	Parts per trillion
PLE	Pressurized liquid extraction
QC	Quality control
RASFF	Rapid Alert System for Food and Feed
r^2	Regression coefficient
t_R or R_t	Retention time
RSD	Relative standard deviation
R_f	Retention factor
rpm	Resolution per minute
SEM	Standard Error Mean
SPE	Solid phase extraction
tBME	Methyl tertiary butyl ether
TP	Total protein
TLC	Thin layer chromatography
v/v	Volume by volume
UA	Uric Acid
ULOQ	Upper limit of quantification
UV-Vis	Ultraviolet visible
UHPLC	Ultra- performance liquid chromatography
USFDA	United States Food and Drug Administration
w/w	Weight by weight
w/v	Weight by volume

Introduction

I. INTRODUCTION

Awareness of detection of mycotoxins has increased mainly in the last 50 years since the discovery of aflatoxins in the 1960's. Although, they have accompanied mankind from the very beginning and were probably associated with several mysterious diseases known from history (Richard, 2007).

Mycotoxin occurrence in nature is worldwide global problem, now recognized as prevalently abiotic hazards produced as secondary metabolites by various fungi and excreted into their substrates. These substrates frequently contaminate most of the agricultural commodities under favorable environmental conditions. Mycotoxins are injurious to vertebrates upon ingestion, inhalation, or skin contact. The diseases they cause, known as mycotoxicoses, do not need to involve the toxin-producing fungus. Thus, they are abiotic hazards but with biotic origin (Marin *et al.*, 2013).

Mycotoxins have acute and chronic effects resulting in teratogenic, carcinogenic, and oestrogenic or immunesuppressive and other toxic effects on both animal and human health. Direct consequences of consumption of mycotoxin-contaminated animal feed include reduced body weight gain, feed intake, poor feed conversion, increased disease incidence (due to immune-suppression), reduced reproductive capacities, high morbidity and mortality (Bennett and Klich, 2003; Radmilam *et al.,* 2009; Marin *et al.,* 2013), which leads to severe economic losses and includes reduction of livestock production, agricultural production, healthcare, veterinary and regulatory costs (Peraica *et al.*, 1999; Schatzmayr *et al.,* 2006; Rubert *et al.*, 2010).

Mycotoxins have carry over effect, can appear in the food chain because of fungal infection of crops, either being consumed directly by humans or used as livestock feed. The metabolism of ingested mycotoxins could result in mycotoxin accumulation in different organs or tissues, entering into the food chain through meat, milk, eggs or their products (Shreeve *et al.,* 1979; McLean and Dutton, 1995; Beretta *et al.,* 2002; Radmilam *et al.,* 2009; Inger *et al.,* 2011; Marin *et al.,* 2013).

India's animal wealth is huge in terms of its population with 485 million livestock and 489 million poultry, India ranks first in global livestock population. Compared with the rest of the livestock sector, the poultry industry in India is one of the more scientific and fastest growing sectors. India is emerging as the world's 2nd largest poultry market with an annual growth of more than 14 per cent, producing 61 million tonnes or 3.6 percent of global egg production.

Apart from this, India ranks 6th in broiler production (125 billion Rupees) with an annual output of 2.39 million tonnes of broiler meat, as per the estimates of the Ministry of Agriculture, Govt. of India. The total poultry industry is valued at about 350 billion rupees. The per capita consumption per year is approximately 2.4 kg, which is much lower than the National Institute of Nutrition's recommendations of 11 kg.

The poultry industry is probably more severely affected than any of the livestock industries because poultry appear to be more susceptible to the effects of dietary mycotoxins and losses can be quite severe, hence there is need to protect from mycotoxicoses (Hamilton, 1971).

It is a common practice of feed mill owners, that damaged and moldy food grains rejected as unfit for human consumption are mixed in poultry and animal feed. Similarly, other cereal grains, oilseed cakes and their products, particularly corn and its byproducts contaminated with mycotoxins are mixed in poultry ration (Zahid *et al.*, 2008).

To date, around 400 different mycotoxins with toxic potentials already have been described (Kabak *et al.*, 2006). However, only few of them have distinct toxic effects, no region of the world escapes from the problem of mycotoxins. The Food and Agriculture Organization of the United Nations (FAO) estimated that approximately 25 % of the cereals produced in the world are contaminated by mycotoxin, leading to 5-10 % of commodities being discarded and there is consequently huge pressure to solve this issue (Rice and Ross, 1994).

Many international agencies are trying to achieve universal standardization of regulatory limits for mycotoxin. Currently, over 100 countries have regulations regarding mycotoxin in the feed industry, out of which 13 mycotoxin are of concern (Van Egmond *et al.*, 2007).

Mycotoxin presence is not only related to the effect they might have on consumer health, but may also have an impact on world trade. According to the annual report of the Rapid Alert System for Food and Feed (RASFF), in 2012 approximately 56 % of rejections at the European Union (EU) border were due to mycotoxins, which are ten times higher than any other hazard category. So this is the ‘number one’ issue in the safe transport of bulk raw commodities.

Despite being classified as categorically undesirable, their occurrence usually is not completely preventable even when using good manufacturing practice (GMP) and good agriculture practice (GAP). Even though, up to a certain level they might not pose any direct health concern to the consumer, they are still adding to the overall exposure. Today, mycotoxins are believed to play an important role in food safety and hygiene next to viral and bacterial agents (Inger *et al.,* 2011).

Also, different types of mycotoxins produced by a single or several fungal species may be present simultaneously in a commodity, which is of importance since exposure to mixtures of mycotoxins may have unexpected synergistic or additive toxic effects differing from those of an individual compound (Yiannikouris and Jonany, 2002).

The control measures to ensure mycotoxin-free food includes chemical analysis of these contaminants in a great variety of samples. Further complicated by the structural diversity of mycotoxins, which call for development of different analytical methods (Irena and Helena, 2009). The public health concerns resulting from the finding of mycotoxins and the observation of both acute and chronic effects in animals has prompted the research effort focusing on analytical methods development to analyze the mycotoxins residues in food animals and their products thereby to reduce the risk in animals and human.

Analysis of mycotoxin is essential to minimize the consumption of contaminated food and feed. Classical analytical methods for mycotoxins include thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC); gas chromatography (GC) and mass spectrometry (MS).

Aflatoxin B_1 (AFB_1) and Ochratoxin A (OTA) are important food borne mycotoxins having hepatotoxic and nephrotoxic effects, respectively in vertebrates including humans (Marin *et al.,* 2013).

Aflatoxin produced by the genus *Aspergillus* (*A. flavus, A. parasiticus, A. nominus*) (Sargeant *et al.,* 1961), classified as potent human carcinogen Group 1B and OTA produced by the genus *Aspergillus* (*A. ochraceus, A. sulphureus* and others) and *Penicillium,* especially *P. viridicatum* (Lai *et al.,* 1970), classified as a possible human carcinogen (Group 2B) (Peraica *et al.,* 1999; Marin *et al.,* 2013) by the International Agency for Research on Cancer (IARC, 1993).

Carry-over of AFB_1 and OTA from feed to animal products has been demonstrated in poultry, pig, fish, dog, ruminants as well as in other species including human (Shreeve *et al.,* 1979; Micco *et al.,* 1987; Peraica *et al.,* 1999; Marin *et al.,* 2013).

The hatching eggs of coloured broiler parental lines were obtained during 1994 from AICRP on poultry breeding for meat, Ludhiana centre and subjected to selection and acclimatization at Bangalore centre. These birds are named as RAJA-II (coloured broiler) obtained by crossing PB1 cock with the PB2 hen. These birds are ideal for small scale and backyard farming, resemble local birds and do have high adaptability, survivability and better carcass quality. There are no published reports on the effect of mycotoxicoses and tissue residues in these birds.

By considering above points, the present study was conducted to know the pattern of mycotoxicoses and residual effect with respect to age, dose and duration of exposure and mycotoxin interaction in coloured broiler chicken with the following objectives.

1. Experimental induction of mycotoxicosis in coloured broiler chicken with various dietary concentrations with a special emphasis to aflatoxin and ochratoxin.

2. To study the serum biochemical parameters and pathological changes.

3. To assess the residual analysis of mycotoxins in coloured broiler chicken tissues.

Review of Literature

II. REVIEW OF LITERATURE

2.1 Aflatoxin

Moldy feed toxicosis was recognized as a serious livestock problem in the 1950's but it was only in 1960 during the investigations in the United Kingdom of moldy feed toxicosis which was called Turkey "X" disease, that *A. flavus* and *A. parasiticus* were identified as the organisms responsible for the elaboration of the toxin in the feed. The earliest symptoms of the disease are lethargy and muscular weakness followed by death (Sargeant *et al.*, 1961).

In the poultry industry, AFB1 is called "the silent murderer" because its chronic consumption at levels below 20 ppb does not induce evident clinical symptoms; however, it reduces the absorption of food and causes immunosuppression. The final result was a low productivity, because birds show a low growth and low stance. Additionally, due to induced immunosuppression, birds were much more susceptible to opportunistic infectious agents and respond poorly to vaccination programs (AgroBioTek, 2009).

Aflatoxins persist to some extent in food even after the inactivation of the fungi by food processing methods, such as ultra high temperature products, due to their significant chemical stability (Park *et al.*, 1994; Yazdanpanah *et al.*, 2005). Aflatoxins are common in humid climatic conditions like those existing in Asian and African countries and certain parts of Australia. Factors that influence aflatoxin toxicity residue levels in animal species include: species and breeds of animals and poultry, levels and duration of exposure, nutrition and health of animals, age, sex and diseases, drugs and other mycotoxins (FDA, 1979).

IARC then combines the ratings for animal and human data giving an overall rating of group 1 (‘human carcinogen’-sufficient animal and human evidence); group 2A (‘probable human carcinogen’); group 2B (‘possible human carcinogen’); group 3 (‘inadequate information’); and group 4 (‘no evidence’) (Magan and Olsen, 2004).

2.1.1 Production

Aflatoxins are polyketide based potent liver carcinogenic, mutagenic and immunosuppressive compounds, primarily produced by food-borne fungi, mainly *Aspergillus* species such as *flavus, parasiticus, niger, nomius, pseudotamari* and *bombycids,* etc. *A. flavus* is common in agriculture and others encountered much less frequently (Ito *et al.*, 2001; Bennett and Klich, 2003; Strosnider *et al.*, 2006; Pildain *et al.*, 2008; Turner *et al.*, 2009).

These fungi are ubiquitous in the environment can colonize on variety of products such as corn, maize, oilseeds, spices, groundnuts and tree nuts, including animals products like cheese, panner etc., under favorable conditions produce aflatoxin in warm (30-35°C) temperature with moisture above 14 % and high-humidity (90-95%) conditions (Royes and Yanong, 2002).

The occurrence of aflatoxins in agricultural commodities depends on region, season and the conditions under which a particular crop is grown, harvested or stored (Margarita *et al.*, 2006). Crops grown under warm and moist weather in tropical or subtropical countries were more prone to aflatoxin contamination than those in temperate zones. Aflatoxin production was also stimulated by high zinc concentration, riboflavin in diet (Pattison *et al.*, 2008).

2.1.2 Physicochemical properties

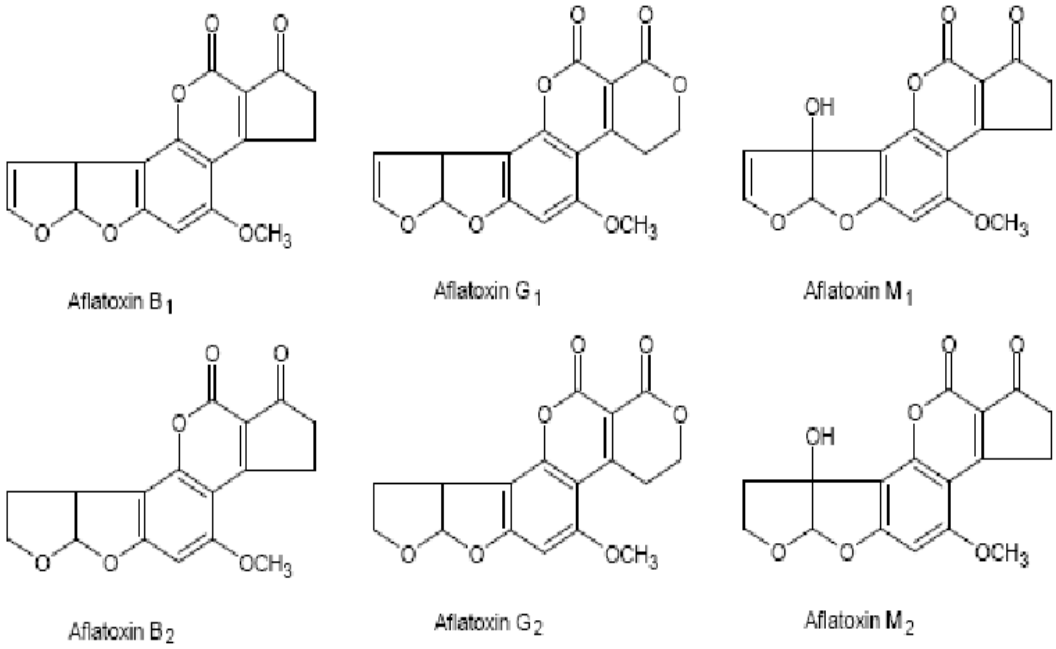
There are almost 20 different types of aflatoxins identified until now, the health issues related to aflatoxins are equally complex and demand more research. Chemically, aflatoxins are difurocoumarolactones (difurocoumarin derivatives) that fluoresce under ultraviolet light. Their structure consists of a bifuran ring fused to a coumarin nucleus with a pentenone ring in B and M aflatoxins or a six membered lactone ring in G aflatoxins.

Even though 20 different aflatoxins have been identified, only four aflatoxins have been detected as natural contaminants of feeds and feedstuffs. Depending upon color of the fluorescence, AFs are divided into aflatoxin B1 and B2 (AFB1, AFB2) for blue, and G1 and G2 (AFG1, AFG2) for green (Sargeant *et al.,* 1961; Hartley *et al.,* 1963). Aflatoxin M1 and M2 (AFM1, AFM2), known as milk-AF's, were isolated from urine and milk and identified as mammalian metabolites of AFB1 and AFB2 respectively (Carnaghan *et al.,* 1963; Patterson *et al.,* 1978). The structure of AFB1 resembles closely with the structure of pyrrozolidone which has got basic carcinogenic activity and most acutely toxic to various species compared to other aflatoxins and other metabolites of AFB1 were $B2_a$, aflatoxicol, aflatoxicol H_1 and aflatoxins P_1 and Q_1 have been identified (FDA, 1979).

The molecular formula of AFB1, AFG1 was established as $C_{17}H_{12}0_6$ and $C_{17}H_{12}0_7$ with molecular weight 312 and 328 respectively. AFB2 and AFG2 were found to be the dihydro derivatives of the parent compounds with molecular formula $C_{17}H_{14}0_6$ and $C_{17}H_{14}0_7$ having molecular weight 314 and 330 respectively (Hartley *et al.,* 1963).

Aflatoxins very slightly soluble in water (10-30 μg/mL), insoluble in non-polar solvents, freely soluble in moderately polar organic solvents (e.g., chloroform and methanol) and especially in dimethyl sulfoxide. Unstable to ultraviolet light in the presence of oxygen, to extremes of pH (< 3, > 10) and to oxidizing agents, melting point of AFB_1 is 268-269°C. The lactone ring is susceptible to alkaline hydrolysis. Aflatoxins were also degraded by reaction with ammonia or sodium hypochlorite.

Chemical structures of aflatoxin B1, B2, G1, G2, M1 and M2 as follows:



2.1.3 Mechanism of action

Liver is the primary site of metabolism for AFB1 and where the metabolites binds to nucleic acids and proteins. Kidneys also take part in detoxification of aflatoxins and are also among the organs where most of the aflatoxin residues are detected (Sawhney *et al.*, 1973; Fernandez *et al.*, 1994). CytochromeP450 enzymes (CYP) (including CYP1A2,

CYP3A4 and CYP2A6) in the liver and other tissues convert AFB_1 to epoxides (AFB1-8,9-exo-epoxide, and AFB1-8,9-endo-epoxide), and to AFM1, AFP1, AFQ1, and its reduced form aflatoxicol.

Of the epoxides, the AFB1-8,9-exo-epoxide (not the AFB1-8,9-endo-epoxide) can form covalent bonds with DNA and serum albumin resulting in AFB1-N7-guanine and lysine adducts, respectively. Like AFB1, AFM1 can also be activated to form AFM1-8,9-epoxide that binds to DNA resulting in AFM1-N7-guanine adducts. These guanine and lysine adducts have been noted to appear in urine. The metabolites AFP1, AFQ1, and aflatoxicol are thought to be inactive and are excreted as such in urine, or in the form of glucuronyl conjugates from bile in feces.

In case of chicken exposed to AFB1 contaminated rations, AFB1, AFM1, and aflatoxicol have been detected in liver, kidneys, and thigh muscles (Micco *et al.,* 1988; Fernandez *et al.,* 1994). Besides these, AFB2a has also been detected in livers of both broilers and layers on a ration contaminated with a mixture of aflatoxins (AFB1 80 %; AFB2 2.6 %; AFG1 16.8 %; and AFG2 0.1 %) (Fernandez *et al.,* 1994). Recent studies have shown that CYP2A6 and to a lesser extent CYP1A1 are responsible for bioactivation of AFB1 into epoxide form in the liver of chicken and quail (Diaz *et al.,* 2010).

2.1.4 Aflatoxicosis in poultry

Susceptibility of chickens to toxic effects of AFB1 varies with several factors such as breed, strain, age, general health, immune status , as well as environmental factors and nutritive status, gender, dose and duration of toxin intake and also the

capacity of liver microsomal enzymes to detoxify AFB_1 (Edds, 1973; Veltmann, 1984; Binder, 2007). A wide variation in LD_{50} values has been obtained in animal species tested with single doses of aflatoxins. The susceptibility ranges from ducklings > turkey poults > goslings > pheasant chicks > chickens (Muller *et al.,* 1970). For most species, the LD_{50} value ranges from 0.5 to 10 mg/kg body weight. LD_{50} of a day old duckling is 0.3 mg/kg BW, in chicken embryo 0.025 mg/kg, in chicken vary from 2-6.3 mg/kg.

2.1.4.1 Growth performance

Aflatoxin levels above 0.5 mg/kg diet decreased food intake and food conversion and retarded growth in Australian broiler chickens. The severity of the response and the ability of the birds to recover was dose related and dependent on the strain and sex of the chicken, males being more susceptible (Bryden *et al.,* 1980).

It was estimated that with each mg/kg increase of aflatoxin in the diet the growth rate would be depressed by 5 % when dietary concentrations containing 1 mg/kg of aflatoxin in broilers (Dersjant *et al.*, 2003).

Afzal and Saleem (2004) observed that feeding of broilers with aflatoxin (170 ppb) contaminated feed for seven weeks reduced the body weight gain and FCR, also mortality (52 %) noticed. Addition of mycotoxin detoxifier Mycotox® (5 kg/ton) improved negative effect of aflatoxin on body weight gain and FCR, even reduced mortality.

Bintvihok and Kositcharoenkul (2006) have reported that addition of calcium propionate (0.25 percent + 0.50 percent) to a diet containing low levels of aflatoxin B_1

(50 ppb and 100 ppb) for six week improved the broiler performance (body weight gain, feed consumption and feed conversion ratio) in comparison to aflatoxin treated and control groups.

Zahid *et al.* (2008) investigated that when broiler chicks of 7 days of age were offered feed containing 0, 1600, 3200 and 6400 µg/ kg aflatoxin B1 for 7 days where significant decrease in body weight was observed in all groups which were in a dose related manner and it was more severe in group fed 6400 µg/kg AFB1 in feed, also mortality was high (0 %, 10 %, 20 % and 36.25 % respectively).

It has been reported that broilers exposed to aflatoxin B_1 (1 mg/kg) containing AflaDetox (1, 2 and 5 g/kg) as toxin binder reduced the feed intake, body weight gain, and caused poor feed conversion rate as compared to AFB_1 group, also reduced the toxic effects of AFB_1 on the relative weights of liver. However reaching values not significantly different from the control diet and no effects when supplemented to the uncontaminated diets (Denli *et al.*, 2009)

Magnoli *et al*. (2011) studied the effect of aflatoxin B_1 (50 µg/kg) on broiler productivity (body weight gain, feed consumption, and feed conversion ratio) in the presence of sodium bentonite (Na-B ,0.3 %) and monensin (MON, 100 mg/kg) where birds exposed to dietary treatments for 28 day (day 18 to 46) did not show significant differences among treatments.

A significant reduction in feed intake, poor performance reported by Yang *et al.* (2012) who conducted an experiment where broilers fed daily with corn naturally contaminated with AFB_1 and AFB_2 (134 and 23.6 μg/kg respectively).

A significant decrease in average daily weight gain and increased feed requirements, with a gain ratio increasing from day 8 to 42 were noticed in an experiment where broilers fed with a moldy peanut meal naturally contaminated with AFB_1 @ 330 μg/kg (Fan *et al.,* 2013).

Feeding of ducks with maize naturally contaminated AFB_1 (196.8 μg/kg) diets for 35 days decreased ADG and ADFI linearly and quadratically during the whole experiment, whereas over all F/G was reduced linearly. The mortality of ducks was linearly and quadratically increased (He *et al.,* 2013).

2.1.4.2 Serum biochemistry

Aflatoxins damage the liver thereby reduces synthesis of serum proteins mainly decrease the level of albumin. Hypoalbuminaemia noticed in chronic hepatic diseases condition, the level of globulins decreases, but not as much as the albumin because not all the globulins are formed in the liver, such as gamma-globulins (Fernandez *et al.,* 1995).

George *et al.* (2007) reported decrease in levels of serum total protein, albumin and increase in levels of ALT and AST by feeding AF at the dietary levels of 50, 150 and 300 ppb in broilers from 0 to 42 days of age.

Mohamed and Mohamed (2009) reported significant increase in serum ALT, AST, in male broiler chicks fed with 4 mg aflatoxins/kg diet than the negative control group fed with standard basal diet from 0-21 days.

Metabolic alterations caused by aflatoxins in chickens result in elevated lipid levels, disruptions in hepatic protein synthesis, which result in several blood coagulation disorders, immunosuppression and decreased plasma amino acid concentrations (Sumit *et al.,* 2010).

Significant increased in enzyme activity (AST, ALT and GGT) were observed in the aflatoxin B_1 (50 ppb and 100 ppb) treated groups, whereas the aflatoxin B1-calcium propionate 0.25 percent + 0.50 percent) supplemented diet groups were slightly decreased, in comparison to control values. Treatment with calcium propionate effectively reduced increased in hepatic enzyme activities in broiler fed with diet containing low levels of aflatoxin B_1 (50 ppb and 100 ppb) for six week (Bintvihok and Kositcharoenkul, 2006).

Denli *et al.* (2009) observed a significant interaction between AFB_1 (1mg/kg) and AflaDetox (1, 2 and 5 g/kg) on serum biochemistry. A significant increase in ALP, AST activity and the concentration of UA, decrease in serum TP and ALB observed in AFB1 treated group compared with control one, were counteracted by AflaDetox addition, whereas no effects of AflaDetox supplementation were observed on uncontaminated diets.

It had been reported that biochemical parameters (total protein, ALB, GLOB, SGOT and SGPT) of birds exposed to aflatoxin B_1 (50 μg/kg), sodium bentonite (Na-B, 0.3%) and monensin (MON, 100 mg/kg) as alone and combined dietary treatments for 28 day (day 18 to 46) did not show significant differences among treatments (Magnoli *et al*. 2011).

ALT, AST, and LDH increased linearly, but activity of ALT showed quadratic response while the levels of TC and TG reduced linearly in ducks which were fed with maize naturally contaminated AFB_1 (196.8 μg/kg) diets for 35 days (He *et al.,* 2013).

2.1.4.3 Gross and histopathology

Hepatic damage is manifested by enlarged and putty-colored liver, petechial hemorrhages, marked vacuolation of hepatic cells and bile duct proliferation. Feed levels of AFB1 as low as 250-500 ppb given to New Hampshire chickens have been reported to result in liver damage, decreased hemoglobin, and hypoproteinemia (Brown and Abrams, 1965).

Experimental trials with naturally contaminated feed containing aflatoxin levels ranging from 1-1.5 ppm have caused growth retardation in chickens. Mortality was low but marked hepatic damage was manifested by enlarged and hemorrhagic liver (Carnaghan *et al.,* 1966).

Relative liver weight and liver lipid concentration were increased and significant changes occurred in liver fatty acid composition at dietary level of AFB1 0.5mg/kg.

Mortality from a single dose of AFB1 occurred within 72 h with an approximate LD_{50} of 15-18 mg AFB1/kg BW for the more susceptible male chickens (Bryden *et al.,* 1980).

Lesions depend on the age of the host and the dose of toxin ingested and can include: hepatosteatosis or fatty liver, kidney hypertrophy, splenomegaly, atrophy of the thymus, testes and bursa of Fabricius. Bruising associated with an increase of capillary fragility and haemorrhagic points (petechial haemorrhages) on the surface of muscles of the leg and breast have been described in aflatoxicosis of broiler chickens (Biro *et al.,* 2002).

Zahid *et al.* (2008) noticed pallor discoloration of liver and enlargement of liver and kidneys, hemorrhages in different organs of the body in broilers (7 day old) which were fed with aflatoxin B1 (6400µg/kg) for 7 days. Microscopically congestion of liver parenchyma, cytoplasmic vaculation/fatty change of hepatocytes, necrosis of hepatocytes, newly formed bile ducts, mononuclear and hetrophilic cell infiltrations were observed. Kidneys were enlarged and microscopically reveal degeneration and necrosis of tubular epithelial cells, congestion and hemorrhages of the parenchyma.

Acute toxicity of aflatoxins in chickens may be characterized by liver necrosis with icterus, distended gallbladder and bile ducts, white pin head sized lesions and paleness in liver. Histopathologically liver damage is manifested as vacuolation of hepatic cells and bile duct proliferation (Sumit *et al.,* 2010).

In aflatoxicosis, a thickened basement membrane in the glomeruli and associated hyaline droplets in the renal tubules were noted. It was not known if the glomerulus was

damaged by toxins or by a leakage of unusual protein from a severely damaged liver. Liver lesions in chicken were characterized by retrogressive and regenerative parenchymal changes (Herenda and Franco, 1996).

Denli *et al.* (2009) reported that there was significant damage in the liver tissues of broilers receiving AFB_1 (1 mg/kg) alone had vacuolar degeneration of hepatocytes, perilobular inflammation (mainly mononuclear cells), bile duct hyperplasia, and hypertrophy compared with uncontaminated diet birds. AflaDetox supplementation (1, 2 and 5 g/kg) to AFB_1 diets significantly avoided these lesions to values not significantly different from the control.

Magnoli *et al*. (2011) reported that when birds exposed to dietary treatments for 28 day (day 18 to 46) no macroscopic change in liver of AFB_1 (50 μg/kg) in presence of sodium bentonite (Na-B, 0.3 %) treated groups and is similar to control diet group, thus Na-B had protective effect on aflatoxicosis. Whereas in presence of monensin (MON, 100 mg/kg) showed slightly clear coloration compared with those of control birds, thus had potential toxic effect on liver. The relative weights of livers, did not show significant differences among treatments.

It had been reported that microscopically, fat vacuoles and hepatocellular necrosis with perilobular location observed in AFB_1 (50 μg/kg) alone. Hepatocytes with lesions of degrees 1 and 2 (i.e., hydropic degeneration and fat microvacuoles) were observed in AFB_1 + Na-B + MON (50 μg + 0.3% +100 mg/kg)) treated group. Liver of AFB_1 + MON showed degree 2 lesions (*i.e* fat vacuoles and hepatocellular necrosis). Livers from broilers consuming the AFB_1+ Na-B diet showed only moderate hydropic degeneration.

Lesions of degree 3 (*i.e* severe) were not observed in any of the treatments (Magnoli *et al*., 2011).

A significant decrease in the ratio of villus height to crypt depth in the duodenum with increasing content of AFB_1 and AFB_2 (134 and 23.6 μg/kg respectively) contaminated corn in the broiler ration reported by Yang *et al.* (2012).

Hepatic portal area around the bile duct cells had different degrees of hyperplasia, a mass or cords, and a lumen formation, and liver cells had different degrees of swelling degeneration was noticed in ducks fed with maize naturally contaminated AFB_1 (196.8 μg/kg) diets for 35 days (He *et al.,* 2013).

Several researchers reported that AFB_1 caused hepatic architecture enlargement, fatty degeneration, bile duct hyperplasia, periportal fibrosis, hepatocytic vacuolation and necrosis (Teleb *et al.,* 2004; Ortatatli *et al.,* 2005; Ellakany *et al.,* 2011)

2.1.5 Aflatoxicosis in other animals

2.1.5.1 Ruminants

Most of ruminants less susceptible to aflatoxicosis than monogastric and poultry. In ruminants, AFs have a wide variety of effects, including weight loss, poor performance, reduced milk production, decreased fertility, abortion, hepatotoxicosis, and immunosuppression (Robens and Richard, 1992; Diekman and Green, 1992; Barton *et al.,* 2000).

Aflatoxin ingested in the feed by cattle is physically bound to ruminal contents, and as little as 2-5% reaches the intestine. Levels of AFB1 in excess of 100 µg/kg of feed are considered to be poisonous for cattle (Radostits *et al.,* 2000). The effects of aflatoxin fed to cattle depend on the level of aflatoxin in the ration, the length of feeding period and the age of animal (Jones *et al.*, 1994).

Young sheep are more susceptible to aflatoxicosis than adult sheep (Fernandez *et al.,* 2000). AF adversely affected spermatologic, biochemical, and testis parameters in ram when exposed to 250 µg/day of total AF and the combined administration of esterified glucomannan (2 g/day) with AF reversibly eliminated these adverse effects in rams (Mehmet *et al.*, 2014). LD_{50} value in sheep is 5 mg/kg BW.

2.1.5.2 Horse

The existing information on aflatoxicosis in the horse is inconclusive, although a total dietary concentration of 500-1000 µg/kg has been shown to induce clinical changes and liver damage, depending on the duration of exposure (Meerdink, 2002). The target organ in horses, as in all affected animals, is the liver, where the toxin induces centrilobular necrosis (Stoloff and Trucksess, 1979).

Horses suffering from aflatoxicosis exhibit non-specific clinical signs, such as inappetence, depression, fever, tremor, ataxia and cough (Larsson *et al.*, 2003).Necropsy findings include yellow-brown liver with centrilobular necrosis, icterus, haemorrhage, tracheal exudates and brown urine (Angsubhakorn *et al.,* 1981; Cysewski *et al.,* 1982; Bortell *et al.*, 1983; Vesonder *et al.*, 1991).

2.1.5.3 Canine

Canine aflatoxicosis was first reported in 1952 by Seibold and Bailey who described a liver disease called hepatitis "X" which was observed in dogs fed moldy contaminated feed. Dogs and cats are extremely sensitive to aflatoxins. The LD_{50} of AFB1 in dogs is 0.5-1.5 mg/kg and in cats is 0.3-0.6 mg/kg, vomiting, increased water consumption, polyuria, polydipsia, jaundice and elevation of serum liver enzymes in acute aflatoxicosis in dogs and cats (Rumbeiha, 2001).

2.1.5.4 Swine

Young swine are extremely sensitive to aflatoxins but susceptibility decreased with age (Diekman and Green, 1992). The toxicity of aflatoxin is both-dose related and time related and age is an important factor in susceptibility (Lawlor and Lynch, 2001). Sows and boars normally tolerate levels > 0.5 ppm in the feed for short periods but, when fed for extended periods, contamination levels in the feed should not exceed 0.1 ppm (Blaney and Williams, 1991). The LD_{50} in young pig dosage was determined to be 0.8 mg/kg (Jones and Jones, 1978).

The clinical syndrome in pigs include rough coat, depression, anorexia, decreased feed conversion, decreased rate of gain, weight loss, muscular weakness and shivering, tremors, bloody rectal discharge and icterus (Sisk *et al.,* 1968; Jones and Jones, 1978; Hoerr and D'Andrea, 1983; Radostits *et al.,* 2000). Aflatoxins also suppress the immune system and thus make pigs more susceptible to bacterial viral or parasitic diseases (Diekman *et al.,* 1992).

2.1.5.5 Fish

Fish have been found susceptible to aflatoxin and trichothecenes. Aflatoxicosis is most prevalent among fishes. The extent of lesions caused by consumption of aflatoxins depends upon the age and species of the fish. Fry are more susceptible to aflatoxicosis than adults and some species of fish are more sensitive to aflatoxins than others (Royes and Yanong, 2002).

Rainbow trout are the most sensitive species to aflatoxin. Feeding trout diets containing less than 1 ppb will cause liver tumors in 20 months (Horn *et al.,* 1989). Diet containing AFB1 at 0.4 ppb for 15 months had a 14 % chance of developing tumors. Feeding trout a diet containing 20 ppb for 8 months resulted in 58% occurrence of liver tumors and continued feeding for 12 months resulted in 83 % incidence of tumors (Royes and Yanong, 2002).

Deaths quickly occur in 50% of stock if dietary levels of 500 to 1000 ppb are consumed. Warm water fishes such as channel catfish (*Ictalurs punctatus*) are much less sensitive than rainbow trout, and the level required to cause 50% mortality is approximately 30 times that of rainbow trout (Horn *et al.,* 1989). Channel catfish fed a diet containing purified AFB1 at 10000 ppb for 10 weeks exhibited decreased growth rate and moderate internal lesions (Royes and Yanong, 2002).

Initial findings associated with aflatoxicosis in fishes include pale gills, impaired blood clotting, poor growth rates or lack of weight gain. Prolonged feeding of low concentrations of AFB1 causes liver tumors, which appear as pale yellow lesions and

which can spread to the kidney. Increased in mortality may be observed (Royes and Yanong, 2002).

2.1.6 Aflatoxin residues and binders

2.1.6.1 Poultry

Arulmozhi *et al.* (2002) reported that broiler chicks exposed to aflatoxin (100 ppb) for six weeks, aflatoxin residues detected on 45 th day in liver, kidney and muscle by HPLC (6.6, 3.2 and 5.1 ng/g respectively)

Domestic fowls fed with aflatoxin B_1 (3 ppm) for seven days. Where residues of aflatoxin B_1 and its metabolite were detected in liver (0.15 ppb), muscle (not detected) of domestic fowls, samples were purified with a Sep-Pak florosil and quantified by HPLC (Bintvihok *et al.*, 2002)

Laying Japanese quail were fed with low levels of aflatoxin B_1 (25, 50 and 100 µg/kg) for 90 days and residues in eggs were detected by HPLC at levels that ranged from 0.01 to 0.08 µg/kg (AFB_1), 0.03-0.37 µg/kg (AFM_1), 0.01-1.03 µg/kg (AFB_{2a}) and 0.01-0.03 µg/kg (AFL). Results indicate that the excretion of aflatoxin residues in quail eggs might occur at relatively low concentrations under conditions of long-term exposure of quail to low levels of AFB_1 (Oliveiray *et al.*, 2003)

Bintvihok and Kositcharoenkul (2006) determined the effect of dietary calcium propionate (0.25 percent+0.50 percent) on aflatoxin residues and its metabolite in broilers fed with low levels of aflatoxin B_1 (50 ppb and 100 ppb) for six weeks. Samples were cleaned-up with sep-pak florisil cartridge and quantified by HPLC-FD (AFB_1, AFB_2,

AFG_1 and AFG_2) with normal phase column, for the determination of AFM_1 and aflatoxicol, HPLC was performed with a reverse phase column. The residual levels of aflatoxin B_1 (0.13 and 0.02 ppb) and aflatoxin M_1 (0.32 and 0.08 ppb) in respective tissues of liver and muscle of AFB_1 treated (100 ppb) were significantly higher as compare to aflatoxin B_1-calcium propionate supplemented groups AFM1 (0.05 and 0.03 ppb), AFB_1 not detected. AFB_1 and AFM_1 detected in liver tissue (0.05 and 0.10 ppb respectively) of AFB_1 50 ppb levels. However, AFB_2, AFG_1, AFG_2 and aflatoxicol residues were not observed in the tissues and results of this study indicate that calcium propionate effectively reduce the AFB_1 toxicity.

Denli *et al.* (2009) reported that the effect of AflaDetox (1, 2 and 5 g/kg of feed) on aflatoxin residues in broilers exposed to aflatoxin B_1 feed (1mg/kg) in liver and breast muscle was detected by HPLC- FD after derivatization with bromine in the Kobra cell by using immunoaffinity column. The AFB_1 residues not detected in the breast muscle of all treatments (LOD: 0.05 μg/kg). Detectable amount of AFB_1 (0.166 μg/kg) was found in the liver of the chickens fed the AFB_1 alone in diet. The results showed that AFB_1 transmission ratio from the feed: liver was approximately 6,000:1.

Magnoli *et al*. (2011) detected and quantified the AFB_1 residues by HPLC-MS/MS method in liver of birds exposed to aflatoxin B_1 (50 μg/kg), sodium bentonite (Na-B, 0.3 %) and monensin (MON, 100 mg/kg) as alone and combined dietary treatments for 28 day (day 18 to 46). Liver tissue extracted by SPE Oasis cartridges, LOQ and LOD were 0.025 and 0.0025 ng/g, respectively, percentage of recovery of AFB_1 was 99 ± 13 %. AFB_1 residues in livers ranged from 0.2 to 1 ng/g and were significantly

higher ($P < 0.05$) in the livers of broilers fed diets containing the combination of AFB1, Na-B, and MON.

The aflatoxins in the tissues were extracted using immune-affinity columns (Aflatest®WB) and estimated by a HPLC-FD method with pre-column derivatization by trifluroacetic acid. Broiler chicks of 7, 14 and 28 days of age exposed to different dietary levels of AFB_1 (1600, 3200 and 6400 µg/kg) for 7 days. Maximum AFB_1 residue (6.97 and 3.27 ng/g in liver and muscle respectively) concentration was high in young age birds those kept on high AFB_1 ration. After withdrawal of AF contaminated rations, residues clearance was slow and AFB_1 was detectable in liver and muscles of birds for longer duration in younger birds fed with high AFB_1 dietary levels (Zahid *et al.*, 2010)

Yang *et al.* (2012) conducted an experiment where broilers fed daily with corn naturally contaminated with AFB_1 and AFB_2 (134 and 23.6 µg/kg respectively) and aflatoxins extracted by AflaStar™ R immunoaffinity columns and determined by HPLC-MS/MS technique. AFB_1 residues in livers and breast muscles were at levels of 0.137 and 0.016 µg/kg, respectively. Also AFM_1 residue was detected in livers at a level of 0.051 µg/kg but it was not detectable in breast muscles.

Fan *et al.* (2013) conducted an experiment where broilers were fed with a moldy peanut meal naturally contaminated with aflatoxins (AFB_1 330 µg/kg) for 42 days and aflatoxin residues in liver and muscle samples were detected by HPLC-FD with post-column photochemical derivation as samples subjected to immunoaffinity columns, where AFB_1 in liver was 0.24 µg/kg, however no residues were observed in muscles.

2.1.6.2 Other food animals

Stubblefeild and Shotwell (1981) developed a method for determination of aflatoxin B_1 and M_1 in animal tissues and blood (beef, swine, chicken and human) which were artificially contaminated with aflatoxin (0.1 to 1 ng/g). 2-D TLC and densitometry was used for detection and recoveries of aflatoxin in meat and blood were 90 and 80 % respectively with detection limit ≤0.1 ng/g.

Gabrijela *et al*. (2007) validated the procedure for the determination of aflatoxin B_1 in animal liver (pig, chicken, turkey, beef, calf) by an HPLC method with fluorescence detection after derivatization with bromine in the Kobra cell. The limit of detection (LOD) and limit of quantification (LOQ) were 2 ng/kg and 7.8 ng/kg respectively. The repeatability of measurements, represented by the standard deviation (RSDr) was 7.5 %, 7.1 %, and 4.8 % at the contamination levels of 0.025 µg/kg, 0.050 µg /kg and 0.075 µg /kg respectively

Meat (poultry, lamb, goat and beef), eggs, milk and feed samples from different local markets in Jordan were screened for determination of aflatoxins and their metabolites by HPLC with both UV and fluorescent detector, showed higher levels of accuracy (92 to 109 %) and lower detection limits (0.05 µg/kg) when using SPE-CN or IAC both (Herzallah, 2009).

Deng *et al.* (2010) reported that long term exposure of Tilapia fish with different levels of AFB_1 (19, 85, 245, 638, 793 and 1641 µg/kg) for 20 weeks, resulted the aflatoxicosis in a dose- and duration-dependent manner, also aflatoxin residues detected only in liver but not in edible flesh.

Meat samples from Nigeria market were screened for various aflatoxins by Olufunmilayo and Akeeb (2011). Aflatoxins B_1, B_2, G_1 and G_2 found positive which were detected and quantified by thin layer chromatography (TLC), an exceptionally high aflatoxin concentration was found in all the kidney samples.

2.1.7 Aflatoxin on human health

Humans can be exposed to aflatoxins by the periodic consumption of contaminated food, commodities and feed stuffs, and relay poisoning from aflatoxins in milk contributing to an increase in nutritional deficiencies, immunosuppresion and hepatocellular carcinoma. Thus aflatoxins play an important role in public health (Coppock and Christian, 2007; Wagacha and Muthomi, 2008).

Even though heavily contaminated food supplies are not permitted in the market place in developed countries, concern still remains for the possible adverse effects resulting from long-term exposure to low levels of aflatoxins in the food supply. Conditions increasing the likelihood of acute aflatoxicosis in humans include limited availability of food, environmental conditions that favor fungal development in crops and commodities, and lack of regulatory systems for aflatoxin monitoring and control.

Evidence of acute aflatoxicosis in humans has been reported from many parts of the world, namely the Third World Countries, like Taiwan, Ouganda, India, and many others. Aflatoxicosis evidenced by the severe outbreak in Kenya in 2004 (Probst *et al.,* 2007). The syndrome is characterized by vomiting, abdominal pain, pulmonary edema,

convulsions, coma, and death with cerebral edema and fatty involvement of the liver, kidneys, and heart (Strosnider *et al.,* 2006).

Human susceptible to liver cancer when exposed to low levels of these important mycotoxins for long-term. In 1988, the IARC placed aflatoxin B_1 on the list of human carcinogens. This is supported by a number of epidemiological studies done (Van Rensburg *et al.,* 1985; Groopman *et al.,* 1988) in Asia and Africa that have demonstrated a positive association between dietary aflatoxins and liver cell cancer (LCC). Additionally, the expression of aflatoxin-related diseases in humans may be influenced by factors such as age, sex, nutritional status, and/or concurrent exposure to other causative agents such as viral hepatitis (HBV) or parasite infestation (Peers *et al.,* 1987; Yeh *et al.,* 1989).

There may be inhalation exposure to aflatoxin among industrial and agricultural workers. The presence of aflatoxin in dust from contaminated corn has been established (Burg and Shotwell 1984; Burg *et al.,* 1981; Silas *et al.,* 1987).

The median lethal dose of aflatoxin Bl 0.36 mg/kg BW was a special range of highly toxic poison (aflatoxin animal half of the lethal dose is found in the strongest carcinogens). Its carcinogenicity is 900 times more than dimethylnitrosamine induced liver cancer in the large capacity, 75 times higher than the 3,4-benzopyrene. AFB1 induces liver cancer in animals, can also induce cancer, renal cancer, colorectal cancer and breast, ovary, small intestine and other sites of cancer in human

Several investigators have suggested aflatoxin as an aetiological agent of Reye's syndrome in children in Thailand, New Zealand etc. Though there is no conclusive evidence as yet. Epidemiological studies have shown the involvement of aflatoxins in Kwashiorkor mainly in malnourished children. The diagnostic features of Kwashiorkor are edema, damage to liver etc. These out breaks of aflatoxicosis in man have been attributed to ingestion of contaminated food such as maize, groundnut etc. Hence it is very important to reduce the dietary intake of aflatoxins.

2.2 Ohratoxin

Ochratoxin A is a potent nephrotoxic mycotoxins, affected animals / birds suffer with renal damage and the syndrome is termed as mold nephrosis or mycotoxic nephropathy, which also has hepatotoxic, teratogenic, embryotoxic, genotoxic, neurotoxic, immunosuppressive, and carcinogenic (IARC Class 2B) effects (JECFA, 2001).Low quality commodities with visible mould growth or with levels of OTA unsuitable for entry to the human food chain may be directed into animal feeds. This raises concerns relating to animal health, including increased susceptibility to secondary infections and decreased performance of food producing animals.

Carry-over of OTA from feed to animal products like milk especially in liver, kidney and blood have been demonstrated in pigs and poultry, as well as in other species (Micco *et al.,* 1987: Inger *et al.,* 2011).This creates public health and veterinary risks and hazards (Rubert *et al.,* 2010).

OTA is a moderately stable molecule that remains unaltered during most processes of food storage and preparation (Schiavone *et al.,* 2008). Furthermore,

processing (e.g. heating and ripening) and storage do not alter OTA levels in meat products (Monaci *et al.*, 2005).

2.2.1 Production

Ochratoxin A (OTA) is a secondary metabolite, first isolated in 1965 from *Aspergillus ochraceus* (*Aspergillus allutaceus var. allutaceus)* (Van der Merwe *et al.*, 1965) These xerophilic fungi are adapted to grain with a moisture content of 9-16%, whereas their optimal temperature for OTA production is between 25 and 30 °C grows at moderate temperatures (Paterson and Lima, 2010) other producers are *Penicillum verrucosum, Penicillium nordicum* in cool temperate regions and *A. carbonarius* in high temperature climates (Joint FAO/WHO, 2001; CAST, 2003).

Some other fungal species, i.e. *A. alliaceus, A. auricomus, A. glaucus, A. melleus,* and *A. niger*, the genera *Petromyces* and *Neopetromyces* (Ciegler *et al.*, 1972; Abarca *et al.*, 1994; Bayman *et al.*, 2002; Guillamont *et al.*, 2005; Anli and Alkis, 2010) are less important OTA producers.

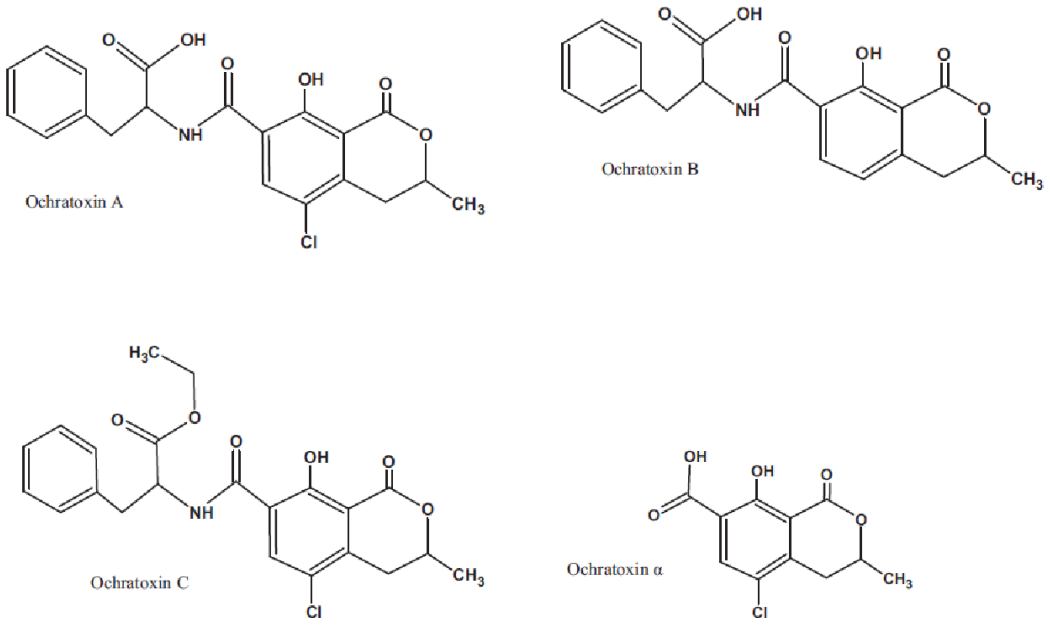
Contamination with OTA can occur in a wide range of raw food commodities including cereals, nuts, nut products, dried fruit, coffee, spices, beans, coffee, wine, beer and grape juice. OTA has been investigated in the following products: cereal and cereal-derived products, figs, grapes and wine. OTA have been recognized on the meat of animals in which consume contaminated feed (Jorgensen, 1998).

2.2.2 Physicochemical properties

Ocharotoxins are weak organic acids with a pKa value of 7.1 and a molar mass of 403.8 $g.mol^{-1}$, molecule posses an intense green fluorescence under UV light in acid medium and blue fluorescence in alkaline conditions (Keeper and Scott, 1989; Miller and Trenholm, 1994; Bredenkamp *et al.*, 1989; Budavari, 1989), consisting of derivative of a group of nine isocoumarin derivatives, polyketide mycotoxin coupled to the amino acid phenylalanine by an amide bond.

Except ochratoxin α (OTα), the ochratoxins comprise a polyketide-derived dihydroisocoumarin moiety linked via the 7-carboxy group to L-β-phenylalanine by an amide bond. The family of ochratoxin consists of four members OTA, OTB, OTC and OTα which differ slightly from each other in chemical structures. OTA ($C_{20}H_{18}O_6NC_l$) with melting point 169ºC is the most toxic member of the ochratoxin group and has the greatest toxicological significance. The particularity of OTA is due to its high stability. It has been shown that it possesses a resistance to acidity and high temperatures. Thus, once foodstuffs are contaminated, it is very difficult to totally remove this molecule.

The four forms differ in that Ochratoxin B (OTB) is a nonchlorinated form of ochratoxin A (OTA) and that ochratoxin C (OTC) is an ethyl ester form ochratoxin A, where the phenylalanine moiety is missing in ochratoxin α (OTα) (Bayman and Baker, 2006). In acid and neutral pH, OTA is highly soluble in polar organic solvents (alcohols, ketones, chloroform), soluble in aqueous sodium hydrogen carbonate, slightly soluble in water and insoluble in petroleum ethers and saturated hydrocarbons (Ringot *et al.*, 2006). Chemical structure of ochratoxins as follows:



2.2.3 Mechanism of action

Ocharotoxin A and ocharotoxin alpha inhibit mitochondrial respiration and oxidative phosphorylation and reduce ATP levels. It also interferes with the functioning of several mRNAs involved in the synthesis of various c-AMP mediated enzymes including phosphoenolopyruvate carboxykinase and other enzymes of gluconeogenesis pathway.

It being a potent nephrotoxin causes organellar damage, especially to tubular epithelial cells and impairs proximal tubular function. These decrease metabolic clearance and urine concentrating ability, inhibit anion transport and cause release of renal brush border enzyme e.g. leucine aminopeptidase. There is periglomerular and interstitial fibrosis, tubular atrophy, thickened basement membranes, glomerular sclerosis and fibrosis. Furthermore, it is considered that OTA represents a teratogenic agent for chickens, but not for other domestic animals. OTA has a genotoxic effect, which can be

explained by an indirect mechanism involving impaired protein synthesis (Bennett and Klich, 2003).

2.2.4 Ochratoxicosis in poultry

The poultry industry is also affected by OTA contamination. Turkeys, chickens and ducklings are susceptible to this toxin. Typical signs of poultry ochratoxicosis were reduction in weight gain, poor feed conversion, reduced egg production, poor egg shell quality and nephrotoxicity.

Outbreaks of clinical ochratoxicosis in food producing animals due to ingestion of contaminated feed have been reported in poultry, pigs and rabbits (Duarte *et al.,* 2011). In fish median lethal dose (LD_{50}) varied between 0.29 (oral) for marine sea bass (El-Sayed *et al.,* 2009) and 4.67 mg/kg of body weight (intraperitoneal) for rainbow trout (Doster *et al.,* 1972). Oral LD_{50} values were shown to be 0.5 mg/kg b.w. for duck, 1 mg/kg b.w. for pig (El-Sayed *et al.,* 2009), 3.3-3.9 mg/kg b.w. for cockerel (Peckham *et al.,* 1971), 5.9 mg/kg b.w. for turkeys, 16.5 mg/kg b.w. for Japanese quail (Prior *et al.,* 1976) and 10 mg/kg b.w. for rabbits (Mir *et al.,* 1999).

Plasma protein binding capacity of OTA has very high i.e up to 99%, the long half-life is sustained not only by the high affinity for proteins, but also by the enterohepatic circulation and biliary excretion which might be responsible for toxin accumulation and prolonged elimination from the body (Galthier, 1991; Roth *et al.,* 1988; Solti *et al.,* 1999) and enters in the food chain (Kuiper-Goodman and Scott, 1989).

2.2.4.1 Body performance

However, numerous studies showed that even exposure to low levels of OTA (0.5 mg/kg feed) altered performance, including decreased feed consumption and growth rate and poor feed conversion efficiency (Prior *et al.,* 1980; Wang *et al.,* 2009)

In the United States of America, Hamilton *et al.* (1982) described five independent episodes of ochratoxicosis in (970,000) turkeys, two episodes in (70,000) laying hens and two episodes in (12,000,000) broiler chickens. All three poultry species developed nephropathy, with turkeys presenting mortality up to 59 %, laying hens a reduced egg production and broiler chickens poor growth rate and feed conversion efficiency.

Abramson *et al.* (1983) noticed that increased mortality (3 times), reduction of weight gain, also pale livers and kidneys, necrotic enteritis were observed in chicks fed with naturally contaminated diet (140 µg/kg).

Elaroussi *et al.* (2008) conducted an experiment where one day old broiler chicks exposed to feed (0-800) µg/kg for 5 weeks. Results showed decreased in body weight, feed conversion ratio, reduced feed consumption and increased mortality.

A significant decreased growth rate, feed conversion ratio, reduced feed consumption, also reduced serum protein and albumin were observed in broiler chickens when exposed to artificially contaminated feed (1000-2000 µg/kg) for 28 days (Sakthivelan and Rao, 2010).

2.2.4.2 Serum Biochemistry

Krogh, (1976) reported that day old chicks fed with OTA contaminated diet (0.3 and 1 ppm) showed impairment in renal function and decreased total plasma protein concentration. OTA reduced the serum protein concentration significantly in broilers at 2 ppm level (Doerr *et al.*, 1982). Reduction in serum total proteins due to ochratoxicosis was reported in by Huff *et al.* (1988).

Ochratoxin A inhibits protein synthesis, produces acute proximal tubular epithelial necrosis in the kidneys and inhibits normal renal uric acid secretion. A decrease in the concentration of proteins, triglycerides, cholesterol, calcium, phosphorus and potassium is followed by an increase in the level of uric acid and creatinine and a decrease in glomerular filtration (Elaroussi *et al.*, 2008).

Sawale *et al.* (2009) reported significant rise in liver enzymes AST and ALT, but decrease in total serum protein (TSP) and albumin in ochratoxin group (1 ppm) in comparison to control group fed with basal diet.

OTA fed at various doses (1-5 mg/kg) to animals of various ages, altered their serum biochemistry, including decreases in cholesterol, total protein, albumin, globulin, potassium, and triglyceride levels, and increases in uric acid and creatine levels and in the activities of serum alkaline phosphatase (ALP) and gamma glutamine transpeptidase (GGT) (Denli *et al.*, 2008; Bailey *et al.*, 1989; Huff *et al.*, 1988; Gentles *et al.*, 1999).

2.2.4.3 Gross and Histopathology

Microscopic examinations reveal acute nephrosis, hepatic degeneration or focal necrosis, and enteritis. Suppression of hematopoiesis in the bone marrow and depletion of lymphoid elements from the spleen and bursa of Fabricius were seen in chicks fed with Sub lethal oral dose of 100 μg of ochratoxin A (Peckham *et al.*, 1971).

Tubular dilatation and hypertrophy, swelling of tubular epithelial cells, localized necrosis, and desquamation of the tubular basement membrane in kidney as signs of tubulonephrosis, also reduction in the number of lymphocytes in the pulpar region of the spleen were reported by Dwivedi and Burns (1984).

In addition to the renal lesions there is mild to moderate glycogen deposition in hepatocytes, mainly at the periphery of the liver lobes at higher levels of dietary OTA (4 and 8 ppm), resulting in yellow enlarged livers. There was also some mild decrease in bursal and thymic size consistent with immunosuppression (Herenda and Franco, 1996).

The enlargement of the liver and kidney in OTA intoxications is caused by the involvement of these organs in detoxification and elimination. Affected kidneys are white to tan, swollen, hard and may have white pinpoint urate crystals. If damage is extensive enough to cause renal failure, dehydration, hyperuricaemia and visceral urate deposition appears at kidney level. Pasty white urates are deposited on pericardial, perihepatic, peritoneal and articular surfaces. More commonly, birds survive in compensated renal failure and kidneys appear enlarged, fibrotic and pale (Biro *et al.*, 2002).

Glomerulonephrosis, tubulonephrosis, focal tubular epithelial cell proliferation and the multiplex adenoma-like proliferation of renal parenchyma are considered to be primarily related to the toxin, while focal intertubular infiltration of lymphocytes and histiocytes can also occur either primarily or secondly as reparation of tubulonephrosis or as a consequence of immune stimulation (Elaroussi *et al.*, 2008).

2.2.5 Ochratoxicosis in other animals

2.2.5.1 Ruminants

Ruminants have a higher tolerance to OTA exposure than monogastric animals because 85-90% of orally administered OTA was converted into phenylalanine and ochratoxin α (OTα) by rumen protozoa which hydrolyze the amidic 37 bond of OTA most of OTα excreted in urine which is generally considered to be non-toxic (Sreemannarayana *et al.,* 1988; Fink, 2008). As a consequence, the risk associated with consumption of animal-derived products is largely limited to monogastric species (Dall *et al.,* 2010).

2.2.5.2 Swine

In swine, the primary syndrome is called porcine nephropathy, which generally occurs after chronic ingestion of diets with concentrations of 0.2 to 4 ppm OTA. The effects on the kidneys are comparable to those seen in a variety of other species (e.g. poultry, rats, fish, and monkeys).

OTA has been linked with endemic nephropathies in livestock as well. OTA was implicated as a causal agent of mycotoxic porcine nephropathy (MPN) reported in

Denmark during the 1960-1970s (Krogh *et al.,* 1976). The high affinity of OTA for proteins, particularly serum albumin, contributes to a high serum half life and promotes bio-accumulation in the organs of animals, the half life of OTA in porcine serum is up to 20-30 times longer than that in poultry serum (Curtui and Gareis, 2001; Jimenez *et al.,* 2001; Schiavone *et al.,* 2008; Duarte *et al.,* 2011). This is reflected in the occurrence of OTA in several animal-derived products (Curtui *et al.,* 2001; Dall *et al.,* 2007). Serum half life of OTA is 72-120 h in pigs, 4.1h in chicken (Galtier *et al.,* 1981) and 77 h in pre ruminant calves (Sreemannarayana *et al.,* 1988)

Among farmed animals, pigs are known to be particularly sensitive to OTA. After absorption through the gastrointestinal tract, the highest concentrations of OTA in pigs are found in the blood, followed by the kidneys, urinary bladder, liver, spleen, muscle and fat (Curtui *et al.,* 2001; Curtui and Gareis, 2001; Chiavaro *et al.,* 2002; Ceci *et al.,* 2007)

2.2.5.3 Canine

Dogs showed a high susceptibility to OTA, for example, a daily dose of 0.2 mg OTA/kg BW for 2 weeks or a single dose of 7.8 mg OTA/kg BW was fatal to young beagle dogs (Szczech *et al.,* 1973). Clinical symptoms of the OTA poisoning included anorexia, weight loss, vomiting, tenesmus, bloody diarrhea, increased body temperature, tonsillitis, dehydration, and prostration. These findings were confirmed by a later study in which dogs showed similar symptoms at OTA doses between 0.2 and 3.0 mg/kg BW (Kitchen *et al.,* 1977).

2.2.5.4 Fish

Exposure of the developing eggs of the zebra fish resulted in a variety of severe abnormalities such as deformities of the head, tail and eyes. These embryos hatched but did not reach the larval stage.

Lovell (1992) reported that the oral LD_{50} for ochratoxin-A in six-month-old rainbow trout is 4.7 mg/Kg. Pathological signs are severe necrosis of liver and kidney tissues, pale kidney, light swollen livers and death.

On Nile tilapia ochratoxicosisA, Srour (2004) showed that increasing OTA levels in the diet resulted in decreasing growth performance and feed utilization parameters.

2.2.6 Ochratoxin residues and binders

2.2.6.1 Poultry

Residue analyses after feeding chickens various diets have been performed by Krogh *et al.* (1976), Prior *et al.* (1980), Golinski *et al.* (1983), Niemiec *et al.* (1988) and Micco *et al.* (1987, 1988). Most residue analyses have been performed after feeding with more than 1000 μg ochratoxin per kg of feedingstuff. In only two studies (Krogh *et al.*, 1976; Micco *et al.*, 1987) were feedingstuff levels near the maximum permitted level used. In these experiments, the residues in the liver were 2 and 11 μg/kg respectively and in muscles 2 μg/kg were indicated.

When almost all diets have contained a concentration of between 1000 and 2000 μg of ochratoxin per kg, the estimate for a feeding stuff concentration of 100 μg/kg becomes highly inaccurate and probably too low. A residue of 11 μg/kg in liver from

chickens having been fed 50 μg of ochratoxin per kg of feeding stuffs (Micco *et al*., 1987) also points in the same direction. A result of these estimates which is worth emphasizing is that the ochratoxin concentration in blood is clearly lower than that found in pigs. The highest levels were found in liver and kidney. Meat/muscle had relatively low levels.

Residues of OTA have been detected in the muscle of hens and chickens, and in eggs. In an experiment Denli *et al.* (2008) evaluated the toxic effects of OTA (OTA, 2 mg/kg of feed) in laying hen, OTA exposure promoted an increase in the content of OTA in the liver (15.1 μg/kg) as compared to control animals.

Toxicological investigation of 90 liver, kidney and gizzard samples originating from chicken farms located in the different agricultural areas of Serbia was conducted by Dragan *et al.* (2011) for preliminary evaluation of the incidence of OTA by HPLC-FL after liquid-liquid extraction procedure with levels ranging from 0.14 to 3.9 ng/g, 0.1 to 7.02 ng/g and 0.25 to 9.94 ng/g in liver, kidney and gizzard respectively. None of the tissue sample contained more than the maximum level (10 ng/g) recommended by the European commission. However, the daily intake of low OTA in meat unlikely to pose an adverse health risk to the consumers in respect to OTA toxicity.

It was demonstrated that after exposure of 28 week old laying hens for 30 days at the concentrations admitted by the current European legislation (100 μg/kg) and at concentrations 20-folds as much the European Legislation limit (2000 μg/kg), OTA was not detectable (HPLC-FD, OchraTest IAC) in the eggs, although a number of eggs were

found to have altered structure or conformation and/or pathological lesions (Bozzo *et al.,* 2011).

A liquid chromatography tandem mass spectrometry (LC-MS/MS) with a pressurized liquid extraction (PLE) was developed for the simultaneous determination of aflatoxins B1, B2, G1, G2, M1 and M2, and ochratoxin A in animal derived foods (muscle, liver, kidney, fat, skin, eggs and dairy milk).The limits of detection defined as CCα varied from 0.07 µg/kg to 0.59 µg /kg.The recoveries of spiked samples from 0.25 µg /kg to 1 µg /kg ranged from 68.3 to 105.7 % with the relative standard deviations of less than 17.6% (Dongmei *et al.*, 2012).

Broilers were fed with two levels (500 and 1000 ppb) of ochratoxin A (OTA) in the presence or absence of a toxin deactivator ((1 and 2 kg/ton of feed containing a mycotoxin deactivating yeast *Trichosporon mycotoxinivorans*) for 42 days. The highest OTA levels were detected in serum > kidneys > liver of OTA treated groups without supplementation of toxin deactivator at day 42 of experiment, while the residues were significantly ($P < 0.01$) lower in treatment groups supplemented with toxin deactivator at 2 kg/ton of feed (Nafeesa *et al.*,2012).

2.2.6.2 Other food animals

Tissue distribution in pigs, rats, chickens and goats generally follows the order kidney > liver > muscle > fat (Harwig et *al.*, 1983), or in some recent studies kidney > muscle > liver > fat (Mortensen *et al.*, 1983; Madsen *et al.*, 1982).

In Denmark, the level of OTA in pork has been indirectly controlled by visual examination of kidneys from slaughtered pigs for macroscopic changes (Jorgensen and Petersen, 2002). If there are changes, the kidneys will be analyzed. A condemnation of the entire carcass when OTA reaches 25 μg/kg in pig kidney is considered to ensure that the amounts of OTA in meat will not exceed 10 μg/kg. This is based on the estimation that the OTA content in pig meat is approximately 40 % of the level in pig kidney (Buchmann and Hald, 1985).

Pig liver and pig kidney are rejected when the amount of OTA ranges between 10 to 25 μg/kg in kidney. In 1998 (after a very wet period during harvesting in 1987), 13.5% of the kidneys having OTA content above 25 μg/kg have induced whole carcass condemnation. In 1995, less than 1% of these organs contained more than 25 μg/kg. Year-to-year variations may reflect differences in the harvesting and climate conditions between years, but also changes of the storage conditions of the foodstuff.

In a recent study in Serbia, Milicevic *et al.* (2008) presented data on the occurrence of OTA contamination in liver and kidney samples from slaughtered pigs (N = 90). Of the 90 pigs, 26.6% of the liver samples contained OTA in the range of 0.22-14.5 μg/kg (mean 0.63 μg/kg), and 33% of the kidney samples in the range of 0.17-52.5 μg/kg (mean, 1.26 μg/kg). OTA has also been detected in meat and offal from pigs in France and Italy (Dragacci *et al.*, 1999; Matrella *et al.*, 2006). Jimenez *et al.* (2001) evaluated the occurrence of OTA in 38 pig derived pâtés. Three of them contained OTA above the detection limit of 0.56 μg/kg. The highest concentration (1.77 μg/kg) was found in a homemade pate.

Ceci *et al*. (2007) estimated the ochratoxin A residues in samples of kidney, urinary bladder, intestine, stomach, liver, lymph nodes and muscles obtained from 5 swine fed with OTA-contaminated feed and evidenced microscopical lesions exclusively in the kidneys and in the urinary bladder with highest concentrations of OTA were observed by HPLC-FLD analysis, 23.9-27.5 μg/kg and 9.8–11.5 μg/kg, respectively.

For milk, little information is available on the rate of transfer of this toxin into milk for dairy cows. In dairy sheep, the carryover is less than 1 %. Low concentrations (10-58 ng/L) have been found in Swedish and Norwegian milk (Breitholtz *et al.,* 1993; Skaug, 1999) but not in German milk (Valenta and Goll, 1996). Ochratoxin from bovine milk may, however, be an important part in the total intake of ochratoxin by human high milk consumers such as children (Olsen *et al.,* 1993).

In a survey conducted in the northwest of France in 2003, Boudra *et al.* (2007) evaluated the presence of aflatoxin M1 (AFM1) and OTA in raw bulk milk (N = 132) from farms based on corn silage and cereal grains grown within the own farm, which represent the maximum risk of mycotoxin contamination. OTA was detected in three milk samples at low levels, 5-8 ng/L, at which they do not appear to pose any particular risk to milk consumers. The concentration of OTA in human or other non-ruminant mammal's milk may be higher than ruminants (Muller *et al.,* 1998).

2.2.7 Ochratoxin on human health

Human exposure can occur through consumption of contaminated food products, particularly contaminated grain and pork products, as well as coffee, wine grapes, and dried grapes.The toxin has been found in the tissues and organs of animals, including

human blood and breast milk (Clark and Snedeker, 2006). Ochratoxin A, like most toxic substances, has large species- and sex-specific toxicological differences (O'Brien and Dietrich, 2005).

OTA was classified as a possible human carcinogen within Group 2B (IARC, 1993). The health hazardous of ochratoxin A (OTA) in human includes hepatotoxic, carcinogenic, teratogenic, genotoxic, nephrotoxic and immunosuppressive. In addition, epidemiological studies have provided evidence of a correlation between high OTA levels in blood and foods, and the development of Balkan Endemic Nephropathy and urinary tract tumors (Toscani *et al.,* 2007: Anli and Alkis, 2010) in human (BEN), a chronic renal disease causing progressive fibrosis and impaired function, where extensive contamination with OTA is described (Petkova-Bocharova *et al.,* 1988 and Vrabcheva *et al.,* 2004).

The suggested molecular target for carcinogenic activity of OTA is histone acetyltransferases (HATs). These enzymes are critical in the regulation of a diverse range of cellular processes, including gene expression, DNA damage repair and mitosis through posttranslational acetylation of histone and nonhistone proteins (Czakai *et al.,* 2011; Mally, 2012).The excretion of OTA in human colostrum (Kovacs *et al.,* 1995) indicates exposure of neonates, who are generally considered as a high-risk group. OTA has a long serum half-life of 35.5 days in humans (Studer-Roher *et al.*, 2000).

3.1 Diatomaceous earth as mycotoxins binder

The use of adsorbent materials is a very common method employed to prevent the mycotoxicoses. These compounds are added to the feed to bind the toxin during digestive

process in gastrointestinal tract results in reduction of toxin bioavailability. Adsorption of mycotoxin requires suitable polarity and suitable position of functional groups. Due to this fact only very few mycotoxins adsorb efficiently without affecting essential feed ingredient.

Diatomaceous earth also known as D.E., diatomite, or kieselgur/kieselguhr, is a naturally occurring, soft, siliceous sedimentary rock that is easily crumbled into a fine white to off-white powder. It has a particle size ranging from less than 3 μm to more than 1 mm, but typically 10 to 200 μm. Diatomaceous earth consists of fossilized remains of diatoms, a type of hard-shelled algae. It is used as a filtration aid, mild abrasive in products including toothpaste, mechanical insecticide, absorbent for liquids, matting agent for coatings, reinforcing filler in plastics and rubber, anti-block in plastic films, porous support for chemical catalysts, cat litter, activator in blood clotting studies, a stabilizing component of dynamite, and a thermal insulator.

Natural freshwater diatomaceous earth or "Food Grade Diatomaceous Earth" is used as an anti-caking agent, as well as an insecticide in agriculture for grain storage and is approved by the US department of agriculture as a feed supplement to prevent caking. It is also used as a natural anthelmintic (dewormer) and anti-caking agent in livestock and poultry feed (Lartigue *et al.,* 2004). Diatomaceous earth has shown the potential *in vitro* to bind aflatoxin, sterigmatocystin, T-2 toxin, zearalenone and ochratoxin (Natour and Yousef, 1998).

OcraTox is an additive resulting from the modification and activation of diatomaceous earth, which is a natural material extracted from a quarry with a maximum

of 70% silicon dioxide, has been tested as a detoxifying agent of OTA in 28 layer hens for 5 weeks, where OTA level and OcraTox level in feed was 2mg/kg and 5g/kg respectively (Denli *et al.,* 2008). Addition of OcraTox to the contaminated diet alleviated the negative effects resulting from OTA, reaching values not significantly different from the control diet for most of the parameters except the relative weight of the liver, showing that OcraTox counteracted the deleterious effects caused by OTA.

4.1 Mycotoxins legislations

4.1.1 Aflatoxins

The U.S. Food and Drug Administration has regulated and enforced limits on concentrations of mycotoxins in foods and feed industries since 1985. In order to reduce the toxic and economic impact of mycotoxins, many countries established regulations and legislative limits of 20ppb was set for total aflatoxins for all food and feedstuff including poultry feed and human food (CAST, 2003; FAO, 1995)**.** However with the exception of milk which has a maximum permissible level of 0.5 ppb for aflatoxin M1. The maximum legal limit allowed for AFB_1 in infant food in the European Union is 0.1 μg/kg (EC, 2006). EU recommended the minimal risk levels (MRLs) of AFM1, AFB1 and aflatoxins to be 0.05, 2 and 4 μg/kg, respectively. In lactating cows and more sensitive young and weak animals, the permissible concentration in combined feed products (for suckling calves, suckling pigs and first stage broiler chicks) is 10 ppb.

Similar to some EU Member States, country India also established a general maximum limit of 30 ppb for total aflatoxins in foods for which no specific maximum limits was set. One of the strictest controls is in Japan where the total aflatoxins level in

all foodstuffs must be blow 10 µg/kg. In South Africa, a general maximum limit for total aflatoxins is set at 10 µg/kg and additionally a general maximum limit for aflatoxin B1 is set at 5 µg/kg for all foodstuffs.

4.1.2 Ochratoxin

Although there has been no advisory or regulatory level for ochratoxin issued by the FDA, many agree that levels between 10-20 ppb for commodities destined for human or animal consumption may cause health problems and economic losses. Some foreign markets have set regulation limits ranging from 5 to 50 ppb.

The toxicology and human health risks of OTA have been assessed at both European and international levels by the European Commission Scientific Committee on Food (SCF) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA), who have established tolerable intakes of OTA from food (JECFA, 2001). In 1998, the SCF noted that tolerable daily intake (TDI) estimates derived by other bodies ranged from 1.2-14 ng/kg body weight. However, due to concerns about the potential genotoxicity of OTA, the SCF recommended that exposures should be kept towards the lower end of the range (e.g. below 5 ng/kg body weight). In 2001, JECFA established a provisional tolerable weekly intake (PTWI) of 100 ng/kg bodyweight.

EU regulatory limit established at 2 ng/g. In India, a maximum limit for OTA is established for the same foodstuffs as in Codex, however the limit was set at 20 µg/kg. No specific limits for OTA in foodstuffs are set in USA, Canada, Australia and New Zealand, Japan, Mexico and South Africa. As for the OTA, the MRLs range from 0.5 to 25 µg/kg in foods and 5 to 300 µg/kg in feeds (FAO, 1997).

Materials and Methods

III. MATERIALS AND METHODS

The present experiment was carried out in the poultry house of Veterinary College, Bangalore, Karnataka Veterinary, Animal and Fisheries Sciences University (KVAFSU), Bidar, after necessary approval from the Institutional Animal Ethics Committee (IAEC), Veterinary College, Hebbal, Bangalore, India, to study the residual analysis of certain mycotoxins in coloured broiler chicken tissues.

3.1 Mycotoxin production

3.1.1 Source

Aspergillus flavus (MTCC 2798) and *Aspergillus ochraceus* (MTCC 10276) cultures were obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh. Aflatoxin B_1 and Ochratoxin A used in the present study was produced in the Mycotoxicology Laboratory of the Department of Veterinary Pharmacology and Toxicology, Veterinary College, Hebbal, Bangalore.

3.1.2 Aflatoxin production

Cultures of *Aspergillus flavus* (MTCC 2798) inoculated on Potato Dextrose Agar (PDA) slants/ plates and incubated at 28^{o}C for 7 days. Aflatoxin B_1 was produced by the inoculation of these cultures on polished rice as described by Shotwell *et al.* (1966). Fermentation was carried out in 500 ml Erlenmeyer flasks containing 50 g of rice, to which 25 ml of distilled water was added, and the mixture was allowed to stand for 2 hrs with frequent shaking. The flasks were tightly plugged with non absorbable cotton and

autoclaved at 121^{o}C at 15 psi for 15 min and cooled at room temperature. They were then inoculated with 3ml spore suspension in a sterile environment, placed on an orbital shaker at 200 rpm and incubated at 28°C. At 24 and 48 hr, sterile water (3-5 ml) was added in the flask, quantity of water adjusted in a manner that individual kernels do not adhere with each other.

If the rice did pack in clumps, the material was loosened by vigorous shaking, and if required, clumps were smashed with the help of a sterile rod within sterile environment to make sure that individual kernel should be kept free from others. On day 7-8 the flasks were again autoclaved at 121^{o}C at 15 psi for 15 min, and placed in a hot air oven at 60^{o}C for 24 hr till all the moisture was removed. The aflatoxin containing rice kernels were grinded to powdered form and stored in a dark place.

3.1.3 Ochratoxin production

Cultures of *Aspergillus ochraceus* (MTCC 10276) inoculated on Potato Dextrose Agar (PDA) slants/ plates and incubated at 28^{o}C for 7 days. Ochratoxin A was produced by the inoculation of these cultures on polished wheat using this fungal culture as suggested by Trenk *et al.* (1971). Fermentation was carried out in 500 ml Erlenmeyer flasks containing 50 g of wheat, to which 25 ml of distilled water was added, and the mixture was allowed to stand for overnight. Then autoclaved at 121^{o}C at 15psi for 15 min, cooled at room temperature and inoculated with 3 ml fungal spore suspension.

The flasks were incubated for 7-14 days at room temperature in dark place with vigorous shaking, quantity of water adjusted in a manner that individual kernels do not adhere with each other. On seventh day, the flasks were autoclaved at 121°c (15 psi) for

15 min. The fermented wheat was collected and dried in a hot air oven at 60°C for 24 hr till all the moisture was removed. The OTA containing wheat kernels were grinded to powdered form and stored in a dark place.

3.2 Mycotoxins quantification in feed and culture

The feed and cultural material was analyzed for the presence of aflatoxins (B_1, B_2, G_1 and G_2), ochratoxin, trichothecenes, fumonisins, by LC-MS/MS method in the Department of Veterinary Pharmacology and Toxicology, Veterinary College, Hebbal, Bangalore.

3.2.1 Method development

3.2.1.1 Preparation of standard solutions

The mycotoxin standards mainly aflatoxins (B_1, B_2, G_1 and G_2), ochratoxin A, HT-2 toxin, were used (Sigma Aldrich Chemicals). T-2 toxin, fumonisin B1 and fumonisin B2 were also used (Ermentek, Israeil). Stock solutions of all mycotoxins (1 mg/ml) were prepared in methanol, stored at −20ºC in amber glass vials, and brought to room temperature (22-25ºC) before use. A series of working standard solutions of AFB1, AFB1, AFG1, AFG2, OTA (1-100 ng/ml), T2, HT2 (0.5-50 ng/ml) and FB1, FB2 (1-200ng/ml) were prepared by suitable dilution of stocks.

These solutions were kept at 4°C and renewed weekly. They were used to calibrate the LC detector response and recovery studies. Acetonitrile, water and methanol were of HPLC grade purchased from E. Merck, India. Formic acid and methyl tertiary butyl ether (tBME) were of the highest analytical grade. Glassware used for standards or

samples soaked in 5% aqueous sodium hypochlorite to destroy mycotoxins residue before cleaning and re-use.

3.2.1.2 Calibration / Linearity of aqueous standards

For the determination of linearity, standard calibration curves containing at least nine points (non zero standards) were plotted and analyzed in triplicate.

	AB1	AB2	AG1	AG2	OCA	T2	HT-2	FB1	FB2
STD-1	1	1	1	1	1	0.5	0.5	1	1
STD-2	2	2	2	2	2	1	1	2	2
STD-3	5	5	5	5	5	2.5	2.5	10	10
STD-4	10	10	10	10	10	5	5	20	20
STD-5	20	20	20	20	20	10	10	40	40
STD-6	40	40	40	40	40	20	20	80	80
STD-7	60	60	60	60	60	30	30	120	120
STD-8	80	80	80	80	80	40	40	160	160
STD-9	100	100	100	100	100	50	50	200	200
LQC	3	3	3	3	3	1.5	1.5	3	3
MQC	50	50	50	50	50	25	25	100	100
HQC	90	90	90	90	90	45	45	180	180

3.2.1.3 Extraction and clean up

A new simple robust method was developed for multimycotoxin analysis in feed. 250 mg of feed sample was weighed and 900 µl of HPLC grade water was added and vortex by using cyclomixer at 80 motor speed to mix well for 10 min. To the above mixture add 500 µl of 1% v/v formic acid in water and the tube was subsequently capped and vortex for about 10 min briefly to mix the content of tube. Then 400 µl of tBME was

added to the above mixture and vortex for 10 min and the contents were centrifuged (Eppendorf Centrifuge 5810R, Germany; fixed angle rotor) at 4000 rpm for 10 min.

2 ml of supernatant of above mixture was collected in a tube, 100 µl of internal standard working solution (Meloxicam-70 µg/ml) was added and vortex to mix well. Samples were evaporated to dryness in a gentle stream of nitrogen under water bath maintained at 70°C. The residue left was reconstituted with 400 µl of mobile phase, and then transferred to autosampler HPLC vials and a 20 µl of the extract was injected into LC- MS/MS system equipped with reverse phase C18 column (Thermo Scientific BDS Hypersil C18 RP, 100x4.6 mm, 5 µm) with a flow rate of 700 µl. Mobile phase consisted of acetonitrile, 0.1% formic acid water, v/v (80:20).

3.3 Mycotoxins residual analysis study

3.3.1 Experimental birds and feed

The study was conducted in coloured broilers which were developed by Department of Poultry Science, Veterinary College, KVAFSU, Bangalore, India and named as RAJA-II obtained by crossing PB1 cock with the PB2 hen. Day old unsexed coloured broiler chicks were procured from Department of Poultry Science, Veterinary College, KVAFSU, Bangalore, India.

Necessary approval from the Institutional Animal Ethics Committee (IAEC), Veterinary College, Hebbal, Bangalore, India was obtained (No. LPM/IAEC/127/2012) before conducting the present experiment work.

Poultry feed was procured from Department of Poultry Science, Veterinary College, KVAFSU, Hebbal, Bangalore. The basal control diet was formulated and compounded to meet the nutritional requirements of commercial broilers based on the recommendations of Bureau of Indian Standards (BIS, 1992) during the starter and finisher period was presented in Table 1. The feed sample was tested for the presence of mycotoxins level before conducting the experiment, level of mycotoxins in feed was corrected and desired level of contamination of experimental feed with AFB1 and OTA was maintained by mixing the appropriate quantity of contaminated rice and wheat culture. Diatomaceous earth was used as mycotoxins binder for aflatoxin and ochratoxin in the present experiment and it was purchased from Sigma Aldrich, USA with purity of $\geq$ 95%.

Table 1: Composition and nutrient value of the experimental diets

Feed ingredients (in kg)	**Starter (1-21 day)**	**Finisher (21-42 day)**
Yellow maize	300	300
Soyabean meal	185	62
Mineral mixture	15	18
DORB	-	120
Feed additives (kg/500kg)	**Starter (1-21 day)**	**Finisher (21-42 day)**
DL-methionine	0.900	0.300
Salt	2.0	2.0
Vit AB_2D_3K	0.125	0.125
Vitamin B complex	0.100	0.100
Cygro	0.250	0.375
ALBMD	0.150	0.150
Liv- 52	0.500	0.500

3.3.2 Experimental design

Chicks were reared under deep litter system and fed a commercial rearing diet for first two days to allow them to adapt to their surroundings. On day third broiler chickens were individually weighed (BW of 48 ± 0.4 g), wing banded and randomly assigned to 10 experimental/treatment groups (Group 2-11) and the negative control group (Group1) each group consist of 66 birds with four replicates of 16-17 birds each and exposed to dietary treatment for six weeks. All broilers were placed in wire-bottomed aluminum cages and housed in an environmentally controlled house equipped with central heatings (Plate 1E).

Table 2: Different experimental groups and their treatment.

Groups	Aflatoxin B1 (ppm)	Ochratoxin A (ppm)	Diatomaceous earth (g/kg)
Group 1	-	-	-
Group 2	-	-	0.5
Group 3	-	-	1
Group 4	0.5	-	-
Group 5	1	-	-
Group 6	-	0.5	-
Group 7	-	1	-
Group 8	0.5	0.5	-
Group 9	1	1	-
Group 10	0.5	0.5	0.5
Group 11	1	1	1

The temperature was maintained at 30°C for the first week, and then was gradually decreased to 21°C until the broilers had reached 24 days of age and maintained

thereafter. The relative humidity ranged from 65 to 70 %. Broilers received 24 h of incandescent lighting for the first three days and 23 h of light and 1 h of darkness from four days of age onward. Ventilation was controlled by negative pressure using fans. The chicks were offered feed and water *ad libitum* through separate feeders and waterer throughout the experimental period. Vaccination of broiler chicks for New Castle disease and Infectious Bursal Disease was carried out on seventh and 14th day respectively. Daily birds were observed for any clinical signs of toxicity, behavioral changes, morbidity and mortality were recorded. Weekly body weight (BW), feed intake, and feed: gain ratio (F: G or FCR) were also recorded.

3.3.3 Sample collection

Birds were sacrificed at weekly intervals, six birds from each group were selected randomly and sacrificed by cervical dislocation (exsanguinations), blood samples were collected for serum biochemical analysis, and then plucked and manually eviscerated to obtain breast and leg muscles. Birds were subjected to detailed postmortem examination for any pathological lesions. Organs were collected for histopathological study. For mycotoxins residual analysis liver, kidney, breast muscle and leg muscle samples were collected in clean plastic container separately from each bird and stored at -20°C until analysis.

3.3.4 Body weight and feed conversion ratio (FCR)

Body weight of representative birds of each group was recorded at the weekly (seven days) interval up to the end of the experimental study. Feed consumption was

recorded at the weekly intervals and feed conversion ratio was calculated using following formula.

$$\text{Feed conversation ratio} = \frac{\text{Average feed consumption per bird during the week (g)}}{\text{Average weight gain per bird during the week (g)}}$$

3.3.5 Serum biochemical analysis

Serum biochemical parameters were estimated from the serum samples collected on day 7, 14, 21, 28, 35 and 42 during the study period using clinical biochemical analyzer - Microlab 300 (Vitalab Scientific, The Netherlands). The serum biochemical parameters were estimated using commercially available diagnostic kits from ERBA Mannheim (Transasia Biomedicals Ltd, HP) by following the manufacturer instructions furnished in the leaflet supplied along with the diagnostic kit.

3.3.6 Gross and histopathological examination

Immediately after sacrifice of the bird from each group, liver, kidney and lymphoid organs namely spleen, bursa of Fabricius and thymus were collected and washed with normal saline, all organs were weighed individually using electrical balance and the weight of each organ was recorded. Organ to body weight ratio i.e organ weight/body weight was calculated and expressed in percentage.

The liver, kidney, spleen, bursa of Fabricius and thymus were processed for histopathology by cutting sections of five microns thickness and stained with Haematoxylin and Eosin (Luna, 1968).

3.4 Mycotoxins residual analysis in tissues

3.4.1 Method development

3.4.1.1 Preparation of standard solutions

Aflatoxins and Ochratoxin A standards were purchased (Sigma Aldrich Chemicals), stock solutions of aflatoxin B1 (1 mg/ml) and OTA (1 mg/ml) were prepared in methanol, stored at -20ºC in amber glass vials, and brought to room temperature (22-25°C) before use. A series of working standard solutions of AFB1 and OTA (1-100 ng/ml) were prepared by suitable dilution of stocks. These solutions were kept at 4°C and renewed weekly. They were used to calibrate the LC detector response and recovery studies. Acetonitrile, water and methanol were of HPLC grade purchased from E. Merck, India. Concentrated formic acid was of the highest analytical grade. Ultra-pure water was produced from distilled water using a Milli-Q water purification system (Milli-Q gradient, Millipore).

3.4.1.2 Calibration / linearity of spiked samples

Linearity was tested for both Aflatoxin B1 and Ochratoxin A in the concentration range from 1-100 ng/ml. For the determination of linearity, standard calibration curves containing at least nine points (non zero standards) were plotted and analyzed in triplicate.

Conc (ng/ml)	AFB1	OCA
STD-1	1	1
STD-2	2	2
STD-3	5	5
STD-4	10	10
STD-5	20	20
STD-6	40	40
STD-7	60	60
STD-8	80	80
STD-9	100	100
LQC	3	3
MQC	50	50
HQC	90	90

3.4.1.3 Extraction and clean up

A new simple method was developed for simultaneous detection and quantification of aflatoxin B1 and Ochratoxin A residues in liver, kidney, breast muscle and leg muscle of coloured broilers**.** Each 1g of defrosted liver, kidney, breast muscle and leg muscle weighed and homogenized with 4 ml of normal saline in blender (Heidolph SilentCrusher M) at 9000 rpm for 10 min and centrifuged (Eppendorf Centrifuge 5810R, Germany; fixed angle rotor) at 8000 rpm for 10 min.

750 µl of above mixture was collected in a micro centrifuge tube, 100 µl of internal standard working solution (Meloxicam-70µg/ml) was added and vortexed to mix well. 250 µl of 1% v/v formic acid in water was added to the above mixture and the tube was subsequently capped and vortex for about 10 min by using cyclomixer at 80 motor speed briefly to mix the content of tube. Then tubes were centrifuged at 5000 rpm for 10 min.

The supernant was collected in separate tube and samples were loaded to previously conditioned (conditioned with 1ml of 5 % methanol and 2 ml of HPLC grade water) SPE HLB cartridges (OROCHEM, 30 mg/ml, DVB-LP with particle size 15 µm and average pore size 180-200 Å). Cartridges were washed with 2 ml of HPLC water, followed by 1 ml of 10% v/v methanol in water. The cartridges were eluted with 0.5ml of elution solution (mobile phase- Acetonitrile: 0.1% v/v formic acid in water) and collect in the tubes, and then transferred to auto sampler HPLC vials and a 20 µl of the extract was injected into LC- MS/MS system equipped with reverse phase C18 column (Thermo Scientific BDS Hypersil C18 RP, 100x4.6 mm, 5µm) with a flow rate of 0.7 ml. Mobile phase consisted of acetonitrile, 0.1% v/v formic acid in water (70:30) with run time 3.5 min.

3.4.1.4 LC–MS/MS analysis

The chromatography was carried out with LC-MS/MS (Agilent Technologies, Waldbron, Germany) Agilent 1200 RRLC system with a solvent delivery pump, auto-degasser, auto sampler and column oven. Electrospray mass spectrometry (ESI-MS) was carried out using a 3200 Q TRAP triple-quadrupole LC-MS/MS system (Applied Biosystems/MDS Sciex), coupled with a Turbo Ion Spray (TISP) source with ESI mode. Applied Biosystems Sciex Analyst software version 1.5 was employed for data acquisition and processing. The separation was performed on a Thermo Scientific BDS Hypersil C18 RP, 100x4.6 mm, 5 µm. Separation was achieved using a gradient elution with the flow rate of 0.7 ml/min, while the injection volume was 20 µl.

Time (min)	A%	B%	Flow Rate (ml/min)
0.01	80	20	0.7
3.00	70	30	0.7

Where, **A**: Acetonitrile, **B**: 0.1% v/v formic acid in water

The source/gas conditions were as under: the curtain gas (CUR) was set at 40psi, while the ion source gas 1 (GS1) and ion source gas 2 (GS2) were set at 40 psi. The temperature was set at 20 ◦C. The compound conditions were Declustering Potential (50.0), Entrance Potential (10.0), Collision energy (30.0) and Collision cell exit potential (5.0). The mass spectrometer was operated in a multiple reaction monitoring (MRM) mode that selected one precursor ion and two product ions for each target compound.

Source/Gas parameters for analysis of multi mycotoxin analysis		
Curtain Gas (CUR)	:	40
Collision Gas (CAD)	:	Medium
Ion Spray voltage (IS)	:	5000 V
Source Type	:	Turbo spray
Source Temperature (at set point, TEM)	:	500 ºC
Ion Source Gas (GS1)	:	40/50
Ion Source Gas (GS2)	:	40
Interface Heater	:	ON
Set Column oven temperature	:	20ºC

3.4.2 Method Validation

The method was validated for specificity/selectivity, linearity, precision and accuracy, recovery and stability as per USFDA guidelines.

3.4.2.1 Specificity

For the study of specificity, which is the ability to differentiate between target analytes and interference, was assessed by analyzing three blank tissue samples. The analytes were identified by matching retention times of peaks with the values of the corresponding standard analyzed under the same experimental conditions.

3.4.2.2 Selectivity

Selectivity was determined by analyzing three replicates of blank tissue samples spiked with the lowest level of the calibration curve concentration.

3.4.2.3 Linearity

Linearity was tested for both Aflatoxin B1 and Ochratoxin A in the concentration range from 1-100 ng/ml. For the determination of linearity, standard calibration curves containing at least nine points (non zero standards) were plotted and analyzed in triplicate. Blank tissue samples were also analyzed to confirm the absence of direct interference. The acceptance limit of accuracy for each of the back-calculated concentration was ± 15 % except for LLOQ where it was ± 20 %. For a calibration run to be accepted at least 75 % of the calibration standards, including ULOQ and LLOQ were required to meet the acceptance criterion.

3.4.2.4 Precision and accuracy

3.4.2.4.1 Inter-day assay

Inter-day assay precision and accuracy was determined by analyzing six replicates at four different QC levels on two different day batches. The acceptance limit of accuracy

was ± 15 % except for LLOQ where it was ± 20 % and precision of ± 15 % coefficient of variance (% CV) except for LLOQ where it was ± 20 %.

3.4.2.4.2 Intra-day assay

Intra-day assay precision and accuracy was determined by analyzing six replicates at four different QC levels on same day batches. The acceptance limit of accuracy was ± 15 % except for LLOQ where it was ± 20 % and precision of ± 15 % coefficient of variance (% CV) except for LLOQ where it was ± 20 %.

3.4.2.5 Recovery

Recovery of the both the Aflatoxin B1 and Ochratoxin A from the extraction procedure was determined by comparing the peak area of the analytes in spiked tissue samples (extracted samples) (three each of low, medium and high QCs) with those of the analytes in tissue samples prepared by spiking the extracted analyte-free tissue samples with the same amounts of the analytes at the step immediately prior to chromatography (post spiked samples). Similarly recovery of the internal standard was determined by comparing the mean peak areas of the extracted QC samples with that of post spiked quality control samples at the step immediately prior to chromatography. The recovery of the analytes and internal standard should be at least more than 50 % and response should be reproducible.

3.4.2.6 Stability test

Stability tests were determined at room temperature and refrigerated conditions (aqueous at 2-8ºC and plasma samples at -20°C). The acceptance limit of accuracy was ± 15 % and precision of ± 15 % coefficient of variance (% CV) for LQC and HQC samples.

3.5 AFB1 and OTA withdrawal period

To know the withdrawal of AFB1 and OTA in broilers which were exposed to aflatoxin and ochratoxin contaminated diet for six weeks, later birds were given mycotoxins free diet for two more weeks (3, 7, 10, 13 and 18 day) and at different intervals liver, kidney and muscles were collected to know the residual withdrawal period in coloured broiler (RAJA II) birds.

3.6 Statistical analysis

The data obtained from the present study were subjected to statistical analysis and analyzed by using two-way ANOVA, Bonferroni post-test were used for multiple comparisons when the analysis indicated significant differences among treatments. All statements of statistical significance were based on a probability of $P \leq 0.05$. Mean values and standard error of mean were calculated and all the values were expressed as Mean±SEM (GraphPad Prism, 2007).

Results

IV. RESULTS

4.1. Mycotoxin production

4.1.1. Subculture of *Aspergillus flavus* (Aflatoxin)

The physical characteristics of *Aspergillus flavus* subcultured on potato dextrose agar were in confirmity with those of pure culture. After two weeks of incubation in a dark place at room temperature, the mycelial growth of *Aspergillus flavus* on the agar surface appeared dark green in colour (Plate 1A).

4.1.2. Aflatoxin production on rice culture

The rice inoculated with spores of *Aspergillus flavus* incubated for the production of aflatoxin at room temperature reveal white mycelial growth with moisture condensation on the inner wall of the flask by third day. The colour of rice turned to greenish by fifth day (Plate 1C).

4.1.3 Subculture of *Aspergillus ochraceous* (Ochratoxin A)

White coloured fungal growth appeared on PDA agar 48 h after inoculation. After sporulation, the mycelial growth turned yellow on seventh day after inoculation (Plate 1B).

4.1.4 Ochratoxin production on wheat/rice culture

After inoculation of wheat/rice with fungal spores, mycelial growth appeared on fifth day with condensation of moisture inside the flask. On seventh day the inoculated wheat/ rice turned to yellow colour after sporulation at room temperature (Plate 1D).

4.2 Multi-mycotoxin analysis in feed and culture

The fungal infected rice and wheat material were dried in oven and analyzed for the presence of aflatoxins (B_1, B_2, G_1 and G_2), ochratoxin, trichothecenes and fumonisins by LC-MS/MS method (Plate 1F) in the Department of Veterinary Pharmacology and Toxicology, Veterinary College, Hebbal, Bangalore. The results are presented in Table 3.

Table 3: Data of multi-mycotoxin analysis in feed and culture sample (µg/kg or ppb)

Mycotoxins	Sample name		
	Aflatoxin sample	**Ochratoxin sample**	**Feed**
Aflatoxin B1	1256	No peak	0.004
Aflatoxin B2	77	No peak	No peak
Aflatoxin G1	No peak	No peak	No peak
Aflatoxin G2	No peak	No peak	No peak
Ochratoxin A	No peak	94000	0.003
T-2 Toxin	No Peak	No Peak	No Peak
HT-2 Toxin	No Peak	No Peak	No Peak
Fumonisin B1	No Peak	No Peak	No Peak
Fumonisin B2	No Peak	No Peak	No Peak

The aflatoxin contaminated rice culture sample showed the presence of aflatoxin B1 and B2 @ concentration of 1256 and 77 ppb, respectively. The extracted ion chromatogram for aflatoxin B1 in rice sample is shown in Fig 1.

Plate 1A: Potato Dextrose Agar petri plate showing dark green colored *Aspergillus flavus* culture growth

Plate 1B: Potato Dextrose Agar petri plate showing yellow colored *Aspergillus ochraceous* culture growth

Plate 1C: Cultured rice showing dark green colored *Aspergillus ochraceous* growth on fifth day after inoculation.

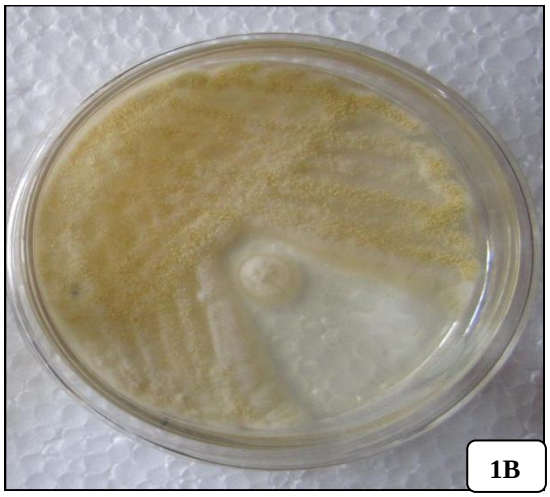
Plate 1D: Cultured rice showing yellow colored *Aspergillus ochraceous* growth on seventh day after inoculation.

Plate 1E: Experimental setup of coloured broiler chicken at fifth week of age.

Plate 1F: 3200 Q TRAP LC-MS/MS analytical setup

PLATE 1

1A



1B

1C

1D

1E

1F

Fig. 1.The extracted ion chromatogram for aflatoxin B1 in rice and wheat sample

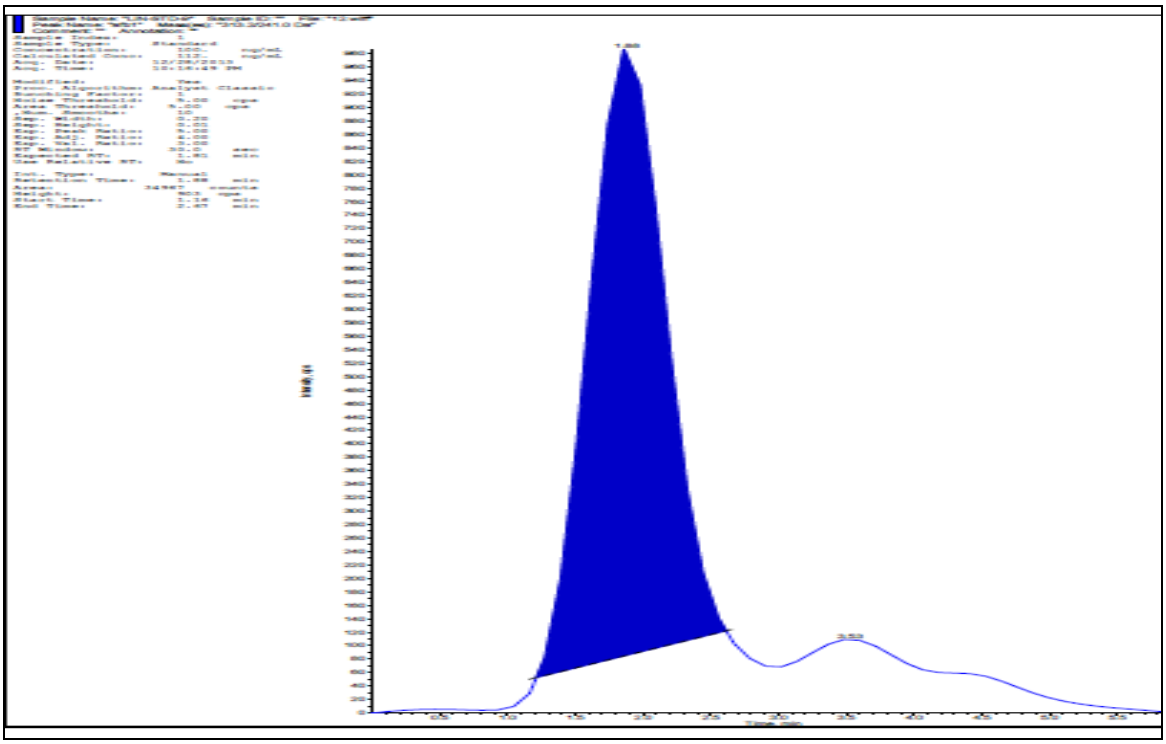
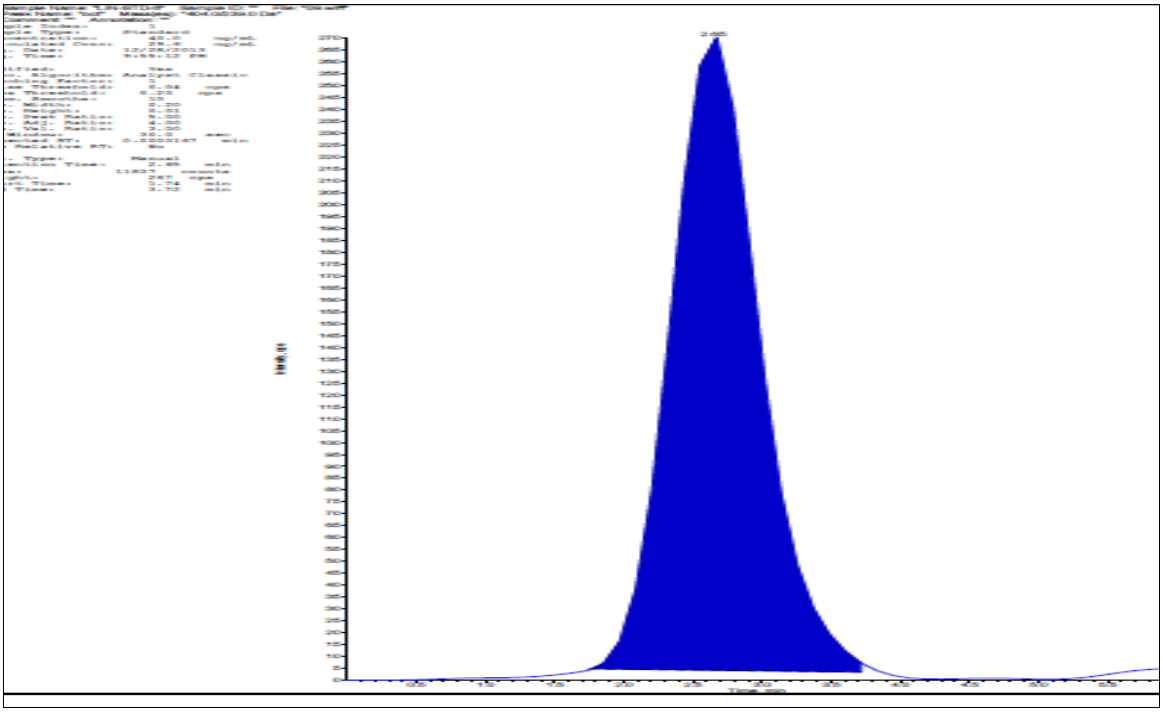


Fig . 2.The extracted ion chromatogram for ochratoxin A in rice and wheat sample



The ochratoxin rice and wheat culture sample showed the presence of ochratoxin and concentration was 94000 ppb. The extracted ion chromatogram for ochratoxin A in rice and wheat sample is shown in Fig 2. The feed material showed the presence of negligible concentration of aflatoxin B1 and ochratoxin A @ 0.004 and 0.003ppb respectively.

4.3 Growth performance

4.3.1 Body weight

The mean body weights of different groups of the experimental birds were measured at weekly and the values have been presented in the Table 4 and the same has been graphically depicted in Fig 3.

A significant ($P<0.001$) decrease in the body weight of the birds were observed in all aflatoxin and ochratoxin groups both in individual and combined mycotoxicoses (Group 4-9) as compared to control (Group1) at the end of day 14, 21 , 28 , 35 and 42 of the experiment. No significant ($P>0.05$) decrease in the body weight was observed in birds fed with mycotoxin binder D.E i.e Groups 2 and 3 as compared to control (Group1) till the end of 42^{nd} day of experiment.

A significant ($P<0.001$) decrease in the body weight was observed in birds fed with combined aflatoxin and ochratoxin groups (Groups 8 and 9, 472.8±1.5 and 370.6±2.5 g, respectively) as compared to individual aflatoxin and ochratoxin groups (Groups 4 to 7, 1004±5.8, 896.5±8.4, 750.6±8.3 and 562.5±9.8 g, respectively). A significant ($P<0.001$) reduction in the body weight gain was observed in OTA groups

(Groups 6-7) as compared to AFB_1 treated groups (Groups 4-5) at 21 day till end of the experiment (Plate 2).

Between the treatment groups significant ($P<0.001$) increase in body weight was observed in birds fed with AFB_1 and OTA along with mycotoxin binder (Diatomaceous earth, DE) treated groups (Groups 10 and 11, 1290±9.7 and 1260±8.7g, respectively) as compared to combined aflatoxin and ochratoxin groups (Groups 8 and 9, 472.8±1.5 and 370.6±2.5g, respectively) without a toxin binder at 21st day until the end of the experiment, but no significant increase in body weight in these groups as compared to control and the values about to reach nearer to control group.

Percent reduction in body weight was recorded. Highest reduction in body weight was noticed in AFB1 and OTA combined toxicity i.e Groups 8 and 9 (66.31and 73.60 per cent respectively) followed by OTA treated groups i.e Groups 6 and 7 (46.47 and 59.89 per cent respectively), reduced body weight was also noticed in AFB1 treated groups i.e Groups 4 and 5 (28.34 and 36.05 per cent respectively) and D.E binder treated combined mycotoxicoses groups i.e. Groups 10 and 11 (7.92 and 10.06 per cent respectively) as compared to negative control group. Birds exposed to combined AFB1 and OTA along with DE binder groups i.e Groups 10 and 11 (63.34 and 70.58 per cent, respectively) showed improved body weight as compared to AFB1 and OTA combined toxic groups i.e. Groups 8 and 9, respectively.

Plate 2A: Combined mycotoxins (AFB1 and OTA) fed bird (left side) compared with negative control bird (right side) on 42 day of the experiment.

Plate 2B: Aflatoxin fed bird (left side) compared with ochratoxin fed bird (right side) on 42 day of the experiment.

Plate 2C: Combined mycotoxins (AFB1 and OTA) fed bird treated with Diatomaceous earth (left side) compared with combined mycotoxins (AFB1 and OTA) fed birds (right side) on 42 day of the experiment.

PLATE 2

2A

2B

2C

4.3.2 Clinical Signs

Birds in Group 1 (negative control) remained normal throughout the period of the experiment. All the birds remained active and alert. Marked depression, reduced feathering, reduced growth rate, leg weakness and reduced bone strength, reduced feed intake, increased water consumption and manure moisture were observed in combined mycotoxicated, ochratoxin followed by aflatoxin groups which were progressive and in a dose related manner.s

4.3.3 Feed Conversion Ratio (FCR)

The mean feed conversion ratio of different groups of the experimental birds was measured at weekly and the values have been presented in the Table 5 and the same has been graphically depicted in Fig 4.

A significant ($P<0.001$) increase in the FCR was observed in Groups 5, 6, 7, 8 and 9 (2.34±0.35, 2.38±0.01, 2.52±0.07, 2.93±0.03 and 3.15±0.17 per cent respectively) as compared to control group (Group1, 2.15±0.08 per cent) from 14 day of the experiment till the end of experimental period (42 day). Group 4 (2.25±0.14 per cent respectively) showed significant ($P<0.001$) increase in FCR as compared to Group 1 from 35 day of the experiment. No significant increase in FCR was observed in birds fed with mycotoxins binder D.E , i.e Groups 2 and 3 (2.16±0.15 and 2.15±0.24 per cent respectively) as compared to control (Group1) till the end of experimental period (42 day).

No significant increase in FCR was observed in Groups 10 and 11 (2.17±0.13 and 2.18±0.05 per cent respectively) as compared to control (Group1) till the end of experimental period (42 day). A significant increase in FCR was observed in respective individual low and high dose of OTA group (Groups 6 and 7, 2.38±0.01 and 2.52±0.07 per cent respectively) as compared to individual low and high dose of AFB1 group (Groups 4 and 5, 2.25±0.14 and 2.34±0.35 per cent respectively) from seventh day till 42 day of the experiment. When combined aflatoxin and ochratoxin groups (Groups 8 and 9) were compared with individual aflatoxin and individual ochratoxin groups (Groups 4 to 7) a significant ($P<0.001$) increase in FCR was observed from day 14 of experiment till the end of experiment on 42 day.

Between the treatment groups, significant ($P<0.001$) decrease in FCR was observed in birds fed with AFB_1 and OTA along with mycotoxins binder (Diatomaceous earth, DE) treated groups (Groups 10 and 11) as compared to combined aflatoxin and ochratoxin groups (Groups 8 and 9) without a toxin binder from day 14 of experiment till the end of experiment on 42 day. Also, no significant increase in FCR was observed in these groups (Groups 10 and 11) as compared to control whereas feed efficiency was improved in toxin binder groups.

4.3.4 Mortality

The rate of mortality was recorded by the end of experimental period and the same has been presented in Table 6. Highest mortality rate was noticed in birds when exposed to combined AFB1 and OTA i.e Groups 8 and 9 (12.21 and 21.21 per cent respectively) as compared to individual AFB1 and OTA groups i.e. Groups 4, 5, 6 and 7

(0, 3.03, 3.03 and 7.57 per cent, respectively) and combined AFB1 and OTA along with DE binder groups i.e. Groups 10 and 11 (1.51 and 1.51 per cent, respectively), highest mortality rate was noticed during first three weeks of experimental period in all treated groups.

Table 6: Individual and combined effect of AFB1 and OTA on mortality (per cent) of coloured broiler chicken and effect of D.E binder on combined mycotoxicoses

Groups	Treatment			
	AFB1 (ppm)	OTA (ppm)	DE (g)	Mortality (%)
G1	-	-	-	0
G2	-	-	0.5	0
G3	-	-	1	0
G4	0.5	-	-	0
G5	1	-	-	3.03
G6	-	0.5	-	3.03
G7	-	1	-	7.57
G8	0.5	0.5	-	12.12
G9	1	1	-	21.21
G10	0.5	0.5	0.5	1.51
G11	1	1	1	1.51

4.4 Relative organ weight (per cent)

4.4.1 Liver

The mean relative weights of liver of different groups of the experimental birds were measured at weekly and the values have been presented in the Table 7.

A significant ($P<0.001$) increase in the relative weight of liver was observed in Groups 4 to 9 as compared to control (Group1) from 2^{nd} week of the experiment to till the end of 6^{th} week of experiment. No significant increase in the relative weight of liver was observed in birds fed with mycotoxins binder D.E , i.e Groups 2 and 3 as compared to control (Group1) till the end of 6^{th} week of experiment.

There was no significant increase in the relative weight of liver was observed in between individual AFB1 and individual OTA groups i.e Groups 4, 5, 6 and 7. When combined AFB1 and OTA groups (Groups 8 and 9) compared with individual AFB1 groups (Groups 4 and 5) there was significant ($P<0.05$) increase in the relative weight of liver was observed at end of experiment. A significant ($P<0.05$) increase in the relative weight of liver was observed in high dose of combined AFB1 and OTA group i.e Group 9 as compared to OTA groups mainly at 2^{nd} and 3^{rd} week of experiment.

Between the treatment groups, no significant increase in the relative weight of liver was observed in birds fed with combined AFB_1 and OTA along with mycotoxins binder (Diatomaceous earth, DE) treated groups (Groups 10-11) as compared to control and combined AFB_1 and OTA groups (Groups 8-9).

Table 4: Individual and combined effect of AFB1 and OTA on body weight (g) of coloured broiler chicken and effect of D.E binder on combined mycotoxicoses (Mean±SEM, n=6)

Groups	Treatment			Days					
	AFB1 (ppm)	OTA (ppm)	DE (g)	7	14	21	28	35	42
G1	-	-	-	111.16±9 ^{abcdefghi}	310.16±8.2^{a}	481.41±4.8 ^{a}	850.83±4.4 ^{a}	997.83±6.6 ^{a}	1401.6±6.5 ^{a}
G2	-	-	0.5	119.83±8 ^{abcdefghi}	312.83±9.4 ^{a}	470.08±4.5 ^{a}	861.33±6.7 ^{a}	991.5±8.7 ^{a}	1385.5±2.5 ^{a}
G3	-	-	1	116.5±9.4 ^{abcdefghi}	314.66±5.1 ^{a}	466.75±8.5 ^{a}	856.66±9.4 ^{a}	999.6±1.5 ^{a}	1373±3.5 ^{a}
G4	0.5	-	-	98.08±2.5 ^{abcdefghi}	236.83±6.4 ^{bcd}	343.18±6.8 ^{bc}	675.58±7.8 ^{bci}	735.58±6.5 ^{bc}	1004±5.8 ^{bhi}
G5	1	-	-	89.5±6.5 ^{abcdefghi}	220.41±11^{bcd}	293.2±5.1^{bcd}	595.25±5.5 ^{bcdhi}	690.83±8.5 ^{bcd}	896.5±8.4^{c}
G6	-	0.5	-	79.41±4.5 ^{abcdefghi}	189.75±4.3^{bcdefg}	221.25±2.3 ^{cdehi}	552.83±1.5^{cd}	636.33±94 ^{cd}	750.6±8.3 ^{d}
G7	-	1	-	68.75±3.9 ^{abcdefghi}	130.33±1.5^{defg}	162.25±8.4 ^{defghi}	380.58±9.7 ^{ef}	486.91±6.3 ^{ef}	562.5±9.8 ^{e}
G8	0.5	0.5	-	80.5±4.21 ^{abcdefghi}	132.83±1.7^{defg}	152.55±6.8^{defgh}	335.08±4.5^{efg}	403.83±13 ^{efg}	472.8±1.5 ^{f}
G9	1	1	-	75.83±7.2 ^{abcdefghi}	94.5±1.1^{efg}	117.58±8.1^{defg}	280.5±7.2^{fg}	331.1±4.5 ^{fg}	370.6±2.5 ^{g}
G10	0.5	0.5	0.5	89.5±5.03 ^{abcdefghi}	198.5±5.1^{bcdehi}	210.25±3.6 ^{cdefhi}	575.16±9.8^{cdhi}	901.25±8.1^{bhi}	1290±9.7^{bchi}
G11	1	1	1	78.41±5.4 ^{abcdefghi}	186.41±6.7 ^{bcdehi}	294.75±2.5 ^{bcdhi}	645.80±6.5 ^{bcdhi}	960.50±3.2^{hi}	1260±8.7^{bhi}

Different superscripts within a row indicate significant differences (P≤0.05)

Table 5: Individual and combined effect of AFB1 and OTA on Feed conversion ratio (per cent) of coloured broiler chicken and effect of D.E binder on combined mycotoxicoses (Mean±SEM, n=6)

Groups	Treatment			Days					
	AFB1 (ppm)	OTA (ppm)	DE (g)	7	14	21	28	35	42
G1	-	-	-	1.39±0.16 ^{abcdefghi}	1.40±0.13 ^{abchi}	1.59±0.04^{abchi}	1.77±0.27 ^{abhi}	1.83±0.05 ^{ahi}	2.15±0.08 ^{ahi}
G2	-	-	0.5	1.36±0.09 ^{abcdefghi}	1.41±0.13 ^{abchi}	1.58±0.11 ^{abchi}	1.75±0.11 ^{abhi}	1.84±0.09 ^{ahi}	2.16±0.15 ^{ahi}
G3	-	-	1	1.38±0.12 ^{abcdefghi}	1.41±0.14 ^{abchi}	1.54±0.13 ^{abchi}	1.76±0.11 ^{abhi}	1.82±0.07 ^{ahi}	2.15±0.24 ^{ahi}
G4	0.5	-	-	1.40±0.04 ^{abcdefghi}	1.46±0.18 ^{abchi}	1.58±0.20 ^{abchi}	1.69±0.26 ^{abhi}	1.94±0.17 ^{b}	2.25±0.14 ^{bhi}
G5	1	-	-	1.46±0.10 ^{abcdefghi}	1.52±0.12 ^{cd}	1.65±0.01 ^{bcd}	1.82±0.12 ^{cd}	2.10±0.10 ^{cd}	2.34±0.35^{cd}
G6	-	0.5	-	1.47±0.10^{abcdefghi}	1.55±0.07 ^{cd}	1.70±0.09 ^{cd}	1.85±0.22 ^{cd}	2.15±0.07 ^{cd}	2.38±0.01 ^{cd}
G7	-	1	-	1.48±0.08 ^{abcdefghi}	1.64±0.13^{e}	1.76±0.23 ^{de}	1.97±0.16 ^{e}	2.24±0.27 ^{e}	2.52±0.07 ^{e}
G8	0.5	0.5	-	1.50±0.14 ^{abcdefghi}	1.75±0.09^{fg}	1.87±0.06 ^{f}	2.10±0.10 ^{f}	2.40±0.04 ^{f}	2.93±0.03 ^{f}
G9	1	1	-	1.52±0.17 ^{abcdefghi}	1.78±0.03^{fg}	1.98±0.06 ^{g}	2.35±0.16 ^{g}	2.76±0.15 ^{g}	3.15±0.17 ^{g}
G10	0.5	0.5	0.5	1.38±0.07 ^{abcdefghi}	1.41±0.09^{abchi}	1.55±0.05 ^{abchi}	1.77±0.2 ^{abhi}	1.85±0.06 ^{ahi}	2.17±0.13 ^{abhi}
G11	1	1	1	1.37±0.05^{abcdefghi}	1.42±0.04^{abchi}	1.57±0.04 ^{abchi}	1.75±0.4^{abhi}	1.86±0.03 ^{ahi}	2.18±0.05 ^{abhi}

Different superscripts within a row indicate significant differences (P≤0.05)

Fig 3. Individual and combined effect of AFB1 and OTA on Body weight (g) and effect of D.E binder on combined mycotoxicosis in coloured broilers

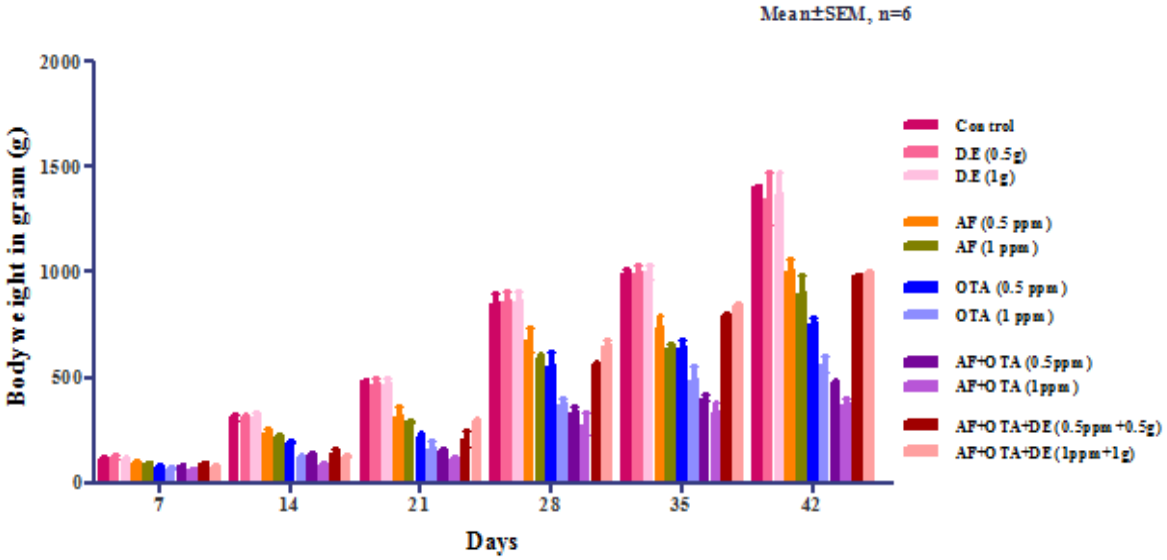
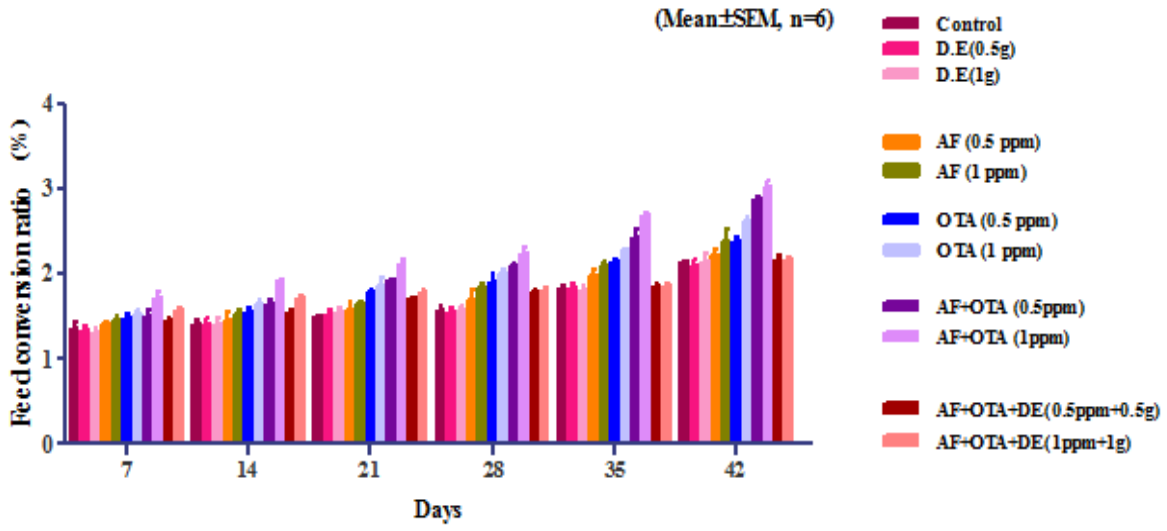


Fig 4. Individual and combined effect of AFB1 and OTA on Feed conversion ratio (per cent) and effect of D.E binder on combined mycotoxicosis in coloured broilers



4.4.2 Kidney

The mean relative weight of kidney of different groups of the experimental birds was measured at weekly and the values have been presented in the Table 8.

A significant ($P<0.001$) increase in the relative weight of kidney was observed in Groups 4 to 9 as compared to control (Group1) at the end of 2^{nd} week of the experiment to till the end of 6^{th} week of experiment. No significant increase in the relative weight of kidney was observed in birds fed with mycotoxins binder D.E only, i.e Groups 2 and 3 as compared to control (Group1) till the end of 6^{th} week of experiment.

There was significant ($P<0.001$) increase in the relative weight of kidney was observed in individual OTA groups i.e Groups 6 and 7 as compared to individual AFB1 groups i.e Groups 4 and 5. When combined AFB1 and OTA groups i.e Groups 8 and 9 compared with individual AFB1 and OTA groups i.e Groups 4, 5, 6 and 7, there was significant ($P<0.001$) increase in the relative weight of kidney from 2^{nd} week till the end of experiment.

Between the treatment groups, no significant increase in the relative weight of kidney was observed in birds with combined AFB_1 and OTA along with mycotoxins binder (Diatomaceous earth, DE) treated groups (Groups 10-11) as compared to combined AFB_1 and OTA groups (Groups 8-9).

4.4.3 Thymus

The mean relative weights of thymus of different groups of the experimental birds were measured at weekly and the values have been presented in the Table 10.

A significant (P<0.01) decrease in the relative weight of thymus was observed in combined AFB1 and OTA groups i.e Groups 8 and 9 as compared to control (Group 1) at 5^{th} and 6^{th} week of experiment. No significant decrease in the relative weight of thymus was observed in birds fed with mycotoxins binder D.E only (Groups 2 and 3) and individual AFB1 (Groups 4 and 5) as compared to control (Group1) till the end of 6th week of experiment, but statistically significant (P<0.05) decrease in the relative weight of thymus was observed in birds fed with individual OTA (Groups 6 and 7) as compared to control (Group1).

A significant (P<0.001) decrease in the relative weight of thymus was observed in combined AFB1 and OTA groups i.e Groups 8 and 9 as compared to individual AFB1 and OTA group (Groups 4, 5, 6 and 7) at 3^{rd} and 6^{th} week of experiment..

There was significant (P<0.05) decrease in the relative weight of thymus was observed in individual AFB1 and OTA groups i.e Groups 4, 5 and 6, 7 as compared to each other at end of experiment. Between the treatment groups no significant decrease in the relative weight of thymus was observed in birds with combined AFB_1 and OTA along with mycotoxins binder (Diatomaceous earth, DE) treated groups (Groups 10-11) as compared to control (Group 1) and combined AFB_1 and OTA groups (Groups 8-9).

4.4.4 Bursa of Fabricius

The mean relative weights of bursa of Fabricius of different groups of the experimental birds were measured at weekly and the values have been presented in the Table 11.

There was no significant decrease in the relative weight of bursa of Fabricius was observed in mycotoxins binder control groups (Groups 2 and 3), individual (Groups 4, 5, 6 and 7), and combined AFB1 and OTA along with mycotoxins binder (Diatomaceous earth, DE) treated groups (Groups 10-11) as compared to control (Group1) at throughout the experimental period, but a significant ($P<0.001$) decrease in the relative weight of bursa of Fabricius was observed in combined AFB1 and OTA (Groups 8 and 9) as compared to control (Group1).

No significant decrease in the relative weight of bursa of Fabricius was observed in individual AFB1 and OTA groups i.e Groups 4, 5 and 6, 7 as compared to combined AFB_1 and OTA till the end of experiment. Between the treatment groups No significant decrease in the relative weight of bursa of Fabricius was observed in birds with combined AFB_1 and OTA along with mycotoxins binder (Diatomaceous earth, DE) treated groups (Groups 10-11) as compared to combined AFB_1 and OTA groups (Groups 8-9).

4.4.5 Spleen

The mean relative weights of spleen of different groups of the experimental birds were measured weekly and the values have been presented in the Table 9.

No significant change in the relative weight of spleen was observed in birds fed with mycotoxin binder D.E only (Groups 2 and 3), individual AFB1and OTA groups (Groups 4, 5, 6 and 7) and significant ($P<0.05$) decrease in the relative weight of spleen in combined AFB1 and OTA groups (Groups 8 and 9) as compared to control (Group1) till the end of 6^{th} week of experiment.

Between treatment groups, no significant change in the relative weight of spleen was observed in combined AFB1 and OTA groups (Groups 8 and 9) as compared to individual AFB1 groups (Groups 4 and 5) and individual OTA groups (Groups 6 and 7) till the end of experiment.

There was no significant change in the relative weight of spleen was observed in individual AFB1 and OTA groups i.e Groups 4 , 5 and 6 , 7 as compared to each other till end of experiment. Between the treatment groups no significant decrease in the relative weight of spleen was observed in birds with combined AFB_1 and OTA along with mycotoxin binder (Diatomaceous earth, DE) treated groups (Groups 10-11) as compared to combined AFB_1 and OTA groups (Groups 8-9).

4.5.1 Serum biochemistry

4.5.1.1 Serum aspartate aminotransferase (AST)

The mean AST activity of different groups of the experimental birds was measured at weekly interval and the values have been summarized in Table 12 and represented graphically in Fig 5.

There was significant ($P<0.001$) increase in the AST activity in Groups 4, 5, 8 and 9 from seventh day to 42 day of the experiment, while individual OTA groups (Groups 6 and 7) showed significant ($P<0.001$) increase in the AST activity from day 21 to day 42 of the experiment as compared to control (Group1). No significant increase in the AST activity was observed in birds fed with mycotoxin binder D.E , i.e Groups 2 and 3 as compared to control (Group1) till the end of 42 day of the experiment.

Table 7: Individual and combined effect of AFB1 and OTA on relative liver weight (per cent) of coloured broiler chicken and effect of D.E binder on combined mycotoxicoses (Mean±SEM, n=6).

Groups	Treatment			Days					
	AFB1 (ppm)	OTA (ppm)	DE(g)	7	14	21	28	35	42
G1	-	-	-	3.92±0.40	3.26±0.18	2.98±0.10	2.77±0.27	2.66±0.15	2.41±0.12
G2	-	-	0.5	3.66±0.36	3.20±0.32	3.10±0.29	2.68±0.12	2.59±0.15	2.29±0.23
G3	-	-	1	3.71±0.34	3.34±0.19	3.13±0.34	2.68±0.17	2.60±0.09	2.27±0.26
G4	0.5	-	-	4.93±0.85	4.88±0.65***	4.71±0.77***	3.94±0.44***	3.72±0.32***	3.32±0.25***
G5	1	-	-	5.51±1.05	5.26±0.63***	4.84±0.58***	4.29±0.24***	4.18±0.24***	3.83±0.38***
G6	-	0.5	-	4.44±0.56	4.29±0.73***	3.92±0.33***	3.68±0.62***	3.60±0.28***	3.47±0.16***
G7	-	1	-	4.70±0.58	4.43±0.35***	4.30±2.19***	3.77±0.43***	3.62±0.67***	3.44±0.40***
G8	0.5	0.5	-	4.81±0.61	4.69±0.69***	4.60±0.50***	4.46±0.21***	4.31±0.33***	4.02±0.19***
G9	1	1	-	5.52±0.79	9.53±1.22***	7.83±0.15***	4.22±1.07***	3.93±0.86***	4.17±0.39***
G10	0.5	0.5	0.5	4.20±0.28	4.13±0.38	3.94±0.71	3.57±0.32	2.92±0.45	2.51±0.21
G11	1	1	1	4.84±0.42	6.28±0.62	5.75±0.58	3.48±0.47	2.70±0.19	2.40±0.14

Mean values with *** ($P<0.001$) as compared to control

Table 8: Individual and combined effect of AFB1 and OTA on relative kidney weight (per cent) of coloured broiler chicken and effect of D.E binder combined mycotoxicoses (Mean±SEM, n=6).

Groups	Treatment			Days					
	AFB1 (ppm)	OTA (ppm)	DE (g)	7	14	21	28	35	42
G1	-	-	-	1.49±0.12	1.14±0.07	0.98±0.06	0.88±0.06	1.02±0.06	0.90±0.06
G2	-	-	0.5	1.40±0.09	1.13±0.08	1.01±0.06	0.87±0.06	1.03±0.06	0.91±0.08
G3	-	-	1	1.43±0.10	1.08±0.06	1.02±0.06	0.87±0.06	1.01±0.05	0.90±0.06
G4	0.5	-	-	1.74±0.14	1.58±0.17***	1.57±0.18***	1.16±0.20***	1.42±0.10***	1.31±0.09***
G5	1	-	-	1.94±0.14	1.59±0.19***	1.64±0.08***	1.36±0.15***	1.71±0.11***	1.60±0.10***
G6	-	0.5	-	2.09±0.39	1.76±0.11***	1.87±0.21***	1.47±0.29***	1.69±0.13***	1.78±0.10***
G7	-	1	-	2.41±0.12	2.93±0.16***	3.11±0.88**	2.13±0.19***	2.38±0.26***	2.60±0.19***
G8	0.5	0.5	-	2.06±0.37	2.53±0.29***	2.70±0.21**	2.38±0.15***	2.65±0.17***	2.84±0.16**
G9	1	1	-	2.75±0.36	4.20±0.19***	4.09±0.54***	2.95±0.82***	3.52±0.42***	3.16±0.36***
G10	0.5	0.5	0.5	1.66±0.23	1.48±0.20	1.38±0.33	1.40±0.15	1.55±0.16	1.07±0.18
G11	1	1	1	1.90±0.33	1.65±0.23	1.44±0.20	1.31±0.24	1.66±0.17	1.14±0.12

Mean values with ** (P<0.01) and *** (P<0.001) as compared to control

Table 9: Individual and combined effect of AFB1 and OTA on relative thymus weight (per cent) of coloured broiler chicken and effect of D.E binder on combined mycotoxicoses (Mean±SEM, n=6).

Groups	Treatment			Days					
	AFB1 (ppm)	OTA (ppm)	DE (g)	7	14	21	28	35	42
G 1	-	-	-	0.46±0.05	0.52±0.09	0.56±0.04	0.40±0.07	0.43±0.03	0.38±0.05
G 2	-	-	0.5	0.44±0.06	0.52±0.11	0.57±0.05	0.40±0.07	0.44±0.03	0.39±0.07
G 3	-	-	1	0.45±0.03	0.51±0.04	0.54±0.03	0.39±0.06	0.44±0.04	0.38±0.04
G 4	0.5	-	-	0.53±0.03	0.68±0.08	0.82±0.12	0.33±0.04	0.37±0.04	0.34±0.04
G 5	1	-	-	0.52±0.16	0.64±0.13	0.86±0.07	0.30±0.04	0.31±0.01	0.30±0.04
G 6	-	0.5	-	0.67±0.06	0.81±0.15	0.98±0.03	0.34±0.05	0.37±0.04*	0.36±0.04*
G 7	-	1	-	0.70±0.13	0.68±0.20	0.89±0.34	0.31±0.02	0.35±0.07*	0.31±0.03*
G 8	0.5	0.5	-	0.59±0.10	0.61±0.15	0.65±0.12	0.29±0.02	0.31±0.03**	0.18±0.04**
G 9	1	1	-	0.79±0.12	0.98±0.33	0.72±0.10	0.28±0.09	0.25±0.08**	0.14±0.03**
G 10	0.5	0.5	0.5	0.59±0.06	0.52±0.13	0.57±0.30	0.43±0.05	0.44±0.05	0.35±0.04
G 11	1	1	1	0.61±0.11	0.63±0.14	0.58±0.24	0.37±0.03	0.40±0.03	0.33±0.05

Mean values with *($P<0.05$) and ** ($P<0.01$) as compared to control

Table 10: Individual and combined effect of AFB1 and OTA on relative bursa of Fabricius weight (per cent) of coloured broiler chicken and effect of D.E binder on combined mycotoxicoses (Mean±SEM, n=6).

Groups	Treatment			Days					
	AFB1 (ppm)	OTA (ppm)	DE (g)	7	14	21	28	35	42
G1	-	-	-	0.91±0.24	0.57±0.03	0.40±0.01	0.25±0.02	0.23±0.03	0.17±0.03
G 2	-	-	0.5	0.89±0.22	0.58±0.05	0.41±0.03	0.24±0.01	0.23±0.03	0.17±0.06
G3	-	-	1	0.90±0.14	0.55±0.05	0.41±0.03	0.21±0.03	0.22±0.02	0.18±0.02
G4	0.5	-	-	0.92±0.19	0.53±0.07	0.42±0.04	0.22±0.01	0.20±0.01	0.15±0.01
G5	1	-	-	0.92±0.17	0.51±0.08	0.42±0.09	0.21±0.02	0.18±0.03	0.12±0.02
G6	-	0.5	-	0.91±0.09	0.53±0.08	0.44±0.08	0.23±0.04	0.18±0.02	0.12±0.01
G7	-	1	-	0.93±0.19	0.56±0.08	0.45±0.15	0.21±0.03	0.17±0.02	0.12±0.02
G8	0.5	0.5	-	0.90±0.11	0.56±0.13	0.43±0.07	0.20±0.02	0.15±0.01	0.04±0.01**
G9	1	1	-	0.92±0.16	0.55±0.13	0.42±0.05	0.18±0.04	0.14±0.02	0.07±0.03**
G10	0.5	0.5	0.5	0.94±0.17	0.59±0.11	0.42±0.10	0.22±0.02	0.15±0.03	0.13±0.01
G11	1	1	1	0.94±0.11	0.56±0.11	0.44±0.03	0.23±0.05	0.17±0.02	0.14±0.02

Mean values with ** ($P<0.01$) as compared to control

Table 11: Individual and combined effect of AFB1 and OTA on relative spleen weight (per cent) of coloured broiler chicken and effect of D.E binder on combined mycotoxicoses (Mean±SEM, n=6).

Groups	Treatment			Days					
	AFB1 (ppm)	OTA (ppm)	DE (g)	7	14	21	28	35	42
G 1	-	-	-	0.12±0.01	0.13±0.01	0.13±0.01	0.14±0.02	0.17±0.01	0.15±0.01
G 2	-	-	0.5	0.11±0.01	0.13±0.01	0.13±0.01	0.14±0.01	0.18±0.01	0.16±0.02
G 3	-	-	1	0.11±0.01	0.12±0.02	0.13±0.01	0.14±0.02	0.17±0.01	0.15±0.01
G 4	0.5	-	-	0.13±0.02	0.16±0.03	0.13±0.03	0.12±0.01	0.18±0.02	0.16±0.01
G 5	1	-	-	0.13±0.02	0.14±0.01	0.12±0.03	0.14±0.02	0.18±0.01	0.16±0.01
G 6	-	0.5	-	0.12±0.01	0.13±0.06	0.14±0.02	0.13±0.04	0.14±0.01	0.15±0.02
G 7	-	1	-	0.14±0.02	0.15±0.06	0.16±0.03	0.14±0.01	0.16±0.03	0.14±0.02
G 8	0.5	0.5	-	0.12±0.01	0.13±0.03	0.15±0.02	0.13±0.01	0.11±0.01*	0.10±0.01**
G 9	1	1	-	0.14±0.04	0.15±0.02	0.17±0.04	0.13±0.01	0.10±0.01*	0.09±0.01**
G 10	0.5	0.5	0.5	0.12±0.02	0.13±0.01	0.14±0.01	0.16±0.03	0.16±0.03	0.16±0.02
G 11	1	1	1	0.13±0.02	0.15±0.01	0.16±0.01	0.15±0.01	0.16±0.01	0.17±0.02

Mean values with *(P<0.05) and ** (P<0.01) as compared to control

There was no significant increase in the AST activity in individual low dose of AFB1 (Group 4) as compared to individual high dose of OTA (Group 7) when compared with each other throughout the experimental period (42 days). There was significant ($P<0.01$) increase in the AST activity was observed in individual high dose of AFB1 (Group 5) as compared to individual low and high dose of OTA (Groups 6 and 7) from day 28 till the end of experiment.

There was significant ($P<0.01$) increase in the AST activity in individual high dose of AFB1 (Group 5) as compared to individual low dose of AFB1 (Group 4) from day 28 till the end of experiment. There was significant ($P<0.05$) increase in the AST activity was observed in individual high dose of OTA (Group 7) as compared to individual low dose of OTA (Group 6) from day 35 till the end of experiment.

A significant ($P<0.001$) increase in the AST activity was observed in both low and high dose of combined AFB1 and OTA groups (Groups 8 and 9) as compared to individual low and high dose of AFB1 and OTA groups (Groups 4, 5, 6 and 7) and low and high dose of combined AFB1 and OTA groups treated with D.E binder (Groups 10 and 11) throughout the end of the experiment (42^{nd} day).

Between the treatment groups, no significant increase in the AST activity was observed in birds fed with the combined AFB_1 and OTA along with mycotoxins binder (Diatomaceous earth, DE) treated groups (Groups 10-11) as compared to combined AFB_1 and OTA groups (Groups 8-9) and the values nearer to control group.

4.5.1.2 Serum alanine aminotransferase (ALT)

The mean ALT activity of different groups of the experimental birds was measured at weekly intervals and the values have been summarized in Table 13 and represented graphically in Fig 6.

A significant ($P<0.001$) increase in the ALT activity was observed in Groups 4, 5, 6,7, 8 and 9 as compared to control (Group1) from seventh day of the experiment till the end of experimental period (day 42). No significant increase in the ALT activity was observed in birds fed with mycotoxin binder D.E, i.e. Groups 2 and 3 as compared to control (Group1) till the end of 42nd day of experiment.

There was no statistical significant increase in the ALT activity was observed in individual AFB1 and OTA groups i.e. Groups 4, 6 and 7 when compared with each other throughout the experimental period (42 days). Whereas significant ($P<0.01$) increase in the ALT activity was observed in individual high dose of AFB1 (Group 5) as compared to individual low and high dose of OTA groups (Groups 6 and 7) at 42 day.

When combined AFB1 and OTA groups i.e. Groups 8 and 9 compared with individual AFB1 groups i.e. Groups 4 and 5 there was significant ($P<0.001$) increase in the ALT activity was observed from day 35 till the end of the experiment. A significant ($P<0.001$) increase in the ALT activity was observed in combined AFB1 and OTA groups (Groups 8 and 9) as compared to low and high dose of individual high dose of OTA groups (Groups 6 and 7) from day 21 till the end of the experiment 42^{nd} day

Between the treatment groups no significant increase in the ALT activity was observed in birds fed with the combined AFB_1 and OTA along with mycotoxins binder (Diatomaceous earth, DE) treated groups (Groups 10-11) as compared to combined AFB_1 and OTA groups (Groups 8-9) and control (Group 1), also the values reaching nearer to control group.

4.5.1.3 Serum gamma glutamyl transaminase (GGT)

The mean GGT activity of different groups of the experimental birds was measured at weekly interval and the values have been summarized in Table 14 and represented graphically in Fig 7.

A significant ($P<0.001$) increase in the GGT activity was observed in Groups 4, 5 8 and 9 from seventh day till the end of the experimental period 42^{nd} day as compared to control (Group1). A significant ($P<0.01$) increase in the GGT activity was observed in Groups 6 and 7 from 21^{st} day till the end of the experimental period 42^{nd} day as compared to control (Group1). No significant increase in the GGT activity was observed in birds fed with mycotoxin binder D.E, i.e Groups 2 and 3 as compared to control (Group1) till the end of sixth week of experiment.

A significant ($P<0.01$) increase in GGT activity was observed in individual AFB1 (Group 4) at seventh day and there was no significant increase in the GGT activity in later period of experiment up to 42^{nd} day as compared to individual OTA groups (Groups 6 and 7).

A significant (P<0.001) increase in GGT activity was observed in individual AFB1 (Group 5) from seventh day till the end of experiment 42nd day as compared to individual OTA group (Group 6). A significant (P<0.001) increase in GGT activity was observed in individual AFB1 (Group 5) from seventh day to 14th day of experiment and no significant increase in the GGT activity was observed in later period of experiment up to 42nd day as compared to individual OTA group (Group 7).

A significant (P<0.05) increase in GGT activity was observed in low dose combined mycotoxicoses group (Group 8) as compared to individual low dose AFB1 group (Group 4) from 21st day up to end of the experimental period (42nd day) and significant (P<0.001) increase in GGT activity was observed in high dose combined mycotoxicoses group (Group 9) as compared to individual AFB1 group (Group 4) till the end of experimental period (42nd day).

There was no significant increase in GGT activity in low dose combined mycotoxicoses group (Group 8) as compared to individual high dose AFB1 group (Group 5) till the end of experimental period (42nd day) and significant (P<0.001) increase in GGT activity was observed in high dose combined mycotoxicoses group (Group 9) as compared to individual high AFB1 group (Group 5) from 14 day till the end of experimental period (42nd day).

A significant (P<0.001) increase in GGT activity was observed in low and high dose of combined mycotoxicoses groups (Groups 8 and 9) as compared to individual low and high dose OTA groups (Groups 6 and 7). Between the treatment groups, there was no significant increase in the GGT activity in birds fed with the combined AFB_1 and OTA

along with mycotoxins binder (Diatomaceous earth, DE) treated groups (Groups 10-11) as compared to combined AFB_1 and OTA groups (Groups 8-9) and control (Group 1) , also the values reaching nearer to control group.

4.5.1.4 Serum creatinine

The mean creatinine concentration of different groups of the experimental birds was measured at weekly intervals and the values have been summarized in Table 15 and represented graphically in Fig 8.

A significant ($P<0.001$) increase in the creatinine concentration was observed in Groups 6, 7, 8 and 9 from seventh day of the experiment till the end of the experimental period (42^{nd} day) and significant ($P<0.001$) increase in the creatinine concentration was observed in Groups 4 and 5 from day 35^{th} to 42^{nd} day of the experiment as compared to control (Group1). No significant increase in the creatinine concentration was observed in birds fed with mycotoxins binder D.E, i.e Groups 2 and 3 as compared to control (Group1) till the end of 6^{th} week of experiment.

There was significant ($P<0.001$) increase in the creatinine concentration in both individual low and high dose of OTA (Groups 6 and 7) as compared to both individual low and high dose of AFB1 (Groups 4 and 5) from day 21^{st} of the experiment till the end of experiment 42^{nd} day. A significant ($P<0.001$) increase in the creatinine concentration was observed in both low and high dose of combined mycotoxicoses (Groups 8 and 9) as individual low and high dose of AFB1 and OTA (Groups 4, 5, 6 and 7).

Table 12: Individual and combined effect of AFB1 and OTA on AST activity (U/L) and effect of D.E binder on combined mycotoxicoses in coloured broiler chickens (Mean±SEM, n=6)

Groups	Treatment			Days					
	AFB1 (ppm)	OTA (ppm)	DE (g)	7	14	21	28	35	42
G 1	-	-	-	153.66±4.9 ^{abdehi}	155.06±5.7 ^{adhi}	157.48±3.0 ^{adhi}	158.66±2.5 ^{adhi}	160.11±3.2^{ahi}	161.33±3.9 ^{ahi}
G 2	-	-	0.5	152.91±5.1 ^{abdehi}	154.4±5.3 ^{adhi}	156.65±3.5 ^{adhi}	157±4.2 ^{adhi}	159.53±3.2^{ahi}	162.38±4.1 ^{ahi}
G 3	-	-	1	153.75±4.3 ^{abdehi}	154.08±5.2 ^{adhi}	155.21±2.8 ^{adhi}	156±5.4 ^{adhi}	158.9±7.0 ^{ahi}	160.46±4.6 ^{ahi}
G 4	0.5	-	-	163.8±4.1 ^{bdehi}	168.68±4.0^{bdehi}	170.01±3.3 ^{bcde}	175.31±1.6 ^{be}	181.5±3.7 ^{be}	190.06±3.8^{be}
G 5	1	-	-	173.58±4.8^{c}	175.41±2.9 ^{bc}	179.15±2.5 ^{bc}	185.81±2.3 ^{c}	193.2±2.3^{c}	201.73±5.3^{c}
G 6	-	0.5	-	156.25±3.2 ^{abde}	159.76±1.3 ^{abdehi}	167.08±3.4 ^{bde}	168.76±3.1^{dehi}	170.06±2.9^{d}	180.48±2.1^{d}
G 7	-	1	-	158.33±3.9 ^{abde}	162.16±4.0^{abdehi}	168.75±5.2 ^{bde}	170.5±5.5 ^{bdehi}	179.98±5.6^{be}	191.33±6.5^{be}
G 8	0.5	0.5	-	176.6±6.3^{cef}	185.73±6.9 ^{f}	191.5±5.5 ^{f}	198.41±5.7 ^{f}	206.5±6.2^{f}	219.08±5.3^{f}
G 9	1	1	-	192.08±3.8^{g}	196.33±1.1 ^{g}	210.66±5.2 ^{g}	231.55±6.0 ^{g}	266.38±6.2^{g}	287.75±3.4 ^{g}
G 10	0.5	0.5	0.5	157.58±2.3^{abdehi}	160.43±1.8^{abdehi}	160.93±1.5^{abdehi}	162±17.2 ^{adehi}	164.11±6.7^{adhi}	167.03±2.9 ^{ahi}
G 11	1	1	1	158.78±8.6 ^{abdehi}	160.4±2.9 ^{abdehi}	164.7±3.2 ^{abdehi}	165.16±4.9^{adehi}	167.31±2.1^{adhi}	168.75±7.8 ^{ahi}

Different superscripts within a row indicate significant differences (P≤0.05)

Table 13: Individual and combined effect of AFB1 and OTA on ALT activity (U/L) and effect of D.E binder on combined mycotoxicoses in coloured broiler chickens (Mean±SEM, n=6)

Groups	Treatment			Days					
	AFB1 (ppm)	OTA (ppm)	DE (g)	7	14	21	28	35	42
G 1	-	-	-	15.08±0.70^{a}	15.37±0.52^{ahi}	15.71±0.30^{ahi}	15.86±0.25^{ahi}	16.01±0.32^{ahi}	16.13±0.39^{ahi}
G 2			0.5	15.05±0.73^{a}	15.32±0.56^{ahi}	15.5±0.42^{ahi}	15.7±0.42^{ahi}	15.95±0.32^{ahi}	16.10±0.34^{ahi}
G 3			1	15.03±0.47^{a}	15.31±0.45^{ahi}	15.52±0.28^{ahi}	15.72±0.48^{ahi}	15.90±0.70^{ahi}	16.04±0.46^{ahi}
G 4	0.5	-	-	16.09±0.36^{bc}	16.80±0.42^{bcedfg}	17.28±0.98^{bced}	17.45±0.81^{bce}	17.81±0.74^{bcde}	17.97±0.24^{bc}
G 5	1	-	-	16.65±0.66^{bc}	16.88±0.36^{bcedfg}	17.37±0.47^{bced}	17.97±0.38^{bce}	18.00±0.08^{bcdefg}	18.36±0.34^{bc}
G 6	-	0.5	-	15.95±0.33^{bcd}	16.35±0.40^{bcedfg}	16.62±0.32^{bced}	16.96±0.46^{bde}	17.20±0.34^{bcde}	17.35±0.1^{bde}
G 7	-	1	-	16.03±0.45^{bce}	16.48±0.53^{bcedfg}	16.74±0.42^{bced}	17.15±0.51^{bcde}	17.37±0.22^{bde}	17.64±0.23^{bde}
G 8	0.5	0.5	-	16.67±0.52^{bcfg}	16.93±0.36^{bcedfg}	17.50±0.32^{bcfg}	18.10±0.41^{bcfg}	18.98±0.27^{fg}	19.15±0.29^{fg}
G 9	1	1	-	16.90±0.36^{bcfg}	17.26±0.23^{bcedfg}	17.97±0.45^{bcfg}	18.50±0.19^{bcfg}	19.17±0.12^{fg}	19.48±0.24^{fg}
G 10	0.5	0.5	0.5	15.76±0.83^{ahi}	16.04±0.78^{ahi}	16.40±0.94^{ahi}	16.56±0.84^{ahi}	16.76±1.32^{ahi}	16.82±0.93^{ahi}
G 11	1	1	1	15.46±1.07^{ahi}	16.05±0.29^{ahi}	16.48±0.89^{ahi}	16.63±0.75^{ahi}	16.84±1.09^{ahi}	16.86±0.89^{ahi}

Different superscripts within a row indicate significant differences (P≤0.05)

Table 14: Individual and combined effect of AFB1 and OTA on GGT activity (U/L) and effect of D.E binder on combined mycotoxicoses in coloured broiler chickens (Mean±SEM, n=6)

Groups	Treatment			Days					
	AFB1 (ppm)	OTA (ppm)	DE (g)	7	14	21	28	35	42
G 1	-	-	-	21.12±0.89^{a}	22.55±0.33^{a}	22.85±0.38^{a}	22.92±0.10^{a}	22.95±0.81^{a}	23.01±0.16^{a}
G 2	-	-	0.5	21.13±0.65^{a}	22.56±0.34^{a}	22.84±0.40^{a}	22.91±0.07^{a}	22.98±0.16^{a}	23.00±0.12^{a}
G 3	-	-	1	21.12±0.37^{a}	22.52±0.32^{a}	22.86±0.58^{a}	22.92±0.08^{a}	22.95±0.06^{a}	23.05±0.11^{a}
G 4	0.5	-	-	22.73±0.36^{b}	23.04±0.24^{ab}	23.90±0.2^{b}	24.28±0.45^{b}	24.86±0.48^{b}	25.26±0.65^{b}
G 5	1	-	-	23.1±0.64^{cb}	23.88±0.45^{c}	24.12±0.55^{cb}	24.91±0.57^{cb}	25.43±0.55^{bc}	26.04±0.70^{bc}
G 6	-	0.5	-	21.19±0.93^{ad}	22.60±0.65^{abd}	23.16±0.50^{abd}	23.50±0.70^{abd}	24.05±0.45^{bd}	24.56±0.45^{bd}
G 7	-	1	-	21.64±0.85^{ade}	22.85±0.25^{abde}	23.84±0.30^{bcde}	24.20±0.45^{bcde}	25.15±0.75^{bcde}	25.91±0.50^{bcde}
G 8	0.5	0.5	-	22.96±0.60^{bcf}	23.86±0.30^{bcf}	24.95±0.55^{cf}	25.24±0.45^{cf}	25.98±0.40^{cf}	26.91±0.60^{cf}
G 9	1	1	-	23.90±0.45^{cg}	24.80±0.35^{g}	25.24±0.30^{g}	26.25±0.40^{g}	27.16±0.45^{g}	28.25±0.55^{g}
G 10	0.5	0.5	0.5	21.47±0.72^{abdeh}	22.78±0.22^{abdeh}	22.88±0.46^{abdh}	23.08±0.38^{abdh}	23.25±0.36^{abdh}	23.47±0.50^{abh}
G 11	1	1	1	21.35±0.80^{abdeh}	22.74±0.23^{abdeh}	22.81±0.34^{abdh}	23.20±0.33^{abdh}	23.34±0.44^{abdh}	23.36±0.37^{abdh}

Different superscripts within a row indicate significant differences (P≤0.05)

Fig 5. Effect of individual and combined AFB1 and OTA on AST activity (U/L)and effect of D.E binder on combined mycotoxicosis in coloured broiler chicken

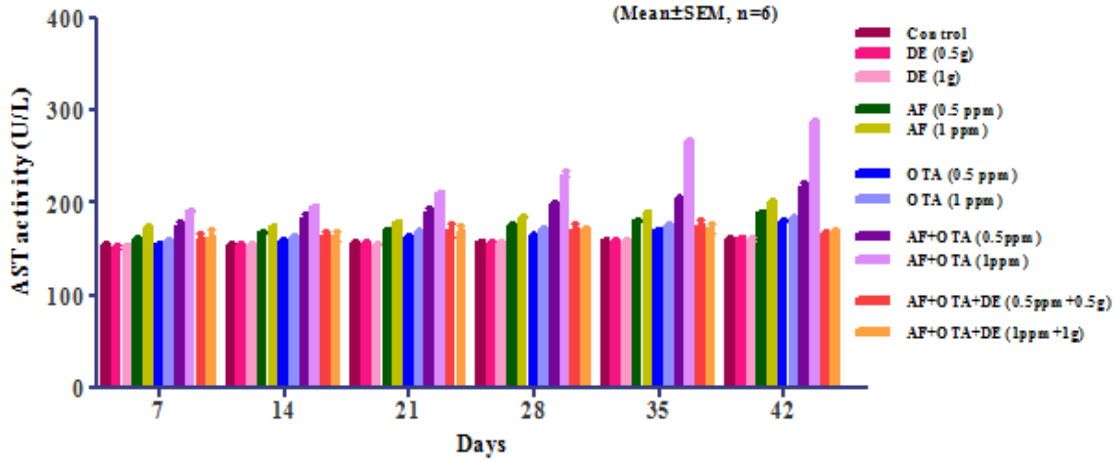


Fig 6. Effect of individual and combined AFB1 and OTA on ALT activity (U/L) and effect of D.E binder on combined mycotoxicosis in coloured broiler chicken

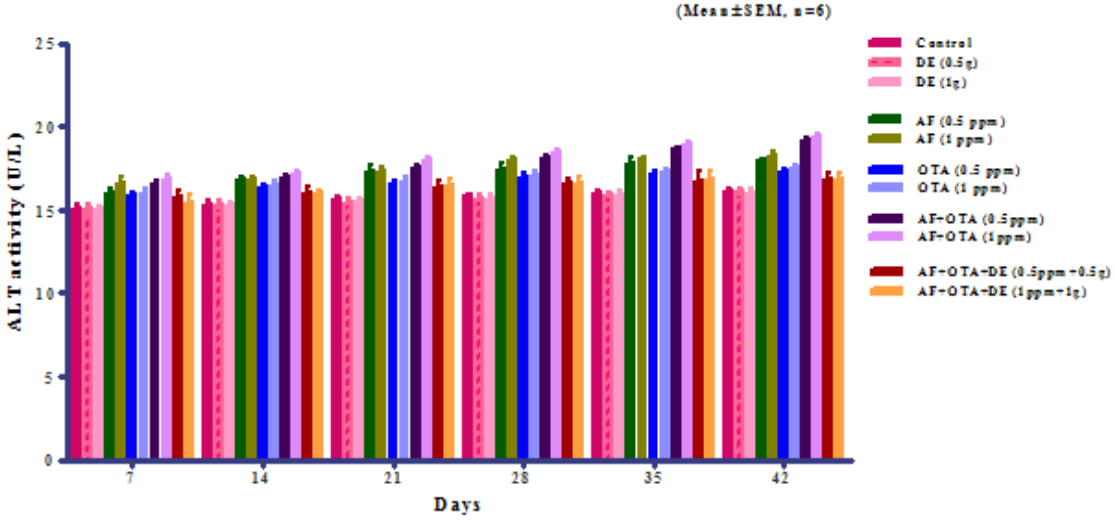
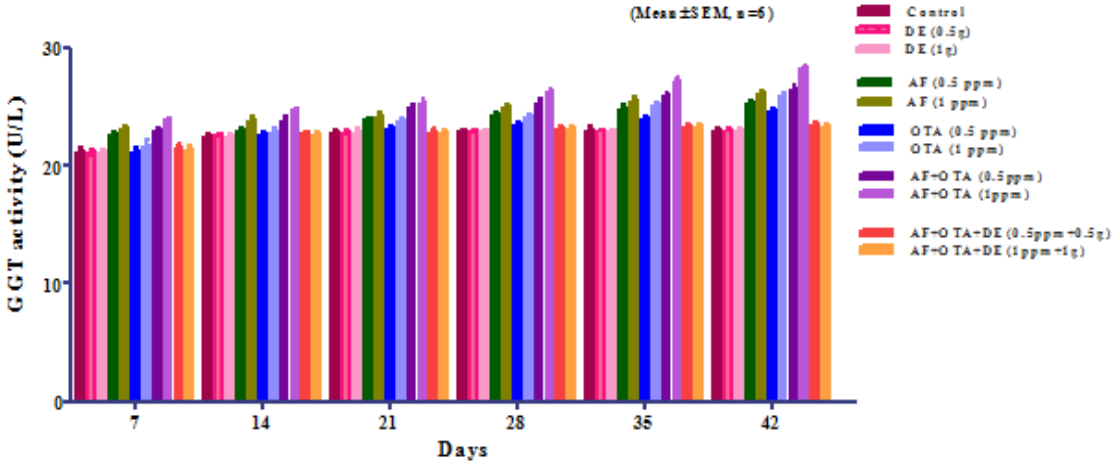


Fig 7. Effect of individual and combined AFB1 and OTA on GGT activity (U/L) and effect of D.E binder on combined mycotoxicosis in coloured broiler chicken



Between the treatment groups, there was no significant increase in the creatinine concentration in birds fed with the combined AFB_1 and OTA along with mycotoxins binder (Diatomaceous earth, DE) treated groups (Groups 10-11) as compared to combined AFB_1 and OTA groups (Groups 8-9) and control (Group 1), also the values reaching nearer to normal values.

4.5.1.5 Serum uric acid

The mean serum uric acid concentration of different groups of the experimental birds was measured at weekly intervals and the values have been summarized in Table 16 and represented graphically in Fig 9.

A significant ($P<0.001$) increase in the uric acid concentration was observed in Groups 6, 7, 8 and 9 from seventh day of the experiment till the end of the experimental period 42^{nd} day and significant ($P<0.001$) increase in the uric acid concentration was observed in Groups 4 and 5 from day 35^{th} to 42^{nd} day of the experiment as compared to control (Group1). No significant increase in the uric acid concentration was observed in birds fed with mycotoxin binder D.E, i.e Groups 2 and 3 as compared to control (Group1) till the end of 6th week of experiment.

There was significant ($P<0.001$) increase in the uric acid concentration in both individual low and high dose of OTA (Groups 6 and 7) as compared to both individual low and high dose of AFB1 (Groups 4 and 5) till the end of experimental period (42^{nd} day). A significant ($P<0.001$) increase in the uric acid concentration was observed in both low and high dose of combined mycotoxicoses (Groups 8 and 9) as individual low and high dose of AFB1 and OTA (Groups 4, 5, 6 and 7)

Between the treatment groups, there was no significant increase in the uric acid concentration in birds fed with the combined AFB_1 and OTA along with mycotoxins binder (Diatomaceous earth, DE) treated groups (Groups 10-11) as compared to combined AFB_1 and OTA groups (Groups 8-9) and control (Group 1), also the values reaching nearer to normal values.

4.5.1.6 Total protein

The mean total protein concentration of different groups of the experimental birds was measured at weekly interval and the values have been summarized in Table 17 and represented graphically in Fig 10.

A significant ($P<0.001$) decrease in the total protein concentration was observed in individual AFB1 and OTA groups (Groups 5, 6 and 7) from 21st day of the experiment and combined mycotoxicoses groups (Groups 8 and 9) from 14th day of the experiment till the end of the experimental period (42nd day) as compared to negative control (Group 1). No significant decrease in the total protein concentration was observed in birds fed with mycotoxin binder D.E, *i.e* Groups 2 and 3 as compared to control (Group1) till the end of 42nd day of experiment.

There was significant ($P<0.001$) decrease in the total protein concentration in low and high dose of individual OTA fed birds (Groups 6 and 7) as compared to low and high dose individual AFB1 from 35th day till the end of the experiment.

There was significant ($P<0.001$) decrease in the total protein concentration in low and high dose combined AFB1 and OTA groups (Groups 8 and 9) when compared with

individual AFB1 and individual OTA groups (Groups 4, 5, 6 and 7) from 14th day till the end of the experiment of 42nd day. Between combined AFB1 and OTA groups (Groups 8 and 9), Group 9 showed statistical significant ($P<0.05$) decrease in the total protein concentration from 28th day of the experiment till the end of 42nd day of experiment when compared to each other.

Between the treatment groups, there was no significant decrease in the total protein concentration in birds fed with the combined AFB_1 and OTA along with mycotoxins binder (Diatomaceous earth, DE) treated groups (Groups 10-11) as compared to combined AFB_1 and OTA groups (Groups 8-9) and control (Group 1), also the values reaching nearer to normal values.

4.6 Gross pathology

The Group I birds (negative control) showed no gross pathomorphological changes in all the organs examined at different days of sacrifice during the experimental study.

4.6.1 Liver

Liver appeared pale to yellowish discolouration, enlarged with fatty changes and friable in consistency in Groups 4, 5, 6 and 7 (Plate 3A and Plate 4A) birds throughout the experimental study. These changes were more severe in the early two weeks, and the changes were progressive with increase in dose and duration of exposure.

Table 15: Individual and combined effect of AFB1 and OTA on creatinine concentration (mg/dL) and effect of D.E binder on combined mycotoxicoses in coloured broiler chickens (Mean±SEM, n=6)

Groups	Treatment			Days					
	AFB1 (ppm)	OTA (ppm)	DE(g)	7	14	21	28	35	42
G 1	-	-	-	0.36±0.01 ^{abchi}	0.37±0.0 ^{abchi}	0.38±0.03 ^{abchi}	0.38±0.00^{abchi}	0.38±0.01 ^{abchi}	0.38±0.04 ^{abchi}
G 2	-	-	0.5	0.36±0.01 ^{abchi}	0.37±0.00 ^{abchi}	0.38±0.02 ^{abchi}	0.38±0.01 ^{abchi}	0.38±0.01^{abchi}	0.38±0.01 ^{abchi}
G 3	-	-	1	0.36±0.00 ^{abchi}	0.37±0.00 ^{abchi}	0.38±0.02 ^{abchi}	0.38±0.01 ^{abchi}	0.37±0.00 ^{abchi}	0.38±0.03 ^{abchi}
G 4	0.5	-	-	0.36±0.01 ^{abchi}	0.37±0.00 ^{abchi}	0.38±0.02 ^{abchi}	0.39±0.02 ^{abhi}	0.40±0.01 ^{b}	0.41±0.00^{b}
G 5	1	-	-	0.37±0.01 ^{abchi}	0.38±0.01 ^{abchi}	0.39±0.01 ^{abchi}	0.40±0.00 ^{bc}	0.41±0.00 ^{c}	0.42±0.00 ^{c}
G 6	-	0.5	-	0.38±0.01 ^{cde}	0.39±0.01 ^{cde}	0.41±0.00 ^{de}	0.42±0.00^{de}	0.42±0.01 ^{d}	0.43±0.00 ^{d}
G 7	-	1	-	0.39±0.02 ^{de}	0.40±0.00^{de}	0.42±0.00 ^{de}	0.43±0.00 ^{de}	0.44±0.01 ^{e}	0.46±0.01 ^{e}
G 8	0.5	0.5	-	0.40±0.01^{f}	0.43±0.01^{f}	0.45±0.00^{f}	0.47±0.00 ^{f}	0.48±0.0 ^{f}	0.50±0.01 ^{f}
G 9	1	1	-	0.42±0.00 ^{g}	0.45±0.00^{g}	0.49±0.00 ^{g}	0.50±0.01 ^{g}	0.52±0.01 ^{g}	0.55±0.01^{g}
G 10	0.5	0.5	0.5	0.37±0.01 ^{abchi}	0.37±0.00 ^{abchi}	0.38±0.00 ^{abchi}	0.38±0.02 ^{abchi}	0.38±0.02 ^{abchi}	0.39±0.01 ^{abchi}
G 11	1	1	1	0.37±0.00 ^{abchi}	0.38±0.00 ^{abchi}	0.38±0.02 ^{abchi}	0.39±0.01 ^{abchi}	0.39±0.02 ^{abchi}	0.39±0.03^{abchi}

Different superscripts within a row indicate significant differences (P≤0.05)

Table 16: Individual and combined effect of AFB1 and OTA on serum uric acid concentration (mg/dL) and effect of D.E binder on combined mycotoxicoses in coloured broiler chickens (Mean±SEM, n=6)

Groups	Treatment			Days					
	AFB1 (ppm)	OTA (ppm)	DE (g)	7	14	21	28	35	42
G 1	-	-	-	6.29±0.02 ^{abchi}	6.30±0.01 ^{abchi}	6.30±0.02 ^{abchi}	6.37±0.03 ^{abchi}	6.39±0.01 ^{ahi}	6.40±0.00 ^{ahi}
G 2	-	-	0.5	6.30±0.02 ^{abchi}	6.30±0.04 ^{abchi}	6.33±0.01 ^{abchi}	6.38±0.01 ^{abchi}	6.39±0.03 ^{ahi}	6.41±0.02 ^{ahi}
G 3	-	-	1	6.29±0.01 ^{abchi}	6.31±0.01 ^{abchi}	6.34±0.03 ^{abchi}	6.39±0.02 ^{abchi}	6.40±0.02 ^{ahi}	6.41±0.05 ^{ahi}
G 4	0.5	-	-	6.28±0.01 ^{abc}	6.32±0.00 ^{abc}	6.35±0.00 ^{abc}	6.38±0.00 ^{abc}	6.48±0.03 ^{bc}	6.51±0.03 ^{b}
G 5	1	-	-	6.29±0.00 ^{abc}	6.34±0.00 ^{abc}	6.37±0.02 ^{abc}	6.39±0.02 ^{abc}	6.49±0.01 ^{bc}	6.56±0.02 ^{c}
G 6	-	0.5	-	7.34±0.02 ^{d}	7.35±0.01 ^{d}	7.38±0.01 ^{d}	7.42±0.03 ^{d}	7.44±0.00 ^{d}	7.48±0.03 ^{d}
G 7	-	1	-	8.35±0.04 ^{e}	8.36±0.02 ^{e}	8.42±0.03 ^{e}	8.46±0.03 ^{e}	8.48±0.00 ^{e}	8.51±0.04 ^{e}
G 8	0.5	0.5	-	9.35±0.03 ^{f}	9.36±0.01 ^{f}	9.41±0.02 ^{f}	9.42±0.04 ^{f}	9.44±0.02 ^{f}	9.50±0.04 ^{f}
G 9	1	1	-	10.36±0.01 ^{g}	10.40±0.04 ^{g}	10.45±0.04 ^{g}	10.47±0.03 ^{g}	10.51±0.03 ^{g}	10.54±0.01 ^{g}
G 10	0.5	0.5	0.5	6.31±0.04 ^{abchi}	6.32±0.05 ^{abchi}	6.33±0.01 ^{abchi}	6.36±0.05 ^{abchi}	6.38±0.01 ^{ahi}	6.51±0.2 ^{ahi}
G 11	1	1	1	6.33±0.02 ^{abchi}	6.33±0.03 ^{abchi}	6.34±0.03 ^{abchi}	6.38±0.01 ^{abchi}	6.40±0.02 ^{ahi}	6.63±0.1 ^{ahi}

Different superscripts within a row indicate significant differences (P≤0.05)

Table 17: Individual and combined effect of AFB1 and OTA on total protein concentration (g/dL) and effect of D.E binder on combined mycotoxicoses in coloured broiler chicken (Mean±SEM, n=6)

Groups	Treatment			Days					
	AFB1 (ppm)	OTA (ppm)	DE (g)	7	14	21	28	35	42
G 1	-	-	-	2.52±0.3 ^{abcdefghi}	2.95±0.4 ^{ahi}	3.83±0.14 ^{ahi}	4.95±0.18 ^{ahi}	5.32±0.13 ^{ahi}	5.52±0.14 ^{ahi}
G 2	-	-	0.5	2.53±0.4 ^{abcdefghi}	2.96±0.3 ^{ahi}	3.85±0.12 ^{ahi}	4.92±0.14 ^{ahi}	5.30±0.13 ^{ahi}	5.52±0.12 ^{ahi}
G 3	-	-	1	2.52±0.3 ^{abcdefghi}	2.95±0.5 ^{ahi}	3.79±0.16 ^{ahi}	4.91±0.15 ^{ahi}	5.28±0.2 $^{a\ ahi}$	5.51±0.3 ^{ahi}
G 4	0.5	-	-	2.51±0.3 ^{abcdefghi}	2.83±0.7 ^{abcde}	3.72±0.6 ^{abcde}	4.62±0.2 ^{b}	4.74±0.1 ^{b}	4.82±0.1 ^{b}
G 5	1	-	-	2.39±0.7 ^{abcdefghi}	2.76±0.1 ^{abcde}	3.55±0.1 ^{bcde}	3.89±0.3 ^{cde}	4.20±0.6 ^{c}	4.26±0.2 ^{c}
G 6	-	0.5	-	2.53±0.3 ^{abcdefghi}	2.83±0.7 ^{abcde}	3.53±0.05 ^{bcde}	3.90±0.1 ^{cde}	4.58±0.1 ^{d}	4.90±0.1 ^{d}
G 7	-	1	-	2.50±0.6 ^{abcdefghi}	2.81±0.7 $^{a\ bcde}$	3.39±0.1 ^{bcde}	3.85±0.3 ^{cde}	4.21±0.1 ^{e}	4.29±0.3 ^{e}
G 8	0.5	0.5	-	2.51±0.3 ^{abcdefghi}	2.56±0.8 ^{fg}	2.76±0.1 ^{fg}	3.13±0.01 ^{f}	3.28±0.2 ^{f}	3.57±0.1 ^{f}
G 9	1	1	-	2.45±0.7 ^{abcdefghi}	2.51±0.2 ^{fg}	2.60±0.2 ^{fg}	2.81±0.07 ^{g}	3.00±0.3 ^{g}	3.20±0.1 ^{g}
G 10	0.5	0.5	0.5	2.50±0.2 ^{abcdefghi}	2.90±0.1 ^{ahi}	3.77±0.3 ^{ahi}	4.78±0.14 ^{ahi}	5.10±0.15 ^{ahi}	5.34±0.14 ^{ahi}
G 11	1	1	1	2.47±0.2 ^{abcdefghi}	2.86±0.3 ^{ahi}	3.72±0.2 ^{ahi}	4.73±0.12 ^{ahi}	5.06±0.16 ^{ahi}	5.30±0.12 ^{ahi}

Different superscripts within a row indicate significant differences (P≤0.05)

Fig 8. Individual and combined effect of AFB1 and OTA on creatinine concentration (mg/dL) and effect of D.E binder on combined mycotoxicosis in coloured broiler chicken

(Mean±SEM, n=6)

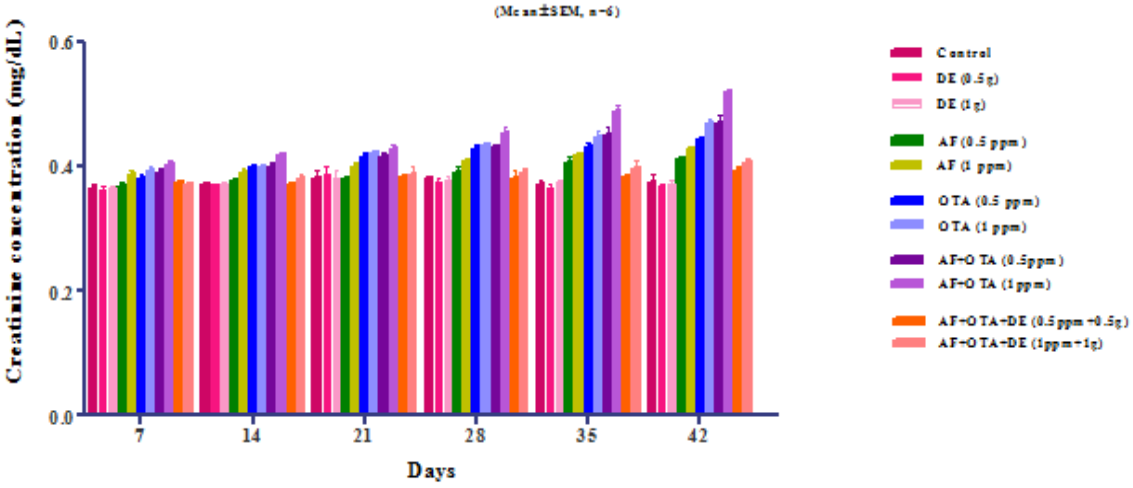


Fig 9. Individual and combined effect of AFB1 and OTA on Serum uric acid (mg/dL) and effect of D.E binder on combined mycotoxicosis in coloured broiler chicken

(Mean±SEM, n=6)

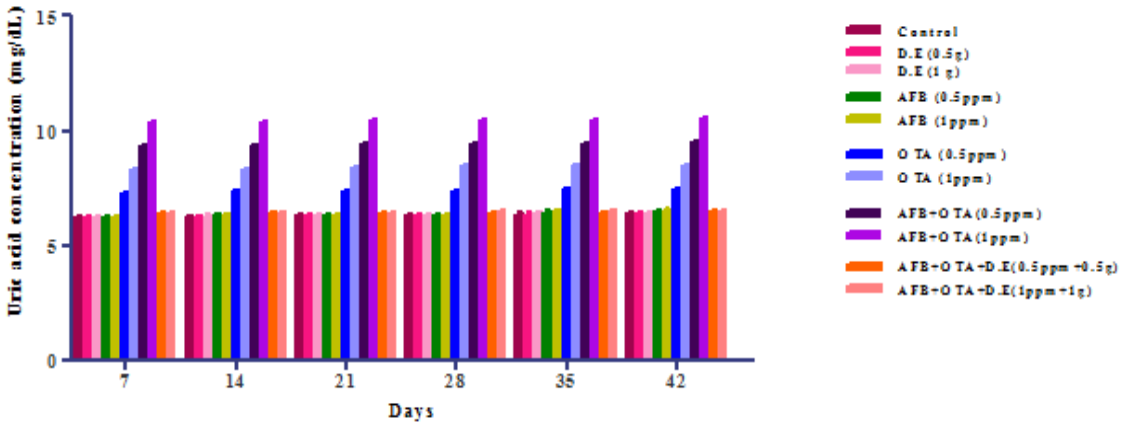
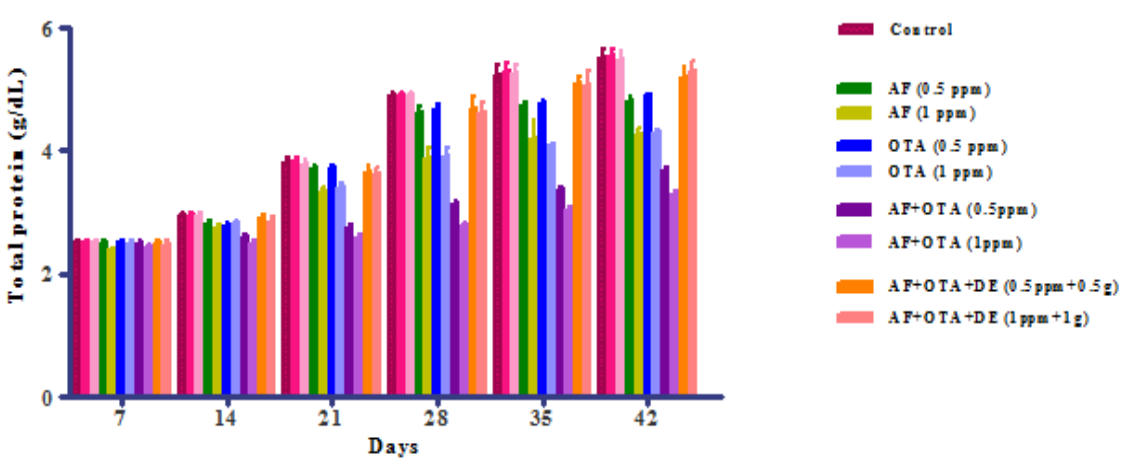


Fig 10. Individual and combined effect of AFB1 and OTA on Total protein concentration (g/dL) and effect of D.E binder on combined mycotoxicosis in coloured broiler chicken

(Mean±SEM, n=6)



Marked paleness, enlarged with fatty changes, friable in consistency, severe congestion and haemorrhage were evident in the liver of combined mycotoxins fed groups (Groups 8 and 9) throughout the experimental study (Plate 5A). In the first three weeks mild paleness, mild congestion and fatty changes were noticed in liver of combined mycotoxins fed groups (Groups 10 and 11) along with D.E toxin binder, later these changes disappeared (Plate 8A).

4.6.2 Kidney

In Groups 4 and 5 birds, the kidneys appeared slightly pale and swollen in the first and second weeks. At the end of third week onwards kidneys appeared mildly congested and moderately congested at the end of the experimental period (Plate 3C).

Enlarged and pale kidneys with congestion and haemorrhages were more pronounced in groups fed with ochratoxin (Groups 6 and 7) (Plate 4C) and aflatoxin-ochratoxin combined mycotoxicoses groups (Groups 8 and 9) (Plate 5C) throughout the experimental study and these changes were marked and severe in subsequent week of exposure to ochratoxin and combined AFB1 and OTA exposure.

In the first three weeks mild congestion and haemorrhages were noticed in kidney of combined mycotoxicoses groups (Groups 10 and 11) along with D.E toxin binder, later these changes were disappeared (Plate 8C).

4.6.3 Lymphoid organs

4.6.3.1 Thymus

Presence of mild focal areas of hemorrhages and congestion were observed in thymic lobes of birds treated with aflatoxin and these changes were more evident after three weeks of experiment ((Plate 3E). In ochratoxin fed groups (Groups 6 and 7), mild congestion and atrophy were noticed and these changes were progressive as the duration of exposure increased (Plate 4E).

Severe congestion and hemorrhages, marked atrophy of thymic lobes were noticed in combined groups (Groups 8 and 9) and these changes were more progressive with increase in the dose and duration of exposure (Plate 5E). Intensity of these gross pathological changes was not noticed in combined mycotoxin groups (Groups 10 and 11) treated with D.E toxin binder (Plate 8C).

4.6.3.2 Spleen and Bursa of Fabricius

Presence of mild focal areas of hemorrhages and congestion, slight atrophy of spleen and bursa of Fabricius were observed in birds treated with aflatoxin (Groups 4 and 5), (Plate 3G) and these changes were more evident after three weeks of experiment. In Ochratoxin groups (Groups 6 and 7), mild congestion and hemorrhages were noticed in bursa of Fabricius and these changes were progressive as the duration of exposure increased.

Severe congestion and hemorrhages, marked atrophy of bursa of Fabricius was noticed in combined mycotoxins fed groups (Groups 8 and 9) and these changes were

more progressive with increase in the dose and duration of exposure. Intensity of these gross pathological changes was less in combined mycotoxin groups (Groups 10 and 11) treated with D.E toxin binder.

4.7 Histopathology

4.7.1 Liver

Sections of liver in Groups 1, 2 and 3 did not reveal any histopathological changes throughout the experimental period (42 day).

Histopathological changes in the liver of birds fed with aflatoxin and combined mycotoxin (Groups 4, 5 and Group 8, 9) reveal degenerative changes in liver parenchyma, including mild degree of congestion, swollen, fatty change with formation of micro and macro fat globules and necrotic changes in individual hepatocyte were noticed in seventh day of the experiment . From third week onwards changes like mild to moderate degree of congestion, glandular arrangement of the hepatocytes, vacuolar degeneration and necrosis, bile duct epithelial hyperplasia were noticed. These changes were more pronounced in the sixth week of the experiment in both aflatoxin and combined mycotoxins fed birds (Groups 4, 5 and Groups 8, 9) (Plate 3B and Plate 5B).

Microscopically, the liver of ochratoxin fed birds (Groups 6 and 7) showed histological changes such as mild to moderate congestion, swollen hepatocytes with vacuolar degeneration, areas of hemorrhages and necrosis along with infiltration of heterophils and lymphoid cells in the portal areas. Fatty vacuoles were observed in the cytoplasm of some birds throughout the experimental period (Plate 4B).Combined

mycotoxin fed groups (Groups 10 and 11) with D.E binder showed mild congestion and degenerative changes.

4.7.2 Kidney

Sections of kidney in Groups 1, 2 and 3 did not reveal any histopathological changes throughout the experimental period.

Histopathological changes in the kidney of birds fed with ochratoxin and co-mycotoxin (Groups 6, 7, 8 and 9) reveal severe tubular distension, occlusion of lumen, inter tubular hemorrhages, swollen tubular epithelium with degenerative changes and desquamation of epithelial cells, in some of the tubules deposition of urate crystals was noticed in some birds (Plate 4D, Plate 5D, and Plate 6C). Additionally, there was infiltration of lymphocytes in renal parenchyma along with accumulation of eosinophilic homogenous material in the lumen, edema (Plate 6E). At later period of observation pathological changes in tubular epithelium were more pronounced.

At 42nd day of age, massive intertubular hemorrhages, varying degrees of degeneration and necrosis, extensive destruction of tubular epithelium with detachment of tubular cells from basement membrane was observed (Plate 6D and Plate 6F). Combined mycotoxicoses treated groups (Groups 10 and 11) with D.E binder showed very mild congestion and distension of tubular epithelium in early stage of exposure later completely disappeared (Plate 8D).

Kidney of birds fed with aflatoxin showed swollen tubular epithelium, presence of intertubular hemorrhages, degenerative changes in tubular epithelium as well as

condensation of nuclear material (Plate 6A). On 42nd day of age, some of the tubules showed necrosis, separation of epithelial cells from basement membrane and areas of hemorrhages (Plate 3D and Plate 6B).

4.7.3 Thymus

Sections of thymus in Groups 1, 2 and 3 did not reveal any histopathological changes throughout the experimental period (42 day).

Section from thymus of aflatoxin and ochratoxin fed Groups (Groups 4, 5, 6 and 7) reveal depletion of lymphocytes from thymic follicle from seventh day of experiment and these changes were more pronounced in later period of exposure (Plate 3F and Plate 4F). Among mycotoxin treated groups, these histopathological changes were more pronounced in combined mycotoxin groups (Groups 8 and 9) as compared to single mycotoxin fed groups. In these groups, moderate degree of hemorrhage, lymphocytolysis and massive depletion of lymphocyte extending from medulla to cortex of thymic follicle resulting in thinning of the cortex and infilteration of heterophils, eosinophillic debries was observed (Plate 5F). D.E binder treated combined mycotoxicoses groups (Groups 10 and 11) showed normal architecture of thymic lobes (Plate 8F).

4.7.4 Bursa of Fabricius

Sections of bursa of Fabricius in Groups 1, 2 and 3 did not reveal any histopathological changes throughout the experimental period (42 day). On seventh day of age, sections from bursa of aflatoxin and ochratoxin Groups (Groups 4, 5, 6 and 7) reveal depletion of medullary lymphocytes from bursal follicle (Plate 3H, Plate 7A and

Plate 7C) and these changes were more progressive with increase in dose and duration of exposure (Plate 7B and Plate 7D).

Among mycotoxin treated groups, these histopathological changes were more pronounced in combined mycotoxins groups (Groups 8 and 9) as compared to single mycotoxins fed groups (Plate 7E). On 42nd day of age, reduction in size of bursal follicles, depletion and necrosis of lymphoid cells from follicle with proliferation of fibrous connective tissue in inter follicular space was observed (Plate 7F). Section of bursa from groups 10 and 11 did not reveal any appreciable changes at 42nd day of age in these groups in contrast to respective D.E binder untreated-mycotoxicated groups (Plate 8H).

4.7.5 Spleen

Sections of spleen in Groups 1, 2 and 3 did not reveal any histopathological changes throughout the experimental period (42 day).

Sections of spleen from aflatoxin and ochratoxin fed groups (Groups 4, 5, 6 and 7) reveal mild to moderate degree of lymphocytolytic activity from seventh day till the end of the expeiment. Among mycotoxin treated groups, these changes were more progressive, massive lymphocytolysis presence of hemorrhages and more proliferation of histiocytes were observed (Plate 5H) in combined mycotoxins fed groups (Group 8 and 9). These changes were not noticed in combined mycotoxins groups treated with D.E binder (Group 10 and 11).

Plate 3A: Gross appearance of liver in a 42 day old coloured broiler chicken fed with high dose of aflatoxin showing paleness, fatty changes, presence of congestion and hemorrhages.

Plate 3B: Section of liver from 42 day old coloured broiler chicken fed (high dose) with aflatoxin showing severe vacuolation and degenerative changes in parenchyma, fatty vacuoles, bile duct hyperplasia, presence of congestion and hemorrhages (H&E, X200).

Plate 3C: Gross appearance of kidney in a 42 day old coloured broiler chicken fed (high dose) with aflatoxin showing swollen areas and hemorrhages.

Plate 3D: Section of kidney in a 42 day old coloured broiler chicken fed (high dose) with aflatoxin showing swollen tubular epithelium, distended tubules and presence of inter tubular hemorrhages (H&E, X200).

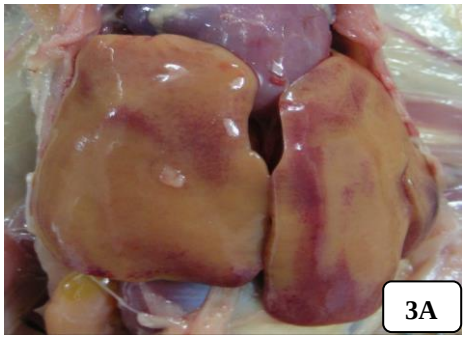
Plate 3E: Gross appearance of thymus in 42 day old coloured broiler chicken fed (high dose) with aflatoxin showing swollen and severely congested thymic lobules.

Plate 3F: Section of thymus lobes in 42 day old coloured broiler chicken fed (high dose) with aflatoxin showing lymphocytolysis, depletion of lymphocytes, hemorrhages and degenerative changes in thymic (H&E, X200).

Plate 3G: Gross appearance of bursa of Fabricius in 42 day old coloured broiler chicken fed with (high dose) aflatoxin showing severe reduction in size and hemorrhages noticed.

Plate 3H: Section of bursa of Fabricius in 42 day old coloured broiler chicken fed with (high dose) aflatoxin showing lymphocytolysis and depletion of lymphocytes, hemorrhages in bursal follicle (H&E, X100)

PLATE 3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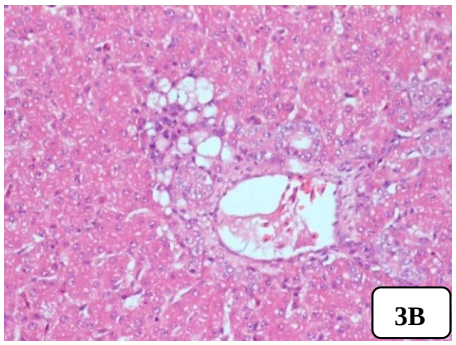
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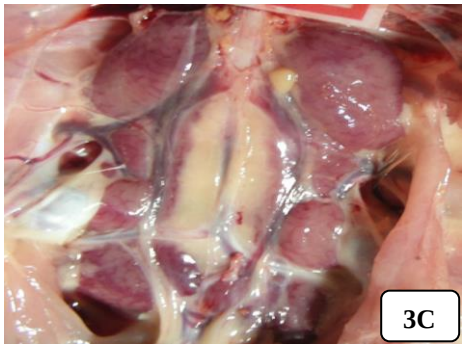
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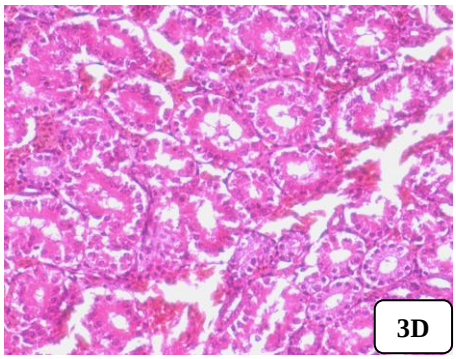
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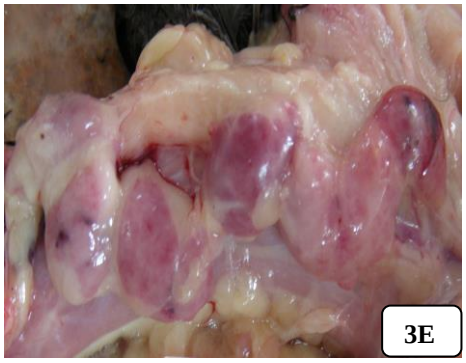
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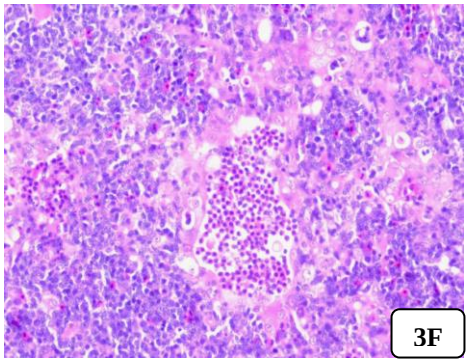
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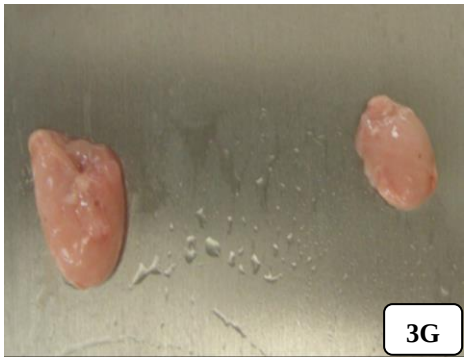
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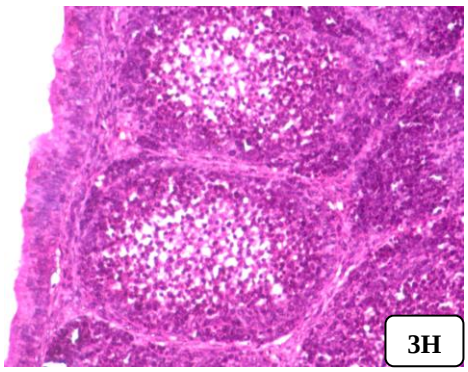

3H

Plate 4A: Gross appearance of liver in 42 day old coloured broiler chicken fed with (high dose) ochratoxin showing enlargement, paleness, fatty changes and presence of focal areas of congestion.

Plate 4B: Section of liver in 42 day old coloured broiler chicken fed with (high dose) ochratoxin showing distended sinusoidal spaces, vacuolation and degenerative changes along with necrosis of individual hepatocytes (H&E, X200).

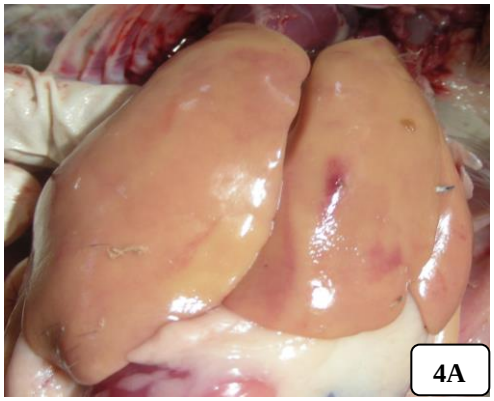
Plate 4C: Gross appearance of kidney in 42 day old coloured broiler chicken fed with (high dose) ochratoxin showing marked swelling, paleness and hemorrhages.

Plate 4D: Section of kidney in 42 day old coloured broiler chicken fed with (high dose) ochratoxin showing massive tubular degeneration and necrosis, presence of inflammatory cells and erythrocytes in interstitial spaces, desquamation of tubular epithelium with hyaline casts in the tubular lumen (H&E, X200).

Plate 4E: Gross appearance of thymus in 42 day old coloured broiler chicken fed with (high dose) of ochratoxin showing atrophy and severe congestion of thymic lobules

Plate 4F: Section of thymus in 42 day old coloured broiler chicken fed with (high dose) ochratoxin showing lymphocytolysis and depletion of lymphocytes, presence of hemorrhages and degenerative changes in thymic lobes (H&E, X200).

PLATE 4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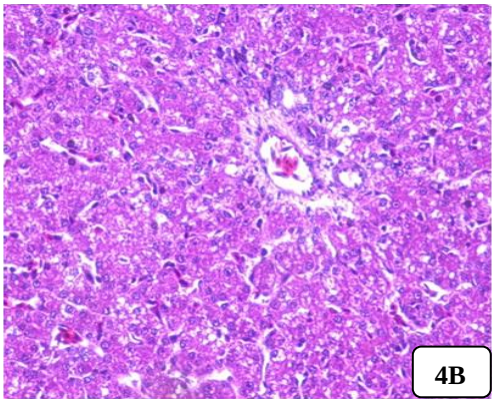
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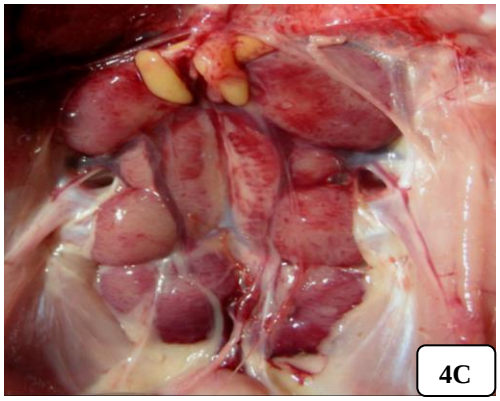
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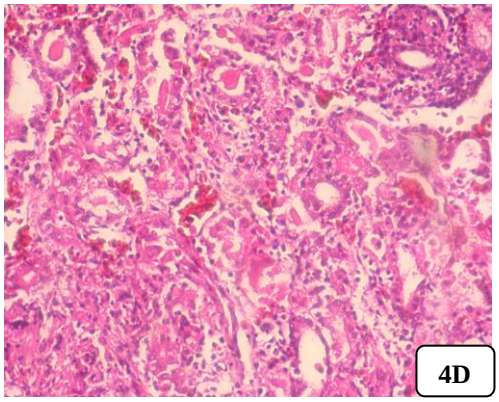
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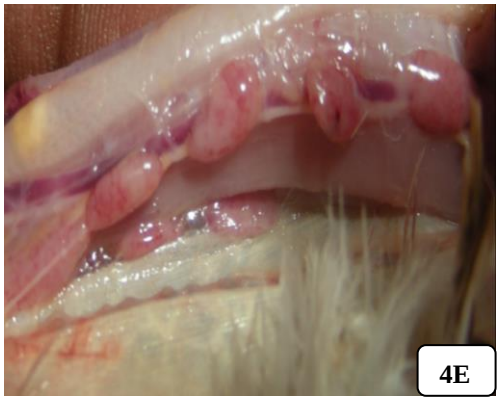
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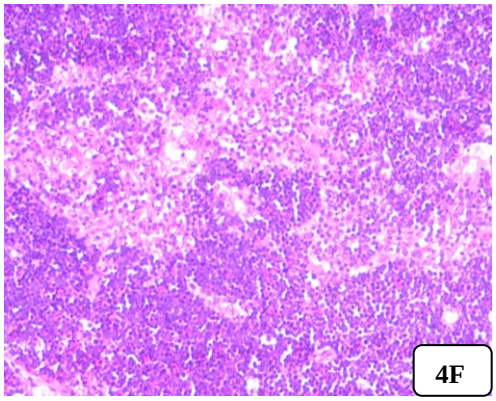

4F

Plate 5A: Gross appearance of liver in a 42 day old coloured broiler chicken fed with (high dose) combined mycotoxin (AFB1 and OTA) showing marked paleness, swollen, presence of congestion and hemorrhages.

Plate 5B: Section of liver in 42 day old coloured broiler chicken fed with (high dose) combined mycotoxins (AFB1 and OTA) showing swollen hepatocytes, degenerative and necrotic changes in parenchyma, bile duct hyperplasia and congestion and hemorrhages (H&E, X200)

Plate 5C: Gross appearance of kidney in a 42 day old coloured broiler chicken fed with (high dose) combined mycotoxin (AFB1 and OTA) showing swollen and distended parenchyma, paleness and mottled appearance.

Plate 5D: Section of kidney in 42 day old coloured broiler chicken fed with (high dose) combined mycotoxin (AFB1 and OTA) showing massive degenerative changes and total desquamation of tubules, presence of intertubular hemorrhages and appearance of hyaline casts in lumen of tubules (H&E, X200).

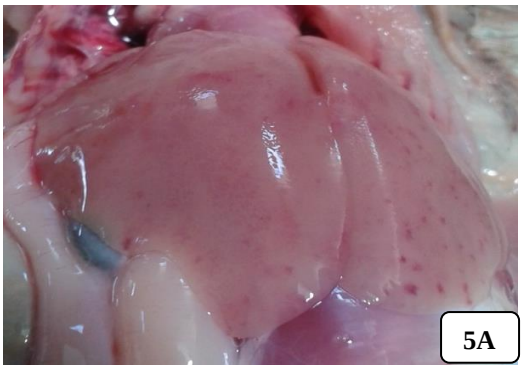
Plate 5E: Gross appearance of thymus in a 42 day old coloured broiler chicken fed with (high dose) combined mycotoxin (AFB1 and OTA) showing marked atrophy with severe congestion of thymic lobules.

Plate 5F: Section of thymus in 42 day old coloured broiler chicken fed with (high dose) combined mycotoxin (AFB1 and OTA) showing severe lymphocytolysis and depletion of lymphocytes, presence of hemorrhages and degenerative changes in thymic lobes (H&E, X400).

Plate 5G: Gross appearance of spleen in a 42 day old coloured broiler chicken fed with (high dose) combined mycotoxin (AFB1 and OTA) showing swelling and massive congestion.

Plate 5H: Section of spleen in 42 day old coloured broiler chicken fed with (high dose) combined mycotoxin (AFB1 and OTA) showing lymphocytolysis and depletion of lymphocytes, presence of hemorrhages and degenerative changes in spleenic follicle (H&E, X200).

PLATE 5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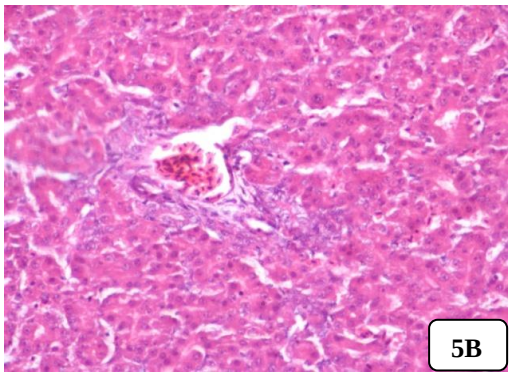
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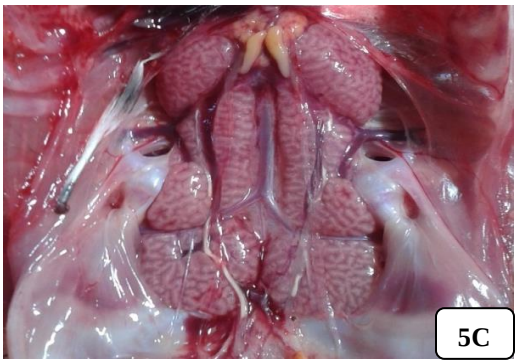
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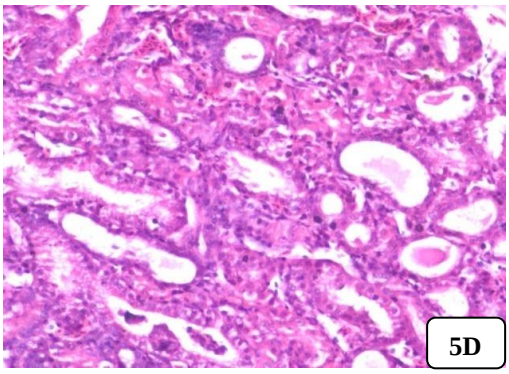
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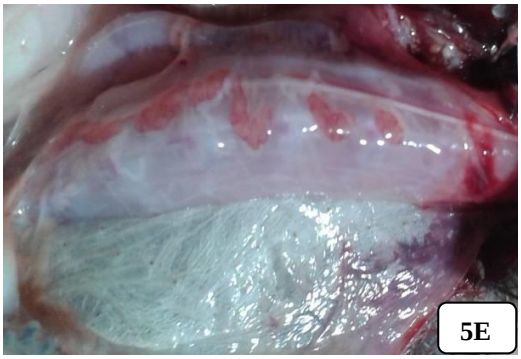
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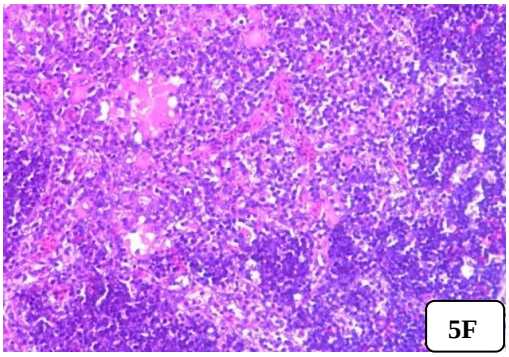
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5G

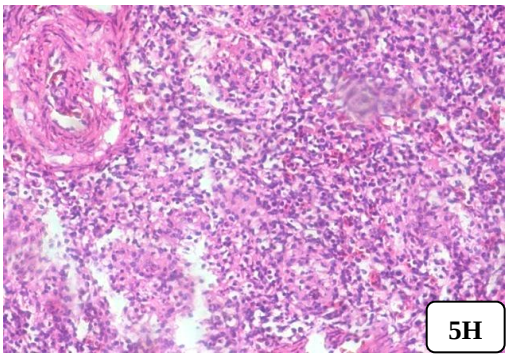

5H

Plate 6A: Section of kidney in seven day old coloured broiler chicken fed with (high dose) aflatoxin showing swollen tubular epithelium and presence of intertubular hemorrhages ((H&E, X200)

Plate 6B: Section of kidney in 42 day old coloured broiler chicken fed with (high dose) aflatoxin showing swollen tubular epithelium, desquamation of tubular epithelium with intertubular hemorrhages (H&E, X400)

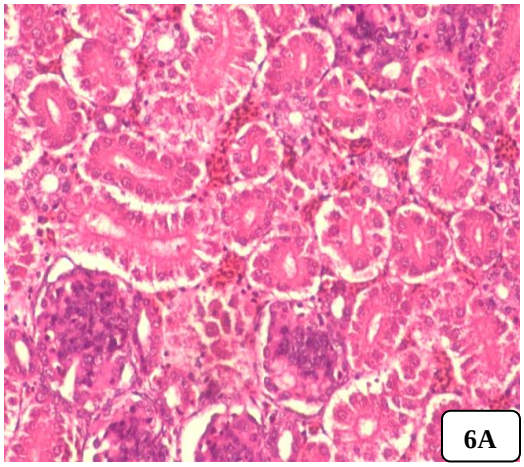
Plate 6C: Section of kidney in seven day old coloured broiler chicken fed with (high dose) ochratoxin showing severe tubular degeneration and desquamation of epithelium, intertubular hemorrhages also note edema and urate crystals ((H&E, X200)

Plate 6D: Section of kidney in 42 day old coloured broiler chicken fed with (high dose) ochratoxin showing swollen tubular epithelium, intertubular hemorrhages, degeneration and desquamation of tubular epithelium (H&E, X400)

Plate 6E: Section of kidney in seven day old coloured broiler chicken fed with (high dose) combined mycotoxin (AFB1 and OTA) showing massive tubular degeneration and desquamation of epithelium, intertubular hemorrhages also note edema and hyaline like mass in tubules ((H&E, X200)

Plate 6F: Section of kidney in 42 day old coloured broiler chicken fed with (high dose) combined mycotoxin (AFB1 and OTA) showing varying stages of degenerative and necrotic changes, presence of intertubular hemorrhages and presence of hyaline masses (H&E, X200)

PLATE 6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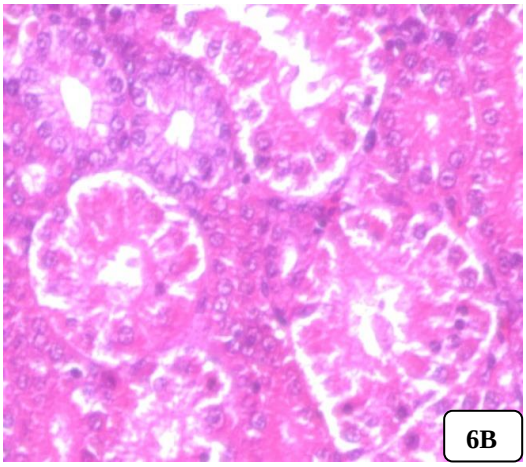
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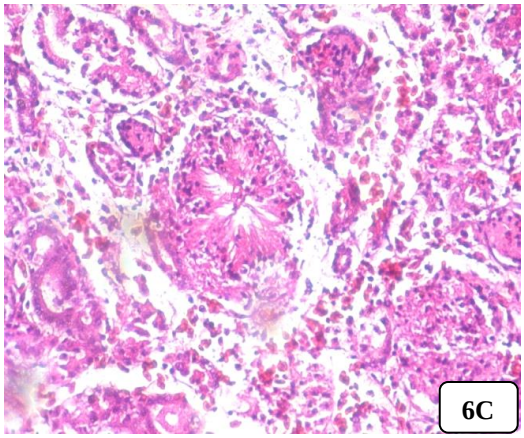
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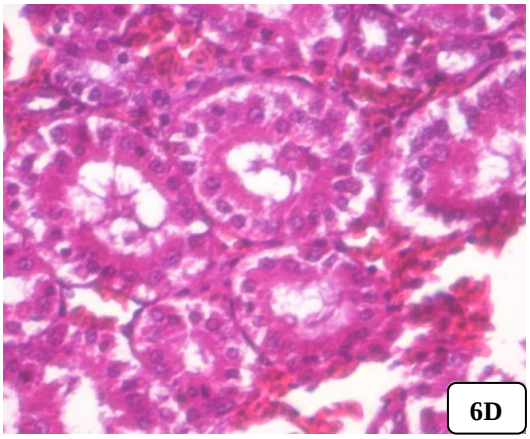
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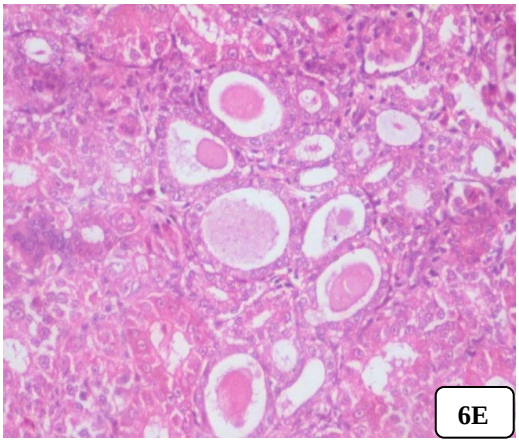
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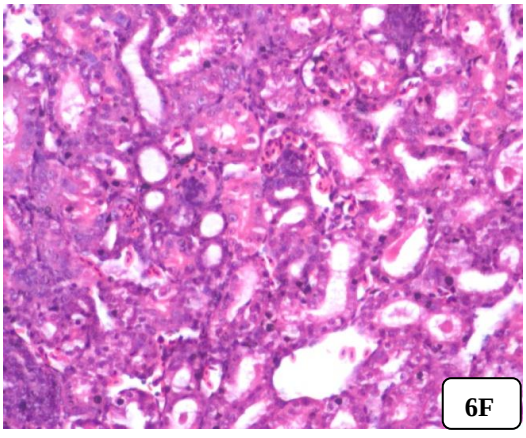

6F

Plate 7A: Section of bursa of Fabricius in seven day old coloured broiler chicken fed with (high dose) aflatoxin showing lymphocytolysis and depletion of lymphocytes with hemorrhages in bursal follicle (H&E, X400).

Plate 7B: Section of bursa of Fabricius in 42 day old coloured broiler chicken fed with (high dose) aflatoxin showing lymphocytolysis and depletion of lymphocytes with hemorrhages. Also note interfollicular fibrosis in bursal follicle (H&E, X400).

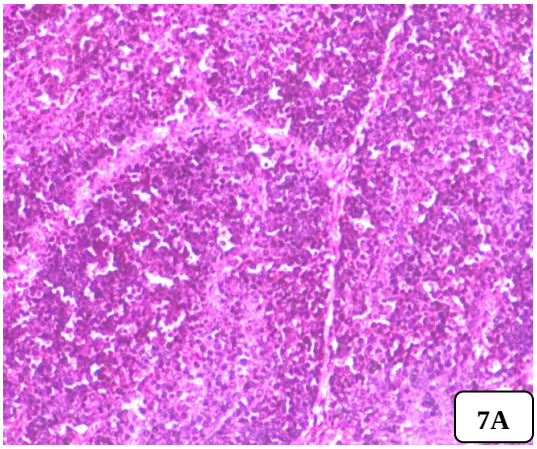
Plate 7C: Section of bursa of Fabricius in seven day old coloured broiler chicken fed with (high dose) ochratoxin showing lymphocytolysis and depletion of lymphocytes with massive hemorrhages in bursal follicle (H&E, X200).

Plate 7D: Section of bursa of Fabricius in 42 day old coloured broiler chicken fed with (high dose) ochratoxin showing lymphocytolysis and depletion of lymphocytes with hemorrhages. Also note interfollicular fibrosis in bursal follicle (H&E, X200).

Plate 7E: Section of bursa of Fabricius in seven day old coloured broiler chicken fed with (high dose) combined mycotoxins (AFB1 and OTA) showing massive lymphocytolysis and depletion of lymphocytes with hemorrhage and empty follicles forming cystic spaces in bursal follicle (H&E, X100).

Plate 7F: Section of bursa of Fabricius in 42 day old coloured broiler chicken fed with (high dose) combined mycotoxins (AFB1 and OTA) showing severe lymphocytolysis and sparcity of lymphocytes, presence of hemorrhages, also note severe interfollicular fibrosis in bursal follicle (H&E, X200).

PLATE 7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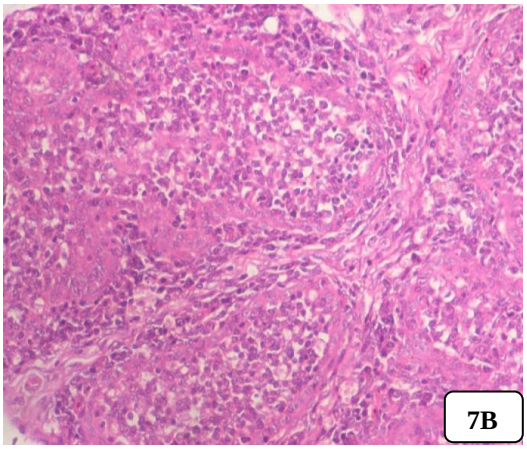
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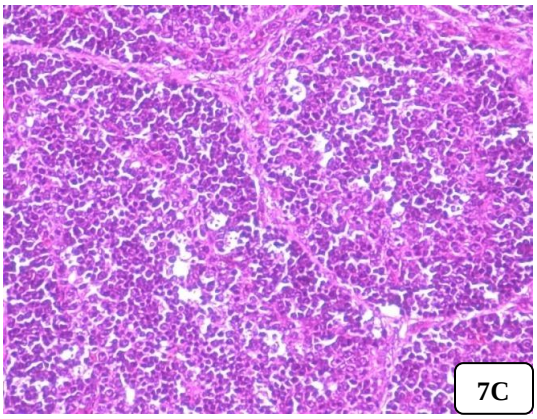
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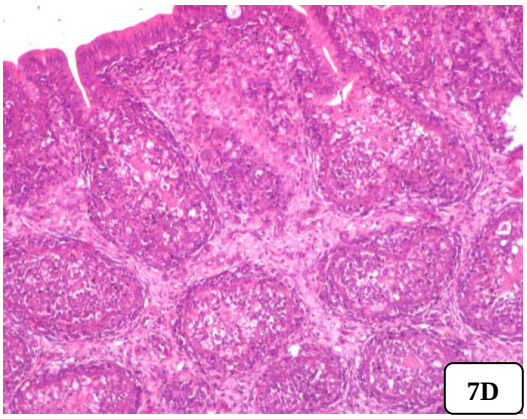
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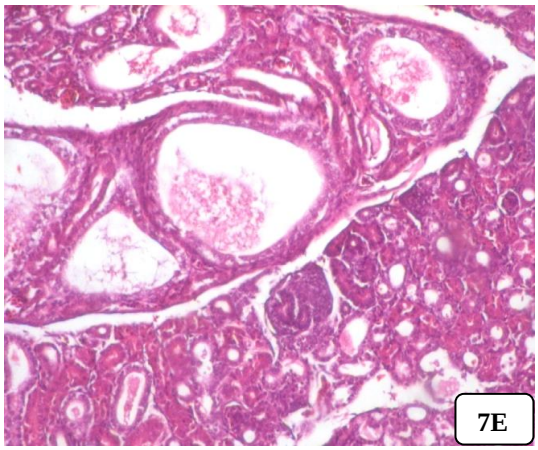
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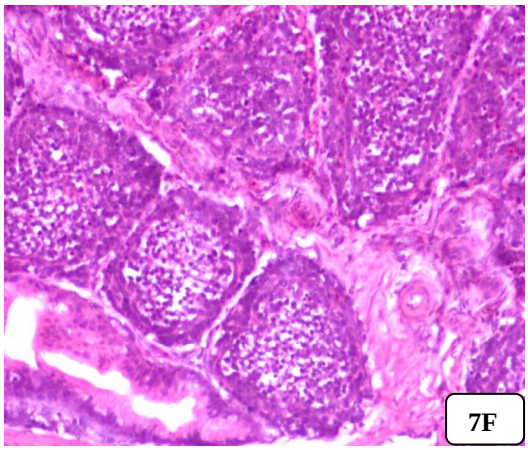

7F

Plate 8A: Gross appearance of liver in a 42 day old coloured broiler chicken fed with (high dose) combined mycotoxins (AFB1 and OTA) along with D.E binder showing mild congestion and fatty change.

Plate 8B: Section of liver in 42 day old coloured broiler chicken fed with (high dose) combined mycotoxins (AFB1 and OTA) along with D.E binder showing normal architecture of liver parenchyma (H&E, X100)

Plate 8C: Gross appearance of kidney in a 42 day old coloured broiler chicken fed with (high dose) combined mycotoxins (AFB1 and OTA) along with D.E binder showing normal appearance of kidney.

Plate 8D: Section of kidney in 42 day old coloured broiler chicken fed with (high dose) combined mycotoxins (AFB1 and OTA) treated with D.E showing normal architecture of tubular epithelium (H&E, X200)

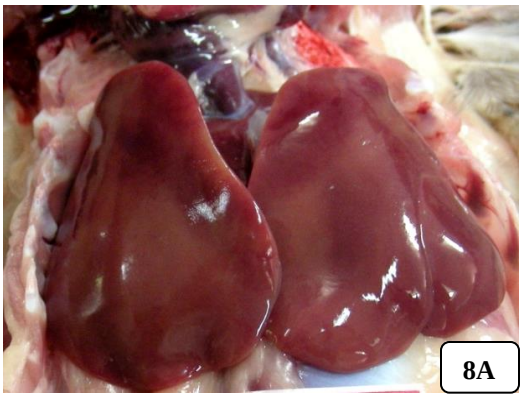
Plate 8E: Gross appearance of thymus in a 42 day old coloured broiler chicken fed with (high dose) combined mycotoxins (AFB1 and OTA) along with D.E binder showing normal appearance of thymic lobes

Plate 8F: Section of thymus in 42 day old coloured broiler chicken fed with (high dose) combined mycotoxins (AFB1 and OTA) along with D.E binder showing normal architecture of thymic lobes (H&E, X200)

Plate 8G: Gross appearance of bursa of Fabricius in a 42 day old coloured broiler chicken fed with (high dose) combined mycotoxins (AFB1 and OTA) along with D.E binder showing normal appearance of bursal follicles.

Plate 8H: Section of bursa of Fabricius in 42 day old coloured broiler chicken fed with (high dose) combined mycotoxins (AFB1 and OTA) treated with D.E showing normal architecture of bursal follicle (H&E, X100).

PLATE 8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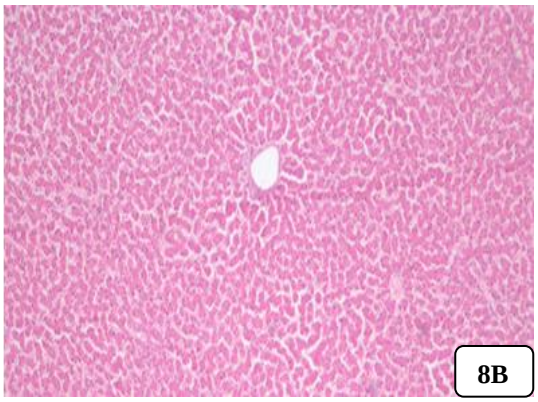
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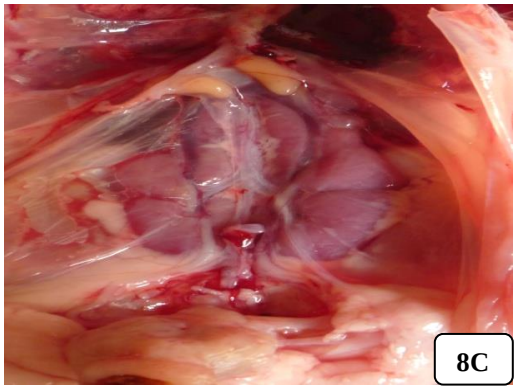
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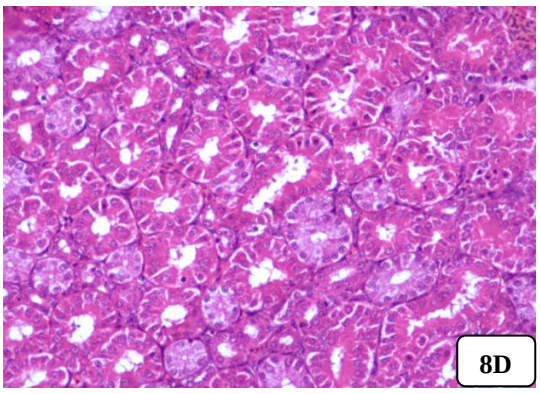
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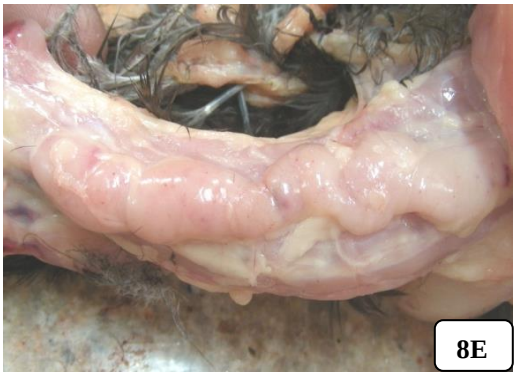
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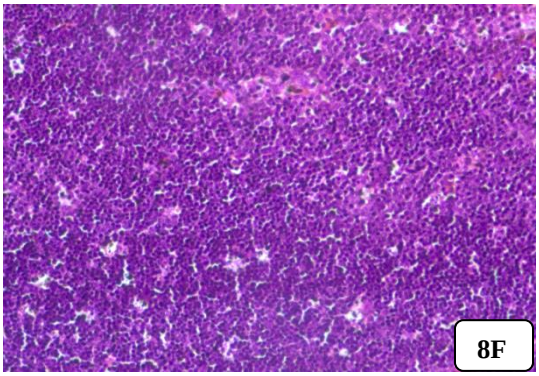
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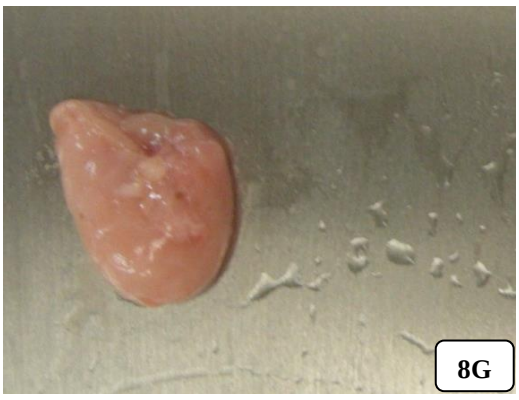
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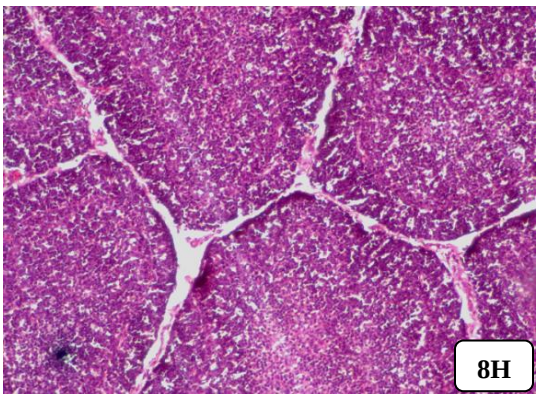

8H

4.8 Validation of method

4.8.1 Selectivity, Specificity and chromatography

The degree of interference by endogenous tissue constituents with the analytes and internal standard was assessed by the inspection of chromatograms derived from the processed blank tissue sample. The respective chromatograms of blank tissue sample, extracted lower limit of quantification and upper limit of quantification samples for both the AFB1, AFB2 and OTA were shown in Fig 11a,11b,11c, 12a,12b,12c and 13a,13b,13c, respectively. No significant endogenous material interference and no interfering peaks were observed in the blank tissue sample at the retention time of the target analytes and internal standard.

For confirmation purposes, specific fragmentation pattern of individual analyte was used for distinguishing the analyte from the matrix interferences thus allowing for greater evidence in compound identification.The parameters of the m/z and collision energy of parent ions and quantitative daughter ions from 3 kinds of mycotoxins are shown in Table 18.

Table 18. Optimum precursor and product ions with the respective collision energy (eV) for MS/MS.

Mycotoxins	t_R min	Dwell time, s	Precursor ion, (m/z)	Product ion, (m/z)	DP (V)	CE (eV)
Aflatoxin B_1 (AFB1)	2.16	0.2	312.3 $[M+H]^+$	313.2/241.0	50	30
Aflatoxin B2 (AFB2)	2.12	0.2	314.3 $[M+H]^+$	315.0/259.1	50	30
Ochratoxin A (OTA)	2.74	0.2	403.2 $[M+H]^+$	404.0/239.0	50	30

Fig. 11a. Chromatogram of blank tissue sample for AFB1

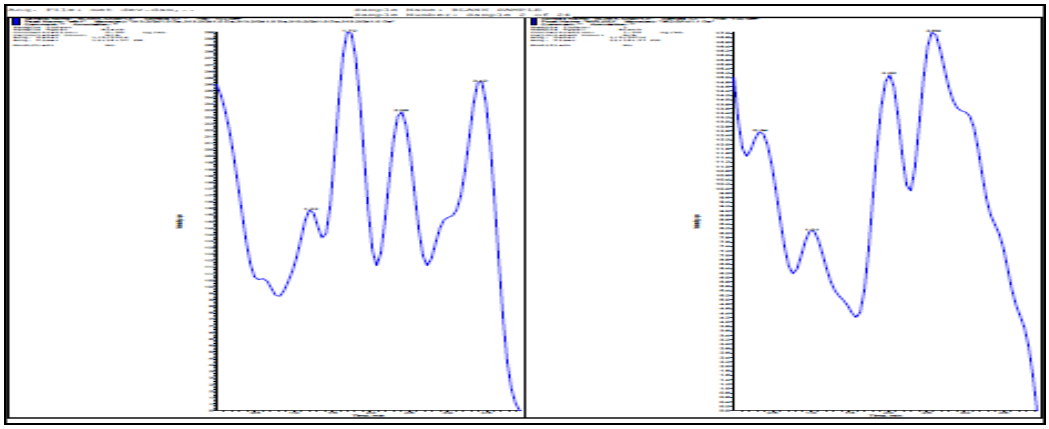


Fig. 11b. Chromatogram of extracted lower limit of quantification sample for AFB1

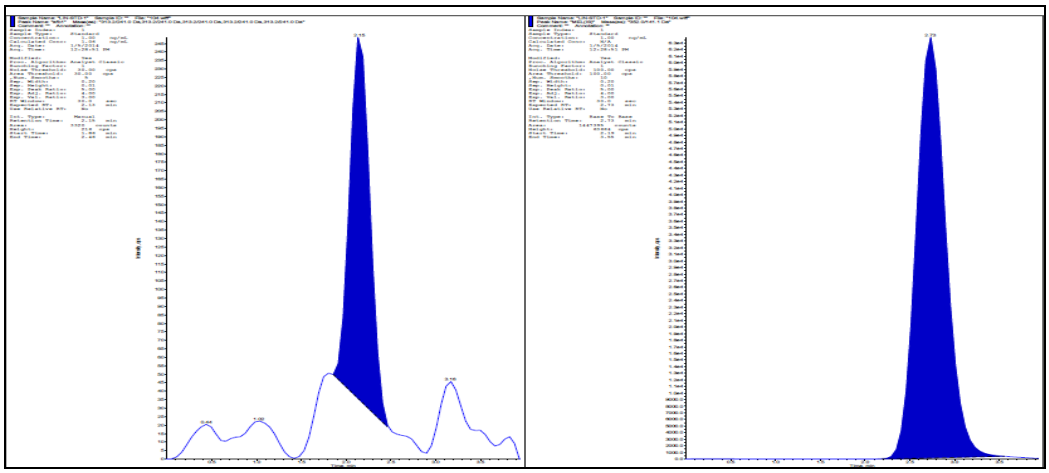


Fig. 11c . Chromatogram of extracted upper limit of quantification sample for AFB1

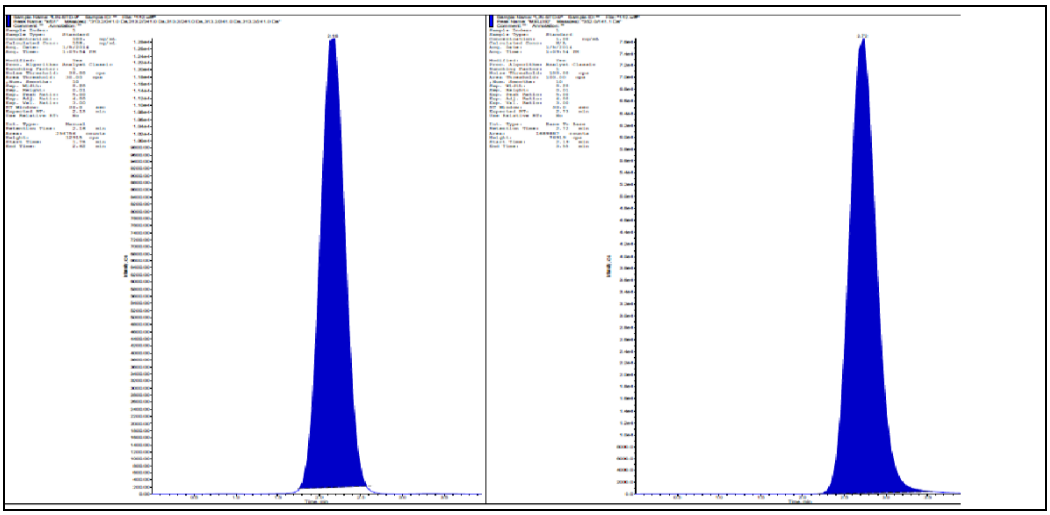


Fig. 12a. Chromatogram of blank tissue sample for AFB2

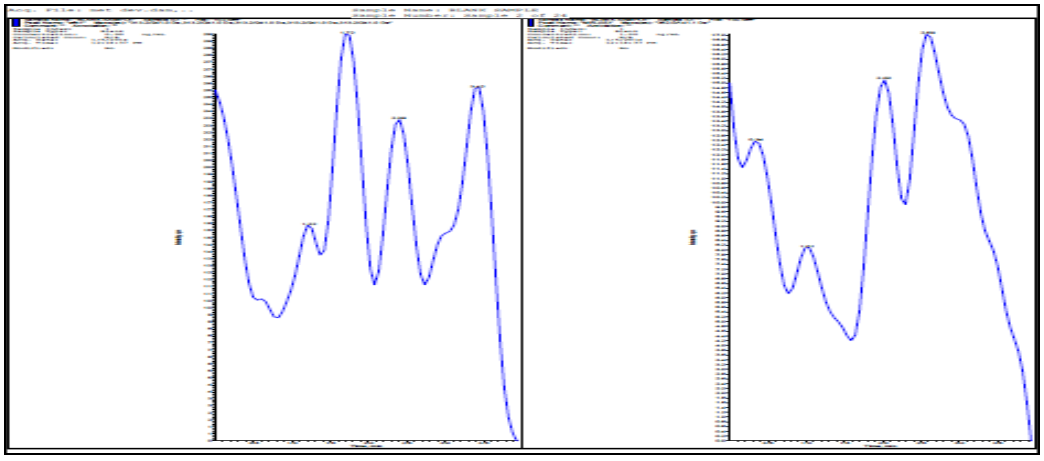


Fig. 12b. Chromatogram of extracted lower limit of quantification sample for AFB2

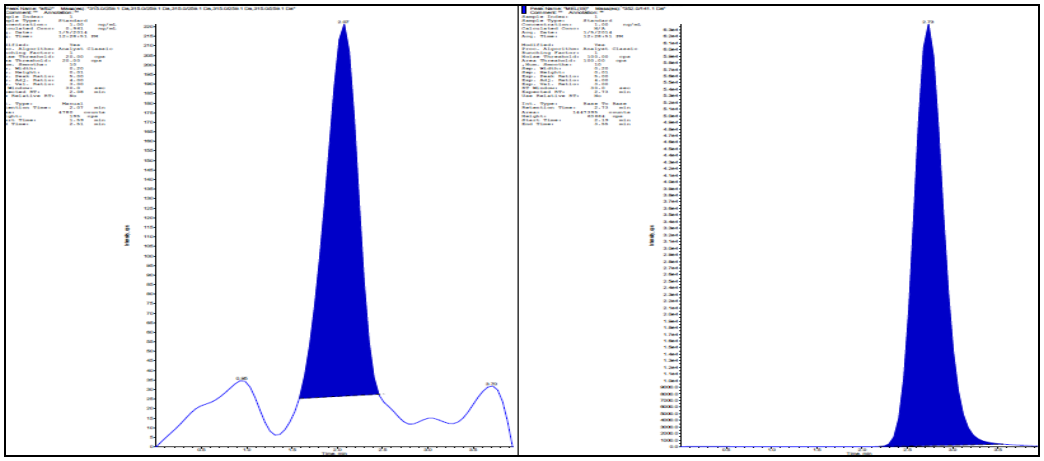


Fig. 12c. Chromatogram of extracted upper limit of quantification sample for AFB2

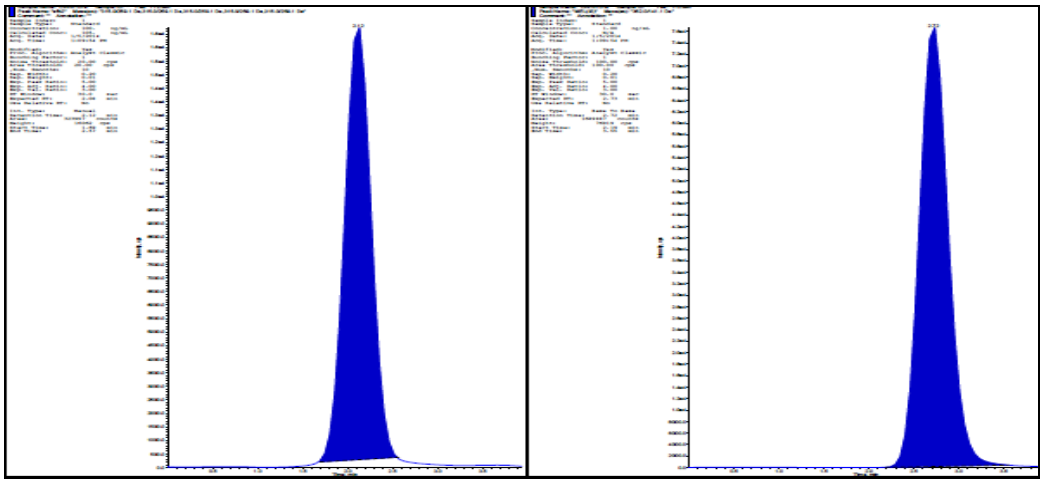


Fig. 13a. Chromatograms of blank tissue sample for OTA

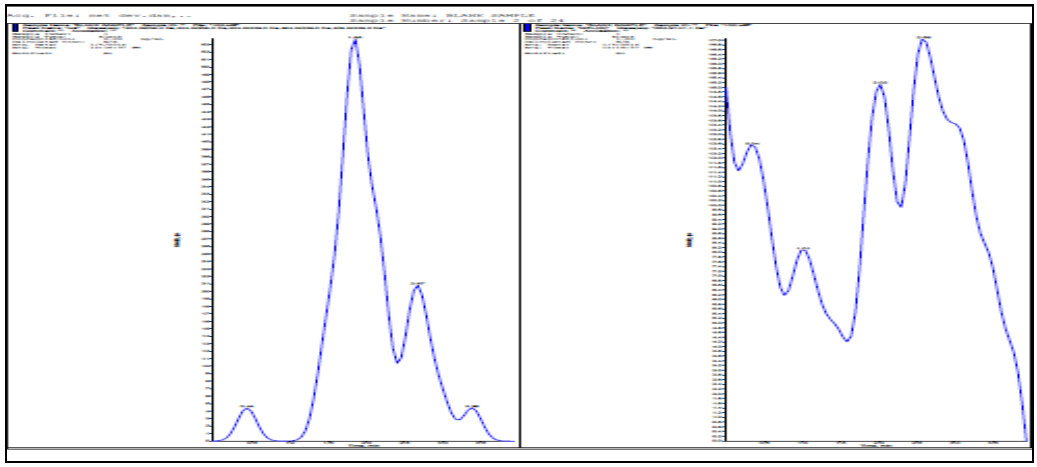


Fig. 13b. Chromatogram of extracted lower limit of quantification sample for OTA

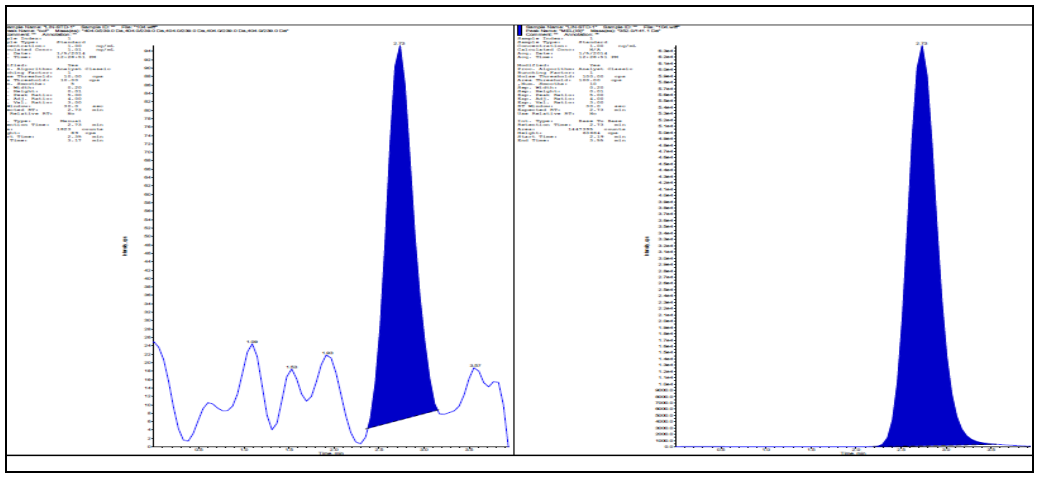
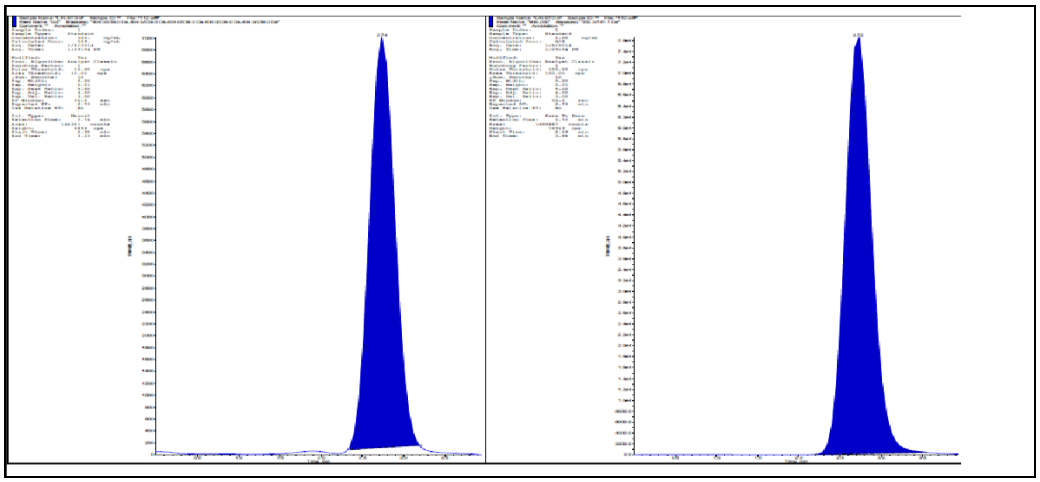


Fig. 13c. Chromatogram of extracted upper limit of quantification sample for OTA



4.8.2 Linearity

The nine point calibration curve was found to be linear over the concentration range of 1-100 ng/ml. After weighing factor of 1/x and $1/x^2$, a regression equation with a weighing factor of $1/x^2$ of analyte to internal standard concentration was found to produce the best fit concentration response relationship for the analyte in coloured broiler chicken tissues. The mean correlation coefficient of the weighted calibration curves generated during the validation for the aflatoxin B1, aflatoxin B2 and ochratoxin A were 0.9950, 0.9968 and 0.9973 respectively. Matrix matched calibration for AFB1, AFB2 and OTA shown in the Fig 14, 15 and 16 respectively.

Mycotoxins	Calibration curve	Coefficient of correlation, R
AFB1	Y=0.00139x+0.000821	0.9950
AFB2	Y=0.00181x+0.000156	0.9968
OTA	Y=0.000781x+0.00047	0.9973

4.8.3 Precision and accuracy

The precision and accuracy of the analytical method adopted was determined by evaluating intra-day and inter-day variation. Intra-day variation was determined by assaying (in-triplicate) of four different tissue standards of known concentrations (1, 3, 50 and 90 ng/ml). Inter-day variation was determined by assaying four tissue standards (1, 3, 50 and 90 ng/ml) on three different occasions at least 24 hours apart between each assay. The precision and accuracy of the method for AFB1, AFB2 and OTA was expressed as per cent co-efficient of variation (CV %) and per cent of mean nominal concentration (%) respectively and presented in Table 19, Table 20 and Table 21.

Fig. 14. Matrix matched calibration for AFB1

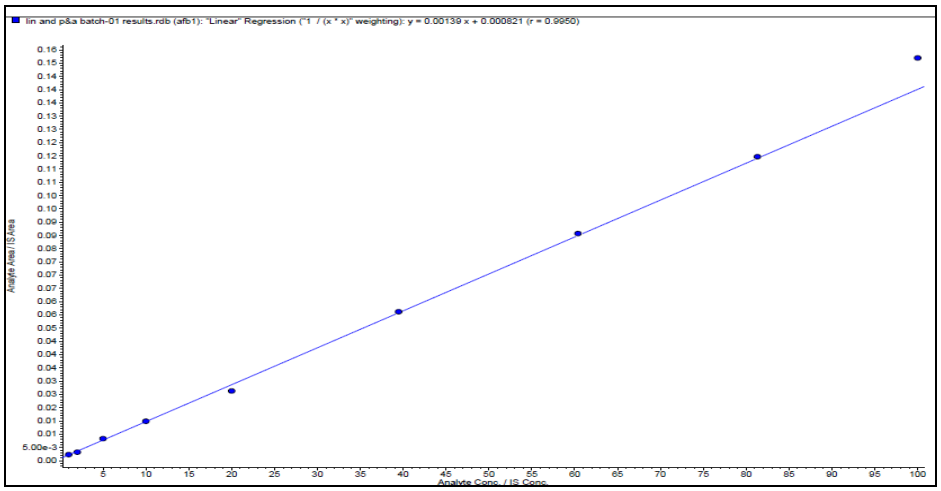


Fig. 15. Matrix matched calibration for AFB2

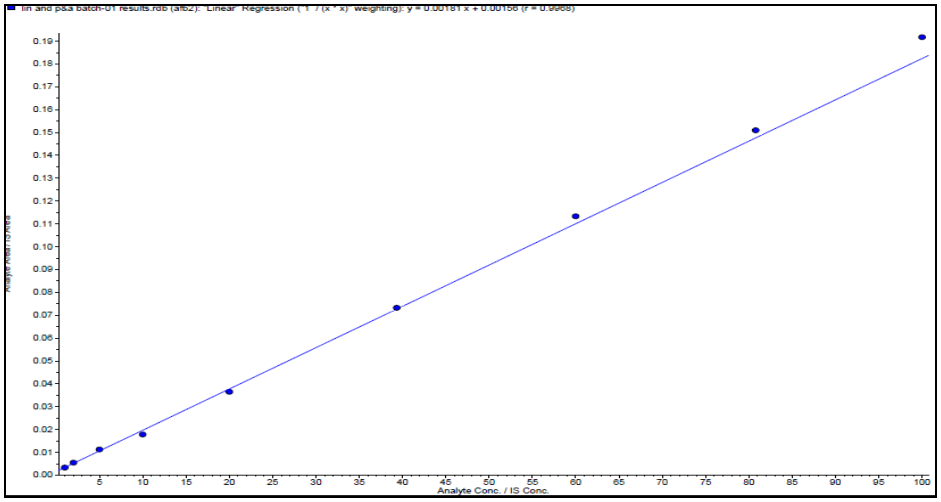


Fig. 16. Matrix matched calibration for OTA

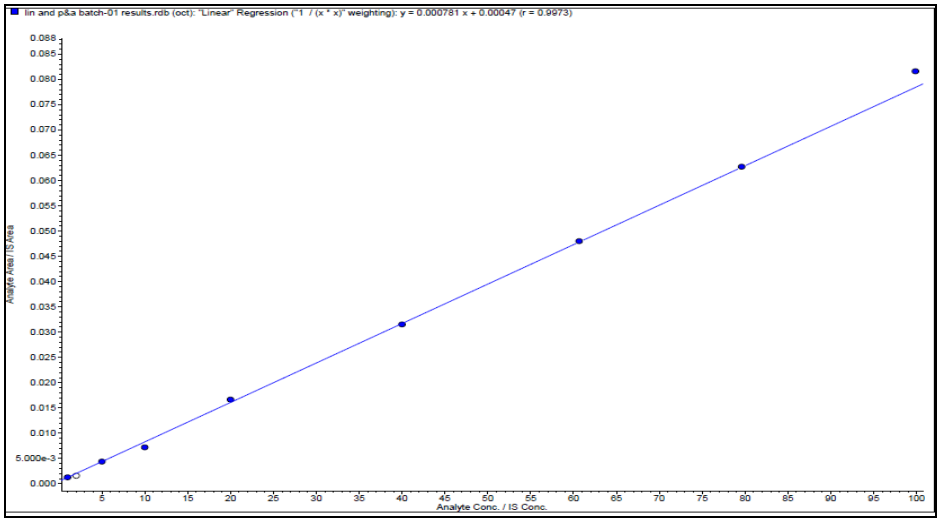


Table 19. Intra- and inter-day assay coefficient of variation (CV%) and nominal concentration (%) of AFB1 residual analysis in coloured broiler chicken tissues (liver, kidney and muscles)

Concentration (ng/ml)	Intra-day assay (n=6)			Inter-day assay (n=6)		
	Mean ±SD (ng/ml)	CV (%)	Mean Nominal concentration (%)	Mean ±SD (ng/ml)	CV (%)	Mean Nominal concentration (%)
1 (LLOQC)	1.03±0.12	12.08	102.67	1.00±0.13	13.41	103.62
3 (LQC)	2.90±0.20	6.87	96.67	2.80±0.21	7.77	96.66
50 (MQC)	53.03±6.34	11.95	106.1	54.00±5.02	9.29	109.7
90 (HQC)	93.28±6.16	6.61	103.6	92.00±7.04	7.65	108.

Accuracy for LLOQC-80-120per cent of their nominal conc
Accuracy for LQC, MQC & HQC-85-115per cent of their nominal conc
%CV for LLOQC should be ± 20%
%CV for LQC, MQC & HQC should be ± 15%

Table 20. Intra- and inter-day assay coefficient of variation (CV%) and nominal concentration (%) of AFB2 residual analysis in coloured broiler chicken tissues (liver, kidney and muscles)

Concentration (ng/ml)	Intra-day assay (n=6)			Inter-day assay (n=6)		
	Mean ±SD (ng/ml)	CV (%)	Mean Nominal concentration (%)	Mean ±SD (ng/ml)	CV (%)	Mean Nominal concentration (%)
1 (LLOQC)	1.05±0.19	18.21	104.62	0.94±0.10	11.50	100.88
3 (LQC)	2.85±0.18	6.47	95	2.80±0.24	8.51	93.33
50 (MQC)	46.33±6.25	6.45	92.67	55.86±6.99	12.52	108
90 (HQC)	95.11±6.13	11.50	105.7	101.00±11.65	11.50	102

Accuracy for LLOQC-80-120% of their nominal conc
Accuracy for LQC, MQC & HQC-85-115% of their nominal conc
%CV for LLOQC should be ± 20%
%CV for LQC, MQC & HQC should be ± 15%

Table 21. Intra- and inter-day assay coefficient of variation (CV %) and nominal concentration (%) of OTA residual analysis in coloured broiler chicken tissues (liver, kidney and muscles)

Concentration (ng/ml)	Intra-day assay (n=6)			Inter-day assay (n=6)		
	Mean ±SD (ng/ml)	CV (%)	Mean Nominal concentration (%)	Mean ±SD (ng/ml)	CV (%)	Mean Nominal concentration (%)
1 (LLOQC)	1.02±0.13	12.39	102.42	1.02±0.13	13.11	102.82
3 (LQC)	2.87±0.23	7.85	95.67	2.7±0.49	18.25	90
50 (MQC)	55.03±5.94	10.82	110.1	59.91±10.76	17.96	109.83
90 (HQC)	96.48±7.30	7.57	107.2	96.00±6.96	7.27	106.5

Accuracy for LLOQC-80-120% of their nominal conc
Accuracy for LQC, MQC & HQC-85-115% of their nominal conc
%CV for LLOQC should be ± 20%
%CV for LQC, MQC & HQC should be ± 15%

4.8.4 Recovery

The mean per cent recoveries of AFB1, AFB2 and OTA at three qc levels (3.0, 50.0 and 90.0 ng/ml) were 75.95, 66.87 and 79.05 % respectively, and the absolute recovery of ISTD was 82.53 %. The extraction efficiency was stable, indicating that the sample preparations were consistent, precise, and reproducible at different levels.

4.8.5 Stability test

Stability tests were determined at room temperature and refrigerated conditions (aqueous at 2-8ºC and tissue samples at -20°C). Samples were found stable for 10 days at -20 ºC which covers the stability days from the date of exposing the birds to the end of sample analysis.

4.8.6 Chromatographic conditions

Mobile phase was optimized through several trials to obtain good resolution. The presence of small amount of formic acid in the mobile phase improved the detection of the analyte. It was found that Acetonitrile: 0.1% v/v formic acid in water (70:30 % v/v) could achieve this purpose and adopted as a final mobile phase. Thermo C-18 RP, 4.6 mm x100, 5µm column gave good peak shapes and response at lowest concentration level. The mobile phase was operated at a flow rate of 0.700 ml/min with a run time of 3.5 min. The retention times of Aflatoxin B1, Ochratoxin A and Meloxicam were 2.16, 2.74 and 2.72 minutes respectively. Several compounds were tried for finding suitable internal standard finally meloxicam was found to be the best for the purpose because its pka was almost similar to the analytes and recovery was good with the current extraction method and response was reproducible.

Table 22. Mean per cent extracted recovery (%) of AFB1 at different QC levels.

LQC			
	Post spiked area	**Extracted Area**	**% recovery**
	10235	6702	65.48
	10654	8016	75.24
	9985	7173	71.84
Mean	10291	7297	
Mean % recovery	70.91		

MQC			
	Post spiked area	**Extracted Area**	**% recovery**
	169758	128479	75.68
	170231	132989	78.12
	157548	122326	77.64
Mean	165846	127931	
Mean % recovery	77.14		

HQC			
	Post spiked area	**Extracted Area**	**% recovery**
	274675	218848	79.68
	334563	267310	79.90
	256574	204791	79.82
Mean	288604	230316	
Mean % recovery	79.80		

% recovery of AFB1 across qc levels	
LQC	70.91
MQC	77.14
HQC	79.80
Mean % recovery	**75.95**

Table 23. Mean per cent extracted recovery (%) of AFB2 at different QC levels.

LQC			
	Post spiked area	**Extracted Area**	**% recovery**
	16035	10244	63.89
	17850	11941	66.90
	16085	10005	62.20
Mean	16657	10730	
Mean % recovery	64.42		

MQC			
	Post spiked area	**Extracted Area**	**% recovery**
	2230450	1592207	71.39
	2446575	1660409	67.87
	2365837	1510261	63.84
Mean	2347621	1587626	
Mean % recovery	67.63		

HQC			
	Post spiked area	**Extracted Area**	**% recovery**
	2284670	1510346	66.11
	2344563	1831505	78.12
	2306574	1413010	61.26
Mean	2311936	1584954	
Mean % recovery	68.56		

% recovery of AFB2 across qc levels	
LQC	64.42
MQC	67.63
HQC	68.56
Mean % recovery	**66.87**

Table 24. Mean per cent extracted recovery (%) of OTA at different QC levels.

LQC			
	Post spiked area	**Extracted Area**	**% recovery**
	5892	4568	77.53
	4250	3150	74.12
	5104	3628	71.08
Mean	5082	3782	
Mean % recovery	74.42		

MQC			
	Post spiked area	**Extracted Area**	**% recovery**
	89866	71770	79.86
	85922	69506	80.89
	83800	68198	81.38
Mean	86529	69825	
Mean % recovery	80.69		

HQC			
	Post spiked area	**Extracted Area**	**% recovery**
	156553	123275	78.74
	152560	126109	82.66
	134658	114699	85.18
Mean	147924	121361	
Mean % recovery	82.04		

% recovery of OTA across qc levels	
LQC	74.42
MQC	80.69
HQC	82.04
Mean % recovery	**79.05**

Table 25. Mean per cent extracted recovery (%) of Meloxicam at different QC levels.

Meloxicam Recovery			
	Post spiked area	**Extracted Area**	**% recovery**
	1898754	1555356	81.91
	1910243	1659348	86.87
	1875142	1462351	77.99
	2075984	1592207	76.70
	1942153	1660409	85.49
	1801236	1510261	83.85
	1789754	1510565	84.40
	1867487	1443150	77.28
	1568594	1413010	90.08
Mean	1858816	1534073	
Mean % recovery	**82.53**		

4.9 Aflatoxin B1 and ochratoxin A residual analysis in coloured broiler chicken tissues

4.9a Aflatoxin B1

4.9a.1 Liver

The mean AFB1 residues in liver of different groups of the experimental birds were measured at weekly interval and the values have been summarized in Table 26 and represented graphically in Fig 17.

AFB1 residues was not detected in negative control (Group 1), mycotoxins binder control groups (Groups 2 and 3). A significant ($P<0.001$) increase in AFB1 residues was observed in both low and high dose of AFB1 and OTA combined mycotoxicoses group (Group 8 and Group 9, 3.28±0.02 and 4.28±0.01 ng/g respectively) as compared to individual low and high dose of AFB1 (Groups 4 and 5, 3.16±0.01 and 4.12±0.03 ng/g respectively) from seventh day of experiment till the end of 42nd day.

A significant ($P<0.001$) increase in AFB1 residue was observed in individual high dose of AFB1 (Group 5) as compared to individual low dose of AFB1 group (Group 4) from seventh day of experiment till the end of 42nd day.

A significant ($P<0.001$) decrease in the AFB1 residues was observed in birds fed with the both low and high dose of combined AFB1 and OTA along with both low and high dose of mycotoxins binder (DE) treated group (Groups 10 and 11, 1.26±0.02 and 1.33±0.01 ng/g, respectively) as compared to individual AFB1 low and high dose group (Groups 4 and 5) and combined mycotoxicoses groups (Groups 8 and 9) from seventh day of experiment till the end of experimental period (42nd day).

Between groups, a significant (P<0.001) increase in AFB1 residue was noticed in high dose of combined mycotoxicoses (Group 9) as compared to low dose of combined mycotoxicoses (Group 8) from seventh day of experiment till the end of 42nd day. No significant increase in between the low and high dose of combined mycotoxicoses treated with low and high dose of mycotoxins binder D.E (Groups 10 and 11).

4.9a.2 Kidney

The mean AFB1 residues in kidney of different groups of the experimental birds were measured at weekly interval and the values have been summarized in Table 27 and represented graphically in Fig 18.

AFB1 residue was not detected in negative control (Group 1), mycotoxins binder control groups (Groups 2 and 3). A significant (P<0.001) increase in AFB1 residue was observed in both low and high dose of AFB1 and OTA combined mycotoxicoses group (Groups 8 and 9, 3.19±0.01 and 3.30±0.01 ng/g, respectively) as compared to individual low dose of AFB1 group (Group 4, 3.09±0.08 ng/g) from seventh day of experiment till the end of experimental period (42nd day).

A significant (P<0.001) increase in AFB1 residues was observed in individual high dose of AFB1 (Group 5, 3.20±0.02 ng/g) as compared to individual low dose of AFB1 group (Group 4, 3.09±0.08 ng/g) from seventh day of experiment till the end of experimental period (42 days). A significant (P<0.001) increase in AFB1 residues was observed in high dose of AFB1 and OTA combined mycotoxicoses group (Group 9, 3.30±0.01 ng/g) as compared to individual high dose of AFB1 group (Group 5, 3.20±0.02 ng/g) from seventh day of experiment till the end of experimental period 42nd day.

Whereas significant ($P<0.001$) decrease in AFB1 residues was observed in low dose of AFB1 and OTA combined mycotoxicoses group (Group 8, 2.08±0.05 ng/g) as compared to individual high dose of AFB1 group (Group 5, 2.78±0.07 ng/g) in the seventh, 14^{th} and 21^{st} day of experimental period only and no significant increase in AFB1 residue was noticed in low dose combined mycotoxicoses (Group 8, 3.19±0.01ng/g) as compared to high dose of individual AFB1 (Group 5, 3.20±0.02 ng/g) till the end of experimental period (42^{nd} day).

A significant ($P<0.001$) decrease in the AFB1 residues was observed in birds fed with the both low and high dose of combined AFB_1 and OTA along with both low and high dose of mycotoxins binder treated group (Groups 10 and 11, 1.22±0.03 and 1.28±0.06 ng/g, respectively) as compared to individual AFB1 groups (Groups 4 and 5, 3.09±0.08 and 3.20±0.02 ng/g, respectively) and combined mycotoxicoses groups (Groups 8 and 9) from seventh day till the end of sixth week of experiment.

Between groups, a significant ($P<0.001$) increase in AFB1 residues was noticed in high dose of combined mycotoxicoses (Group 9) as compared to low dose of combined mycotoxicoses (Group 8) from seventh day of experiment till the end of experimental period (42^{nd} day).

No significant increase in AFB1 residue was observed in between the low and high dose of combined mycotoxicoses treated with low and high dose of mycotoxin binder D.E (Groups 10 and 11).

4.9a.3 Muscles

The mean AFB1 residues in muscles of different groups of the experimental birds were measured at weekly interval and the values have been summarized in Table 28 and represented graphically in Fig 19.

An AFB1 residue was not detected in negative control (Group 1), mycotoxins binder control groups (Groups 2 and 3). A significant ($P<0.001$) increase in AFB1 residues was observed in both low and high dose of AFB1 and OTA combined mycotoxicoses group (Groups 8 and 9, 1.23±0.05 and 2.64±0.03 ng/g, respectively) as compared to individual low dose of AFB1 (Group 4, 0.42±0.01 ng/g) from seventh day of experiment till the end of experimental period (42^{nd} day).

A significant ($P<0.001$) increase in AFB1 residues was observed in individual high dose of AFB1 (Group 5, 1.25±0.03 ng/g) as compared to individual low dose of AFB1 group (Group 4, 0.42±0.01 ng/g) from seventh day of experiment till the end of experimental period (42^{nd} day).

A significant ($P<0.001$) increase in AFB1 residues was observed in high dose of AFB1 and OTA combined mycotoxicoses group (Group 9, 2.64±0.03 ng/g) as compared to individual high dose of AFB1 (Group 5, 1.25±0.03 ng/g) from seventh day of experiment till the end of experimental period day 42. Whereas, low dose of AFB1 and OTA combined mycotoxicoses group (Group 8) showed significant ($P<0.01$) increase in AFB1 residue in only seventh day of experiment (1.03±0.02 ng/g) and no significant increase in AFB1 residue was not noticed until day 42 of the experiment as compared to high dose of AFB1 group (Group 5, 0.97±0.02 ng/g).

A significant (P<0.001) decrease in the AFB1 residue was observed in birds fed with the both low and high dose of combined AFB1 and OTA along with both low and high dose of mycotoxins binder (DE) treated group (Groups 10 and 11, 0.80±0.01 and 0.88±0.01 ng/g, respectively) as compared to individual AFB1 low and high dose group (Groups 4 and 5, 0.42±0.01 and 1.25±0.3 ng/g, respectively) and combined mycotoxicoses groups (Groups 8 and 9) from seventh day of experiment till the end of experimental period (42nd day).

Between groups, a significant (P<0.001) increase in AFB1 residues was noticed in high dose of combined mycotoxicoses (Group 9) as compared to low dose of combined mycotoxicoses (Group 8) from seventh day of experiment till the end of experimental period (42 days). No significant increase in AFB1 residues in between the low and high dose of combined mycotoxicoses treated with low and high dose of mycotoxin binder D.E (Groups 10 and 11).

4.9b Ochratoxin A (OTA) residual analysis

4.9b.1 Liver

The mean OTA residues in liver of different groups of the experimental birds were measured at weekly interval and the values have been summarized in Table. 29 and represented graphically in Fig 20.

OTA residue was not detected in negative control (Group 1), mycotoxins binder control groups (Groups 2 and 3). OTA residues in the liver of different experimental groups (Groups 6, 7, 8, 9, 10 and 11, respectively) were 24.35±2.06, 38.7±2.39,

33.06±2.32, 50.01±2.36, 10.81±0.08 and 11.25±1.80 ng/g (on seventh day) and 38.55±1.039, 51.65±1.08, 52.80±0.70, 71.85±1.90, 17.15±1.72 and 17.45±0.52 ng/g, respectively (on 42 days of the experiment).

A significant (P<0.001) increase in OTA residues was observed in both low and high dose of AFB1 and OTA combined mycotoxicoses group (Groups 8 and 9) as compared to individual low dose of OTA (Group 6) from seventh day of experiment till the end of experimental period (42nd day).

A significant (P<0.001) increase in OTA residues was observed in individual high dose of OTA (Group 7) as compared to individual low dose of OTA group (Group 6) from seventh day of experiment till the end of day 42.

A significant (P<0.001) increase in OTA residues was observed in high dose of AFB1 and OTA combined mycotoxicoses group (Group 9) as compared to individual high dose of OTA (Group 7) from seventh day of experiment till the end of 42nd day. Whereas, a significant (P<0.001) decrease in OTA residue was observed in low dose of AFB1 and OTA combined mycotoxicoses (Group 8) as compared to individual high dose of OTA (Group 7) from seventh day of experimental period only and no significant increase in OTA residues was observed in remaining days of experiment to till the end of experimental period (42nd day), but absolute increase in OTA residues was noticed.

A significant (P<0.001) decrease in the OTA residues was observed in birds fed with the both low and high dose of combined AFB1 and OTA along with both low and high dose of mycotoxins binder (DE) treated group (Groups 10 and 11) as compared to

individual low and high dose of OTA group (Groups 6 and 7) and combined mycotoxicoses groups (Groups 8 and 9) from seventh day of experiment to till the end of experimental period (42nd day).

Between groups, a significant (P<0.001) increase in OTA residues was noticed in high dose of combined mycotoxicoses (Group 9) as compared to low dose of combined mycotoxicoses (Group 8) from seventh day of experiment till the end of experimental period (42nd day). No significant increase in OTA residues in between the low and high dose of combined mycotoxicoses treated with low and high dose of mycotoxin binder D.E (Groups 10 and 11).

4.9b.2 Kidney

The mean OTA residues in kidney of different groups of the experimental birds were measured at weekly interval and the values have been summarized in Table. 30 and represented graphically in Fig 21.

OTA residue was not detected in negative control (Group 1), mycotoxins binder control groups (Groups 2 and 3). OTA residues in the kidney of different experimental groups (Groups 6, 7, 8, 9, 10 and 11, respectively) were 27.35±1.82, 42.4±1.56, 34.91±1.39, 51.15±2.04, 15.23±1.84 and 16.91±3.52 ng/g (on seventh day) and 39.35±0.4, 52.76±0.5, 53.63±0.6, 72.68±1.6, 21.18±3.2 and 21.51±0.9 ng/g, respectively (on 42nd day of the experiment).

A significant (P<0.001) increase in OTA residue was observed in both low and high dose of AFB1 and OTA combined mycotoxicoses group (Groups 8 and 9) as

compared to individual low dose of OTA (Group 6) from seventh day of experiment till the end of experimental period (42 days).

A significant ($P<0.001$) increase in OTA residue was observed in individual high dose of OTA (Group 7) as compared to individual low dose of OTA group (Group 6) from seventh day of experiment till the end of experimental period (42 days).

A significant ($P<0.001$) increase in OTA residues was observed in high dose of AFB1 and OTA combined mycotoxicoses group (Group 9) as compared to individual high dose of OTA (Group 7) from seventh day of experiment till the end of experimental period (42 days). Whereas a significant ($P<0.001$) decrease in OTA residues was observed in low dose of AFB1 and OTA combined mycotoxicoses (Group 8) as compared to individual high dose of OTA (Group 7) from seventh day of experimental period only and no significant increase in OTA residues was observed in remaining days of experiment till the end of experimental period (42 days), but absolute increase in OTA residues was noticed.

A significant ($P<0.001$) decrease in the OTA residues was observed in birds fed with the both low and high dose of combined AFB1 and OTA along with both low and high dose of mycotoxins binder (DE) treated group (Groups 10 and 11) as compared to individual OTA low and high dose group (Groups 6 and 7) and combined mycotoxicoses groups (Groups 8 and 9) from seventh day of experiment till the end of experimental period (42 days).

Between groups, a significant (P<0.001) increase in OTA residues was noticed in high dose of combined mycotoxicoses (Group 9) as compared to low dose of combined mycotoxicoses (Group 8) from seventh day of experiment till the end of experimental period (42 days). No significant increase in OTA residues in between the low and high dose of combined mycotoxicoses treated with low and high dose of mycotoxin binder D.E (Groups 10 and 11).

4.9b.3 Muscles

The mean OTA residues in muscles of different groups of the experimental birds were measured at weekly interval and the values have been summarized in Table. 31 and represented graphically in Fig 22.

OTA residue was not detected in negative control (Group 1), mycotoxins binder control groups (Groups 2 and 3). OTA residues in the kidney of different experimental groups (Groups 6, 7, 8, 9, 10 and 11) were 4.34±0.62, 6.85±0.86, 6.39±0.75, 12.11±0.73, 1.56±0.12 and 1.68±0.91ng/g (on seventh day) and 5.18±0.02, 7.23±0.02, 7.16±0.01, 15.06±0.36, 1.72±0.04 and 1.86±0.04 ng/g , respectively (on 42nd day of the experiment).

A significant (P<0.001) increase in OTA residue was observed in both low and high dose of AFB1 and OTA combined mycotoxicoses group (Groups 8 and 9) as compared to individual low dose of OTA (Group 6) from seventh day of experiment to till the end of experimental period (42 days). A significant (P<0.001) increase in OTA residues was observed in individual high dose of OTA (Group 7) as compared to individual low dose of OTA group (Group 6) from seventh day of experiment till the end of experimental period (42 days).

A significant (P<0.001) increase in OTA residues was observed in high dose of AFB1 and OTA combined mycotoxicoses group (Group 9) as compared to individual high dose of OTA (Group 7) from seventh day of experiment to till the end of experimental period 42 day. Whereas a significant (P<0.05) decrease in OTA residues was observed in low dose of AFB1 and OTA combined mycotoxicoses (Group 8) as compared to individual high dose of OTA (Group 7) from seventh day of experimental period only and no significant (P>0.05) increase in OTA residues was observed in remaining days of experiment to till the end of experimental period (42 days), but absolute increase in OTA residues was noticed.

A significant (P<0.001) decrease in the OTA residues was observed in birds fed with the both low and high dose of combined AFB1 and OTA along with both low and high dose of mycotoxins binder (DE) treated group (Groups 10 and 11) as compared to individual OTA low and high dose group (Groups 6 and 7) and combined mycotoxicoses groups (Groups 8 and 9) from seventh day of experiment till the end of experimental period (42 days).

Between groups, a significant (P<0.001) increase in OTA residues was noticed in high dose of combined mycotoxicoses (Group 9) as compared to low dose of combined mycotoxicoses (Group 8) from seventh day of experiment till the end of experimental period (42 days).

No significant (P>0.05) increase in OTA residues in between the low and high dose of combined mycotoxicoses treated with low and high dose of mycotoxins binder D.E (Group 10 and Group 11).

Table 26: Individual and combined effect of mycotoxins (AFB1 and OTA) on AFB1 residues in the liver (ng/g) and effect of D.E binder on combined mycotoxicoses in coloured broiler chicken and its withdrawal period (Mean±SEM, n=6)

Groups	Treatment			Exposure period (Days)						Withdrawal period (Days)				
	AFB1 (ppm)	OTA (ppm)	DE (g)	7	14	21	28	35	42	3	7	10	13	18
G1	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
G2	-	-	0.5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
G3	-	-	1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
G4	0.5	-	-	2.56 ± 0.10^{a}	2.87 ± 0.02^{a}	3.00 ± 0.06^{a}	3.08±0.07a	3.11±0.05a	3.16±0.01a	2.51 ± 0.09^{a}	1.14±0.02a	0.05^{a}	ND	ND
G5	1	-	-	3.48 ± 0.08^{b}	3.81 ± 0.08^{b}	3.93 ± 0.04^{b}	3.95 ± 0.03^{b}	4.05 ± 0.06^{b}	4.12 ± 0.03^{b}	3.07 ± 0.03^{b}	1.78 ± 0.22^{b}	0.10^{ab}	ND	ND
G8	0.5	0.5	-	2.72 ± 0.09^{c}	2.97 ± 0.05^{c}	3.10 ± 0.01^{c}	3.18 ± 0.02^{c}	3.21 ± 0.02^{c}	3.28 ± 0.02^{c}	2.58 ± 0.02^{c}	1.23 ± 0.05^{c}	0.06^{abc}	ND	ND
G9	1	1	-	3.67 ± 0.04^{d}	3.90 ± 0.04^{d}	4.07 ± 0.04^{d}	4.16 ± 0.02^{d}	4.21 ± 0.02^{d}	4.28 ± 0.01^{d}	3.18 ± 0.03^{d}	1.93 ± 0.05^{d}	0.13^{acd}	ND	ND
G10	0.5	0.5	0.5	1.14 ± 0.04^{ef}	1.18 ± 0.07^{ef}	1.20 ± 0.03^{ef}	1.23 ± 0.02^{ef}	1.25 ± 0.01^{ef}	1.26 ± 0.02^{ef}	0.40 ± 0.04^{ef}	ND	ND	ND	ND
G11	1	1	1	1.18 ± 0.04^{f}	1.21 ± 0.01^{f}	1.25 ± 0.04^{ef}	1.28 ± 0.03^{f}	1.31 ± 0.0^{f}	1.33 ± 0.01^{f}	0.65 ± 0.03^{f}	ND	ND	ND	ND

Different superscripts within a row indicate significant differences ($P\leq0.05$)

Table 27: Individual and combined effect of mycotoxins (AFB1 and OTA) on AFB1 residues in the kidney (ng/g) and effect of D.E binder on combined mycotoxicoses in coloured broiler chicken and its withdrawal period (Mean±SEM, n=6)

Groups	Treatment			Exposure period (Days)						Withdrawal period (Days)				
	AFB1 (ppm)	OTA (ppm)	DE (g)	7	14	21	28	35	42	3	7	10	13	18
G1	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
G2	-	-	0.5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
G3	-	-	1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
G4	0.5	-	-	1.95±0.05^{a}	2.53±0.06^{a}	2.93±0.09^{a}	3.01±0.04^{a}	3.06±0.03^{a}	3.09±0.08^{a}	2.26±0.07^{ab}	1.07±0.03^{ab}	0.01^{ab}	ND	ND
G5	1	-	-	2.78±0.07^{b}	2.91±0.06^{b}	3.12±0.05^{b}	3.16±0.02^{b}	3.19±0.01^{b}	3.20±0.02^{b}	2.31±0.05^{ab}	1.11±0.06^{ab}	0.03^{ab}	ND	ND
G8	0.5	0.5	-	2.08±0.05^{c}	2.61±0.07^{c}	3.03±0.05^{c}	3.13±0.01^{bc}	3.16±0.02^{bc}	3.19±0.01^{bc}	2.62±0.03^{c}	1.19±0.03^{c}	0.06^{abcd}	ND	ND
G9	1	1	-	2.93±0.05^{d}	3.10±0.01^{d}	3.20±0.01^{d}	3.25±0.01^{d}	3.26±0.02^{d}	3.30±0.01^{d}	2.65±0.06^{d}	1.26±0.02^{d}	0.09^{abcd}	ND	ND
G10	0.5	0.5	0.5	1.0±0.06^{ef}	1.10±0.11^{ef}	1.15±0.12^{ef}	1.18±0.09^{ef}	1.20±0.05^{ef}	1.22±0.03^{ef}	0.45±0.07^{ef}	ND	ND	ND	ND
G11	1	1	1	1.06±0.16^{ef}	1.14±0.15^{ef}	1.17±0.14^{ef}	1.21±0.11^{ef}	1.24±0.11^{ef}	1.28±0.06^{ef}	0.65±0.06^{ef}	ND	ND	ND	ND

Different superscripts within a row indicate significant differences (P≤0.05)

Table 28: Individual and combined effect of mycotoxins (AFB1 and OTA) on AFB1 residues in the muscle (ng/g) and effect of D.E binder on combined mycotoxicoses in coloured broiler chicken and its withdrawal period (Mean±SEM, n=6)

Groups	Treatment			Exposure period (Days)						Withdrawal period (Days)				
	AFB1 (ppm)	OTA (ppm)	DE (g)	7	14	21	28	35	42	3	7	10	13	18
G1	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
G2	-	-	0.5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
G3	-	-	1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
G4	0.5	-	-	0.30±0.07^{a}	0.35±0.02^{a}	0.37±0.00^{a}	0.38±0.01^{a}	0.40±0.01^{a}	0.42±0.01^{a}	0.08±0.01^{a}	ND	ND	ND	ND
G5	1	-	-	0.97±0.02^{b}	1.04±0.02^{b}	1.13±0.02^{b}	1.16±0.01^{b}	1.19±0.01^{b}	1.25±0.03^{b}	0.10±0.01^{ab}	ND	ND	ND	ND
G8	0.5	0.5	-	1.03±0.02^{c}	1.09±0.02^{bc}	1.14±0.02^{b}	1.16±0.01^{bc}	1.19±0.01^{bc}	1.23±0.0^{bc}	0.13±0.03$^{b\,c}$	0.05±0.02^{c}	ND	ND	ND
G9	1	1	-	1.89±0.12^{d}	1.93±0.05^{d}	2.0±0.05^{d}	2.23±0.04^{d}	2.52±0.05^{d}	2.64±0.03^{d}	1.26±0.02^{d}	0.15±0.02^{d}	ND	ND	ND
G10	0.5	0.5	0.5	0.57±0.07^{ef}	0.62±0.06^{ef}	0.68±0.02^{ef}	0.73±0.04^{ef}	0.78±0.01^{ef}	0.80±0.01^{ef}	ND	ND	ND	ND	ND
G11	1	1	1	0.60±0.03^{ef}	0.65±0.02^{ef}	0.74±0.02^{ef}	0.76±0.01^{ef}	0.82±0.05^{ef}	0.88±0.01^{ef}	ND	ND	ND	ND	ND

Different superscripts within a row indicate significant differences ($P \leq 0.05$)

Table 29: Individual and combined effect of mycotoxins (AFB1 and OTA) on OTA residues in the liver (ng/g) of coloured broiler chicken and D.E binder effect on combined mycotoxicoses induced birds and its withdrawal period (Mean±SEM, n=6)

Groups	Treatment			Exposure period (Days)						Withdrawal period (Days)				
	AFB1 (ppm)	OTA (ppm)	DE (g)	7	14	21	28	35	42	3	7	10	13	18
G1	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
G2	-	-	0.5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
G3	-	-	1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
G6	0.5	-	-	24.35±2.06^{a}	31.72±1.91^{a}	35.83±1.64^{a}	36.41±1.20^{a}	37.16±1.43^{a}	38.55±1.039^{a}	35.25±1.8^{a}	30.67±1.3^{abc}	19.80±1.0^{abc}	4.67±0.9^{abc}	ND
G7	1	-	-	38.7±2.39^{b}	45.31±2.24^{b}	49.16±2.90^{b}	50.53±2.05^{b}	51.41±1.39^{b}	51.65±1.08^{b}	44.20±2.6^{b}	33.083±3.1^{abc}	22.83±1.5^{abc}	5.37±0.6^{abc}	ND
G8	0.5	0.5	-	33.06±2.32^{c}	46.31±1.76^{bc}	49.36±2.59^{bc}	50.75±1.60^{bc}	51.68±1.01^{bc}	52.80±0.70^{bc}	44.45±1.9^{bc}	31.75±3.1^{bc}	21.55±0.7^{abc}	5.54±0.8^{abc}	0.03
G9	1	1	-	50.01±2.36^{d}	60.07±3.37^{d}	64.81±3.27^{d}	65.71±1.74^{d}	69.7±1.34^{d}	71.85±1.90^{d}	63.83±1.1^{d}	45.71±2.1^{d}	28.83±2.3^{d}	6.77±0.9^{abc}	0.04
G10	0.5	0.5	0.5	10.81±0.08^{ef}	12.83±1.21^{ef}	14.58±1.74^{e}	15.41±1.71^{e}	16.62±0.61^{e}	17.15±1.72^{e}	7.33±1.4^{e}	0.95±0.9^{e}	ND	ND	ND
G11	1	1	1	11.25±1.80^{ef}	13.24±1.47^{ef}	14.93±2.56^{ef}	15.46±0.98^{ef}	16.55±0.56^{ef}	17.45±0.52^{ef}	8.81±1.0^{f}	1.07±0.6^{f}	ND	ND	ND

Different superscripts within a row indicate significant differences (P≤0.05)

Table 30: Individual and combined effect of mycotoxins (AFB1 and OTA) on OTA residues in the kidney (ng/g) of coloured broiler chicken and D.E binder effect on combined mycotoxicoses induced birds and its withdrawal period (Mean±SEM, n=6)

Groups	Treatment			Exposure period (Days)						Withdrawal period (Days)				
	AFB_1 (ppm)	OTA (ppm)	DE (g)	7	14	21	28	35	42	3	7	10	13	18
G1	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
G2	-	-	0.5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
G3	-	-	1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
G6	0.5	-	-	27.35 ± 1.82^{a}	34.25 ± 0.93^{a}	37.33 ± 1.10^{a}	37.75 ± 1.40^{a}	37.97 ± 1.09^{a}	39.35 ± 0.4^{a}	35.41 ± 1.5^{a}	29.58 ± 1.2^{abc}	18.97 ± 0.9^{ab}	4.34 ± 0.6	ND
G7	1	-	-	42.4 ± 1.56^{b}	46.7 ± 2.02^{b}	49.93 ± 1.55^{b}	51.31 ± 1.23^{b}	52.60 ± 1.44^{b}	52.76 ± 0.5^{b}	38.58 ± 0.9^{b}	31.75 ± 2.1^{abc}	19.05 ± 0.9^{abc}	4.47 ± 0.3	ND
G8	0.5	0.5	-	34.91 ± 1.39^{cf}	47.55 ± 0.88^{bc}	50.11 ± 1.85^{bc}	51.97 ± 1.62^{bc}	52.68 ± 1.00^{bc}	53.63 ± 0.6^{c}	46.67 ± 2.1^{c}	31.91 ± 1.0^{abc}	21.6 ± 1.0^{c}	5.67 ± 0.9	0.04
G9	1	1	-	51.15 ± 2.04^{d}	61.38 ± 1.68^{d}	65.48 ± 2.38^{d}	66.21 ± 1.13^{d}	71.11 ± 1.28^{d}	72.68 ± 1.6^{d}	64.25 ± 0.6^{d}	45.91 ± 1.3^{d}	29 ± 2.1^{d}	6.85 ± 0.8	0.05
G10	0.5	0.5	0.5	15.23 ± 1.84^{ef}	16.41 ± 3.30^{ef}	18.01 ± 3.83^{ef}	19.33 ± 2.85^{ef}	20.55 ± 1.31^{ef}	21.18 ± 3.2^{ef}	8.5 ± 1.4^{ef}	0.54 ± 0.4^{ef}	0	0	0
G11	1	1	1	16.91 ± 3.52^{e}	17.25 ± 2.75^{ef}	18.41 ± 1.62^{ef}	19.85 ± 1.80^{ef}	20.03 ± 1.87^{ef}	21.51 ± 0.9^{ef}	9.57 ± 1.0^{ef}	1.32 ± 0.8^{ef}	0	0	0

Different superscripts within a row indicate significant differences ($P\leq0.05$)

Table 31: Individual and combined effect of mycotoxins (AFB1 and OTA) on OTA residues in the muscle (ng/g) of coloured broiler chicken and D.E binder effect on combined mycotoxicoses induced birds and its withdrawal period (Mean±SEM, n=6)

Groups	Treatment			Exposure period (Days)						Withdrawal period (Days)				
	AFB1 (ppm)	OTA (ppm)	DE (g)	7	14	21	28	35	42	3	7	10	13	18
G1	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
G2	-	-	0.5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
G3	-	-	1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
G6	0.5	-	-	4.34 ± 0.62^{a}	4.91 ± 0.13^{a}	5.105 ± 0.07^{a}	5.14 ± 0.04^{a}	5.16 ± 0.03^{a}	5.18 ± 0.02^{a}	3.21 ± 0.52^{a}	0.68 ± 0.47^{a}	ND	ND	ND
G7	1	-	-	6.85 ± 0.86^{b}	6.97 ± 0.04^{bc}	7.12 ± 0.02^{bc}	7.17 ± 0.02^{bc}	7.2 ± 0.03^{bc}	7.23 ± 0.02^{bc}	5.27 ± 0.12^{b}	1.36 ± 0.10^{b}	0.15 ± 0.01^{ad}	ND	ND
G8	0.5	0.5	-	6.39 ± 0.75^{c}	6.88 ± 0.10^{bc}	7.01 ± 0.02^{bc}	7.14 ± 0.02^{bc}	7.15 ± 0.02^{bc}	7.16 ± 0.01^{bc}	4.56 ± 0.37^{c}	1.44 ± 0.05^{c}	0.43 ± 0.01^{ad}	ND	ND
G9	1	1	-	12.11 ± 0.73^{d}	12.61 ± 0.24^{d}	13.08 ± 0.56^{d}	13.94 ± 0.07^{d}	14.56 ± 0.56^{d}	15.06 ± 0.36^{d}	7.10 ± 0.45^{d}	2.98 ± 0.12^{d}	0.63 ± 0.16^{ad}	ND	ND
G10	0.5	0.5	0.5	1.56 ± 0.12^{ef}	1.60 ± 0.06^{ef}	1.63 ± 0.03^{ef}	1.65 ± 0.03^{ef}	1.68 ± 0.12^{ef}	1.72 ± 0.04^{ef}	0.15 ± 0.03^{ef}	ND	ND	ND	ND
G11	1	1	1	1.68 ± 0.91^{ef}	1.72 ± 0.62^{ef}	1.78 ± 0.44^{ef}	1.80 ± 0.42^{ef}	1.83 ± 0.11^{ef}	1.86 ± 0.04^{ef}	0.22 ± 0.30^{ef}	ND	ND	ND	ND

Different superscripts within a row indicate significant differences ($P\leq0.05$)

Fig. 17. AFB1 residues in the liver (ng/g) of coloured broiler chickens exposed to individual, combined mycotoxins and combined mycotoxins with D.E and its withdrawal period (Mean±SEM, n=6)

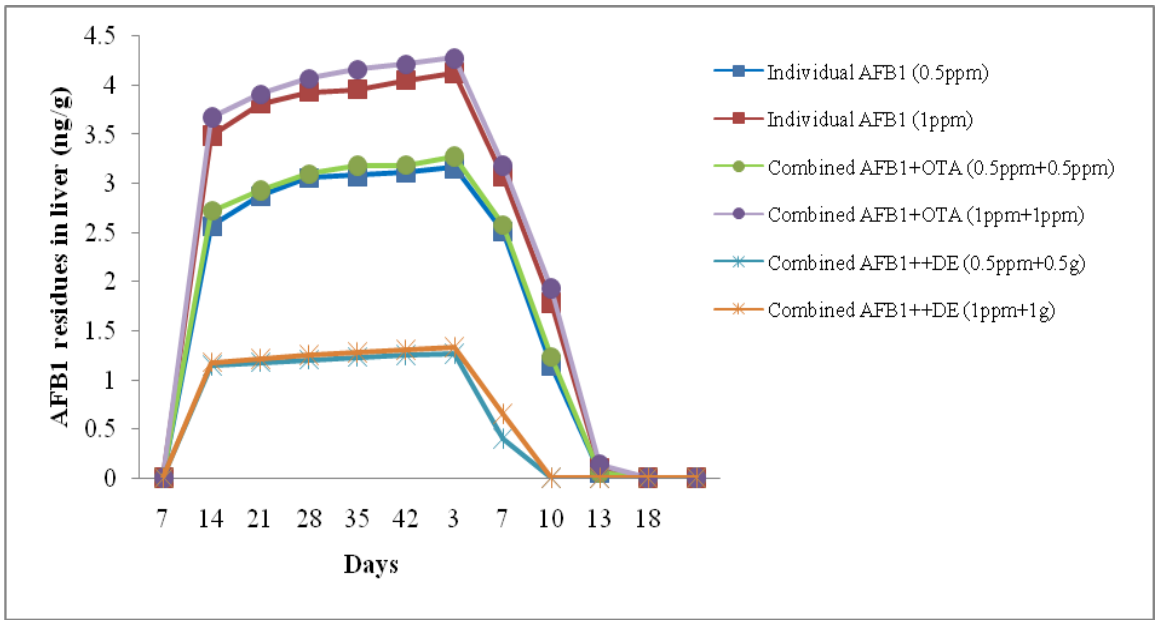


Fig. 18. AFB1 residues in the kidney (ng/g) of coloured broiler chickens exposed to individual, combined mycotoxins and combined mycotoxins with D.E and its withdrawal period (Mean±SEM, n=6)

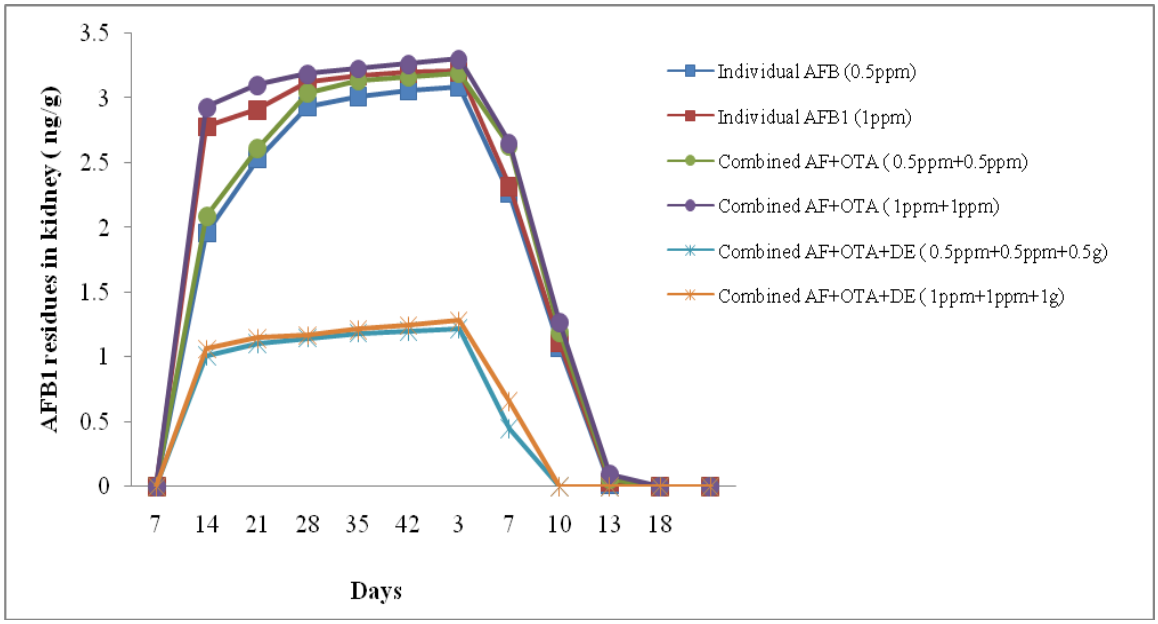


Fig. 19. AFB1 residues in the muscle (ng/g) of coloured broiler chickens exposed to individual, combined mycotoxins and combined mycotoxins with D.E and its withdrawal period (Mean±SEM, n=6)

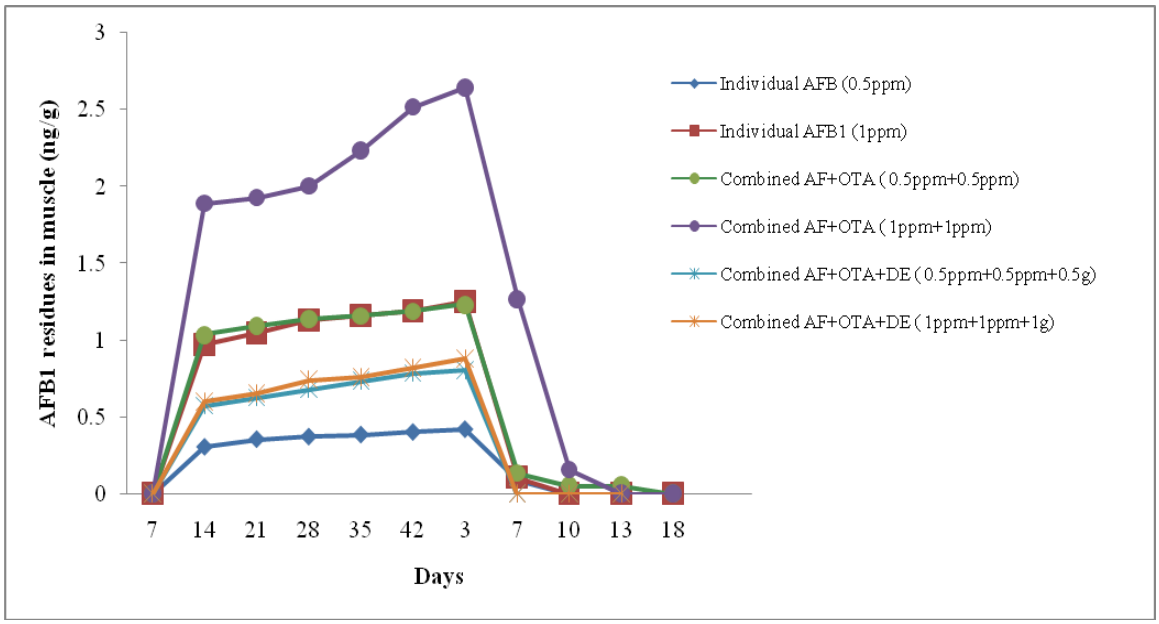


Fig. 20. OTA residues in the liver (ng/g) of coloured broiler chickens exposed to individual, combined mycotoxins and combined mycotoxins with D.E and its withdrawal period (Mean±SEM, n=6)

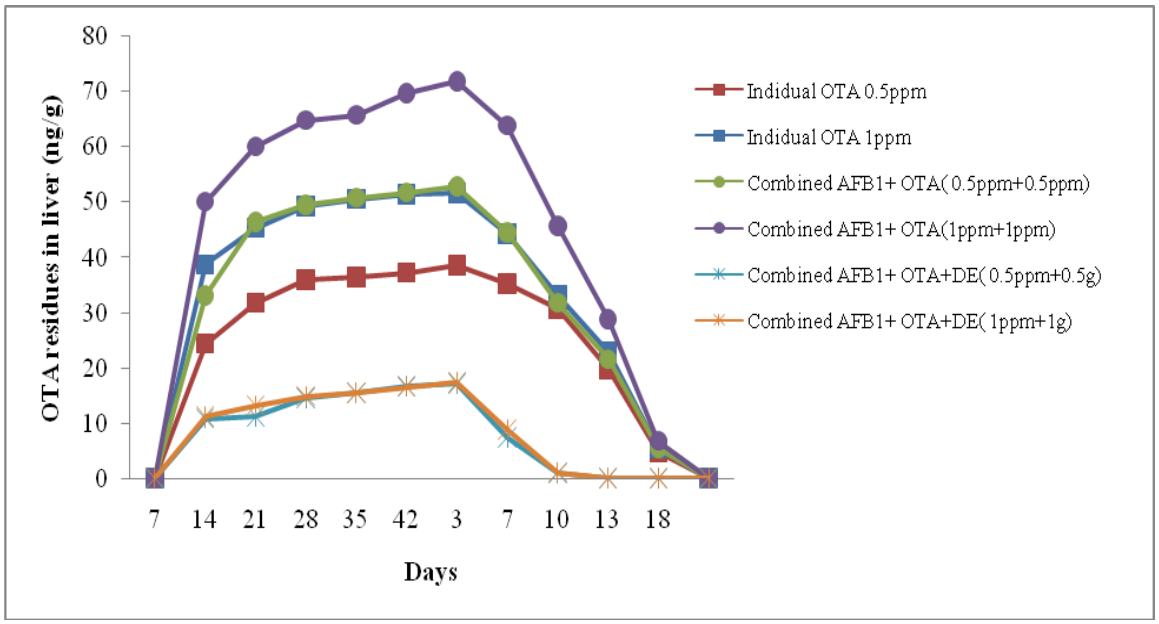


Fig. 21. OTA residues in the kidney (ng/g) of coloured broiler chickens exposed to individual, combined mycotoxins and combined mycotoxins with D.E and its withdrawal period (Mean±SEM, n=6)

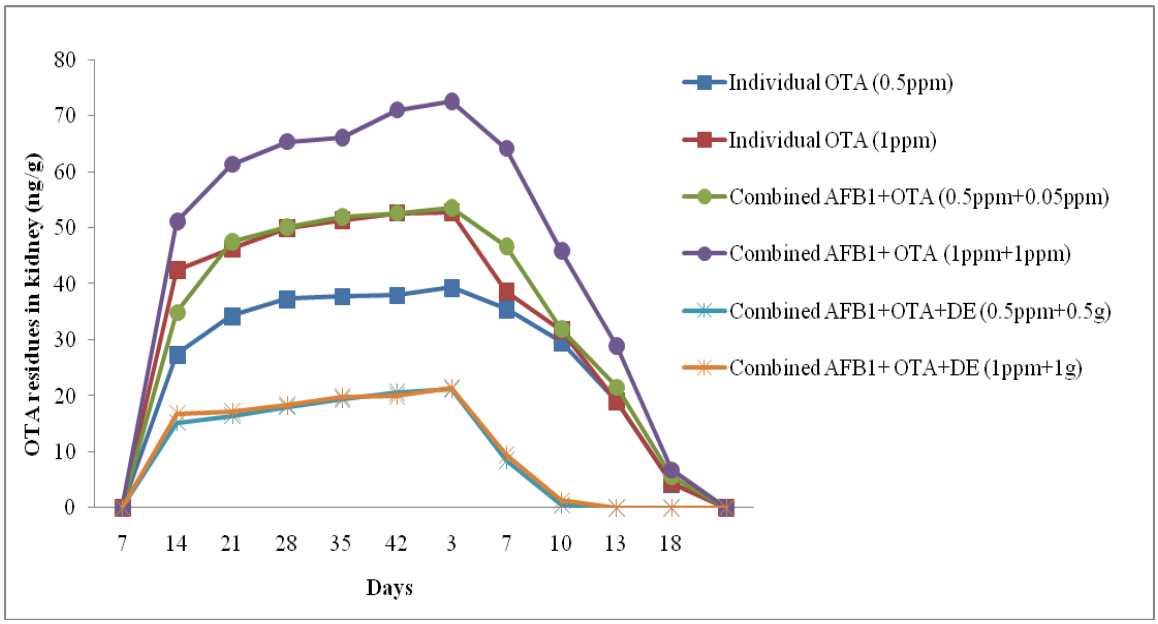
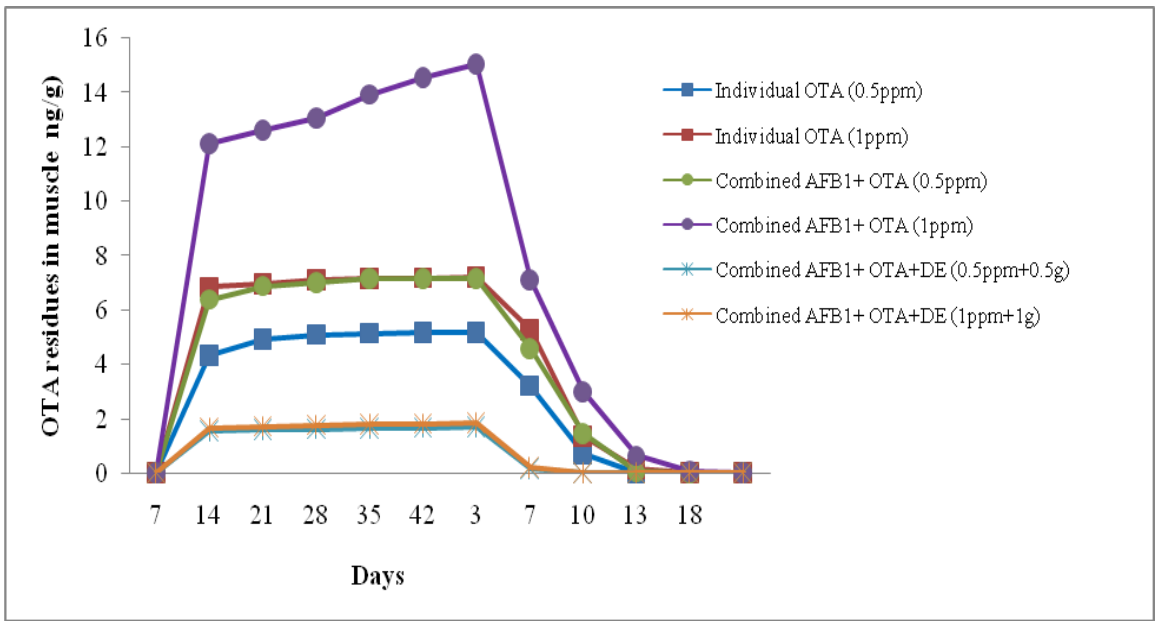


Fig. 22. OTA residues in the muscle (ng/g) of coloured broiler chickens exposed to individual, combined mycotoxins and combined mycotoxins with D.E and its withdrawal period (Mean±SEM, n=6)



4.9c Withdrawal period of AFB1 and OTA

4.9c.1 AFB 1 withdrawal period

4.9c.1.1 Liver

Complete withdrawal of AFB1 residue was noticed in liver of coloured broiler chicken at tenth day of withdrawal of low dose (0.5 ppm) of AFB1 contaminated diet. A small detectable amount of AFB1 residue was noticed in liver of coloured broiler chicken exposed to high dose of AFB1 (1ppm) at tenth day of experiment was 0.10 ng/g and was not detected on 13^{th} day of withdrawal of AFB1 contaminated diet.

Complete withdrawal of AFB1 residue was noticed in liver of coloured broiler chicken at tenth day of withdrawal of low dose of AFB1 and OTA combined mycotoxicoses. A small detectable amount of AFB1 residue was noticed in liver of coloured broiler chicken exposed to high dose of AFB1 and OTA combined mycotoxicoses at tenth day of withdrawal of contaminated diet was 0.13 ng/g and was not detected on 13^{th} day of withdrawal of AFB1 contaminated diet. Complete withdrawal of AFB1 residue was noticed in the liver of coloured broiler chicken exposed to low and high dose of AFB1 and OTA combined mycotoxicosescontaminated diet along with mycotoxins binder of Diatomaceous earth (0.5 and 1g of DE) at seventh day of withdrawal of contaminated diet.

4.9c.1.2 Kidney

Complete withdrawal of AFB1 residues was noticed in the kidney of coloured broiler chicken exposed to low and high dose of (0.5 and 1 ppm) of AFB1 contaminated diet at tenth day of withdrawal of mycotoxin contaminated diet.

A small detectable amount of AFB1 residues was noticed in kidney of coloured broiler chicken exposed to low and high dose of combined AFB1 and OTA contaminated diet at tenth day of experiment were 0.06 and 0.09 ng/g, respectively and were not detected on 13^{th} day of withdrawal of AFB1 and OTA combined mycotoxicoses contaminated diet.

Complete withdrawal of AFB1 residues was noticed in the kidney of coloured broiler chicken exposed to low and high dose of combined mycotoxicoses contaminated diet along with mycotoxins binder of Diatomaceous earth (0.5 and 1g of DE) at seventh day of withdrawal of contaminated diet.

4.9c.1.3 Muscle

A small detectable amount of AFB1 residues was noticed in the muscles of coloured broiler chicken exposed to low and high dose of (0.5 and 1 ppm) AFB1 contaminated diet at third day of withdrawal of contaminated diet were 0.08 and 0.10 ng/g, respectively and complete withdrawal of AFB1 residues was noticed at seventh day of withdrawal of mycotoxins contaminated diet in both low and high dose of AFB1 contaminated groups (Groups 4 and 5). A small detectable amount of AFB1 residue was noticed in muscles of coloured broiler chicken exposed to low dose of AFB1 and OTA combined mycotoxicoses contaminated diet at third day of withdrawal of contaminated diet was 0.13 ng/g and complete withdrawal was noticed on seventh day of withdrawal of contaminated diet.

A small detectable amount of AFB1 residue was noticed in muscles of coloured broiler chicken exposed to high dose of AFB1 and OTA combined mycotoxicosis

contaminated diet at seventh day of withdrawal of contaminated diet was 0.15 ng/g and complete withdrawal was noticed on tenth day of withdrawal of contaminated diet. Complete withdrawal of AFB1 residues was noticed in the muscles of coloured broiler chicken exposed to low and high dose of combined mycotoxicosescontaminated diet along with mycotoxins binder of Diatomaceous earth (0.5 and 1g of DE) within third day of withdrawal of contaminated diet.

4.9c.2 Ochratoxin A (OTA) withdrawal period

4.9c.2.1 Liver

A small detectable amount of OTA residues was noticed in liver of coloured broiler chicken exposed to low and high dose of OTA (0.5 and 1 ppm) contaminated diet at 13^{th} day of withdrawal of contaminated diet were 4.67 and 5.37 ng/g respectively and complete withdrawal of OTA residues was noticed at 18^{th} day of withdrawal of contaminated diet. A small detectable amount of OTA residue were noticed in liver of coloured broiler chicken exposed to low and high dose of combined AFB1 and OTA contaminated diet at 13^{th} day of experiment were 5.54 and 6.77 ng/g, respectively and were not detected on 18^{th} day of withdrawal of AFB1 and OTA combined mycotoxicoses contaminated diet.

A small detectable amount of OTA residues was noticed in liver of coloured broiler chicken exposed to low and high dose of AFB1 and OTA combined mycotoxicosescontaminated diet along with low and high dose of mycotoxins binder of Diatomaceous earth (0.5 and 1 g of DE respectively) at seventh day of withdrawal of

contaminated diet were 0.95 and 1.07 ng/g, respectively and were not detected on tenth day of withdrawal of contaminated diet

4.9c.2.2 Kidney

A small detectable amount of OTA residues was noticed in kidney of coloured broiler chicken exposed to low and high dose of OTA (0.5 and 1 ppm) contaminated diet at 13th day of withdrawal of contaminated diet were 4.34 and 4.47 ng/g, respectively and complete withdrawal of OTA residues was noticed at 18th day of withdrawal of contaminated diet.

A small detectable amount of OTA residue were noticed in kidney of coloured broiler chicken exposed to low and high dose of combined AFB1 and OTA contaminated diet at 13th day of experiment were 5.67 and 6.85 ng/g, respectively and were not detected on 18th day of withdrawal of AFB1 and OTA combined mycotoxicoses contaminated diet.

A small detectable amount of OTA residues was noticed in kidney of coloured broiler chicken exposed to low and high dose of AFB1 and OTA combined mycotoxicosescontaminated diet along with low and high dose of mycotoxins binder of Diatomaceous earth (0.5 and 1 g of DE respectively) at seventh day of withdrawal of contaminated diet were 0.54 and 1.32 ng/g, respectively and were not detected on tenth day of withdrawal of contaminated diet.

4.9c.2.3 Muscles

A small detectable amount of OTA residues was noticed in muscles of coloured broiler chicken exposed to low dose of OTA (Group 6, 0.5 ppm) contaminated diet at seventh day of withdrawal of contaminated diet was 0.68 ng/g and complete withdrawal of OTA residues was noticed at tenth day of withdrawal of contaminated diet. A small detectable amount of OTA residues was noted at seventh and tenth day of withdrawal of contaminated diet in high dose of OTA (Group 7, 1ppm) was 1.36 and 0.15 ng/g, respectively and complete withdrawal of OTA residues noticed at 13^{th} day of experiment.

A small detectable amount of OTA residues was noticed in muscles of coloured broiler chicken exposed to low dose of combined AFB1 and OTA contaminated diet (Group 8) at seventh and tenth day of withdrawal of contaminated diet was 1.44 ng/g and 0.43 ng/g, respectively and complete clearance of OTA residues was noticed at 13^{th} day of withdrawal of contaminated diet.

A small detectable amount of OTA residues was noted at seventh and tenth day of withdrawal of contaminated diet in high dose of combined AFB1 and OTA contaminated diet (Group 9) were 2.98 and 0.63 ng/g, respectively and complete withdrawal of OTA residues noted at 13^{th} day of experiment. A small detectable amount of OTA residues was noticed in muscles of coloured broiler chicken exposed to low and high dose of AFB1 and OTA combined mycotoxicoses contaminated diet along with low and high dose of mycotoxins binder of Diatomaceous earth (0.5 and 1g of DE, respectively) at third day of withdrawal of contaminated diet were 0.15 and 0.22 ng/g, respectively and were not detected on seventh day of withdrawal of contaminated diet.

Discussion

V. DISCUSSION

The results of the present study on the residual analysis of certain mycotoxins in coloured broiler chicken tissues are discussed here under.

5.1 Growth performance

5.1.1 Body weight gain

The results of present study demonstrated that dietary aflatoxin, ochratoxin individually or in combination severely affected the body weight and performance of coloured broilers. There is a general agreement that dietary aflatoxins and ochratoxin reduce weight gain, feed intake and increase feed conversion ratio in birds. The depression in growth upon feeding AF was attributed to reduced protein and energy utilization (Smith and Hamilton, 1970; Verma *et al.,* 2002), which impaired nutrient absorption and reduced pancreatic digestive enzyme production (Osborne and Hamilton, 1981) and consequently reduced appetite (Sharline *et al.,* 1980).

The present findings are supported by several similar studies, where a significant reduction in body weight gain was recorded. Aflatoxin levels 0.5 mg/kg and 1mg/kg or above diet decreased feed intake, poor feed conversion ratio and retarded the growth of broiler chickens (Carnaghan *et al.,* 1966; Bryden *et al.,* 1980; Zahid *et al.,* 2008; Denli *et al.*, 2009; Indresh *et al.*, 2013).

It was estimated that with each mg/kg increase of aflatoxin in the diet the growth rate would be depressed by 5 per cent when dietary concentrations containing 1 mg/kg of aflatoxin in broilers (Dersjant *et al.,* 2003). Contrary to above statement, in the present

study there was 28.34 to 36.05 per cent decrease in body weight gain in individual AFB1 (0.5 and 1 mg/kg) treated groups. The variation in birds to mycotoxin response may be related to variation in species, breeds and genetic lines age, dose and duration of exposure to mycotoxins (Smith, 2002).

Several studies have also shown that dietary AF adversely affected the growth of broilers in a dose-dependent manner (Johri and Sadagopan, 1989; Espada *et al.,* 1992; Beura *et al.,* 1993). Similar reduction in body weight gain was also observed in broilers which were exposed to dietary AF contents at 0.75 mg/kg (750 ppb) and above. The decreases in body weight in toxin fed groups were reported to be dose-dependent (Reddy *et al.,* 1984). Quezada *et al.* (2000) reported that AFB1 markedly reduced body weight gain (20-30per cent) in broilers exposed to 0.5, 1, or 2 µg/g of feed (daily p.o.) for 28 days.

A significant reduction in body weight gain observed in OTA fed birds may be attributed to either decreased feed intake or impaired protein metabolism (Bailey *et al.,* 1989). Further, Creepy *et al.* (1980) and Huff *et al.* (1988) have reported that OTA compete with phenylalanine for binding sites on the phenylalanine tRNA synthetase enzyme, thus inhibiting the protein synthesis leading to decreased body weight gain.

The results are in agreement with the findings of numerous studies reports (Prior *et al*., 1976; Hamilton *et al*., 1982; Huff *et al*., 1988; Gentles *et al*., 1999) where alteration in performance, including decreased feed consumption and growth rate and poor feed conversion efficiency even at low levels of exposure to OTA (0.5 mg/kg feed) (Prior *et al.,* 1980; Wang *et al.,* 2009) and high levels of exposure to OTA have been

noticed (800, 1000, 2000µg/kg) (Elaroussi *et al.* 2006; Sakthivelan and Rao, 2010; Yang *et al.,* 2013).

In the present experiment, significant reduction in body weight was observed in coloured broilers in combined AFB1 and OTA groups i.e Groups 8 and 9 (66.31and 73.60 per cent) showing synergistic effect of AFB1 and OTA on body weight. In agreement with findings of the present study, Verma *et al.* (2004) reported that AF at a dietary concentration of 1 mg/kg or more and OTA at 2 mg/kg or more, either alone or in combination, caused severe reduction in growth and immune response and combination of 2 mg/kg AF and 4 mg/kg OTA exerted the maximum adverse effect on growth indicating a synergistic effect on performance, also Huff *et al.* (1984) made the similar observations.

In the present study, D.E binder counteracted the combined deleterious effect of AFB1 and OTA in coloured broilers. The results are in agreement with findings of Denli *et al*. (2008) who reported addition of OcraTox (DE, 1-5 g/kg) to the contaminated diet alleviated the negative effects resulting from OTA (2 mg/kg).

5.1.2 Feed Conversion Ratio

In the present study, there was significant increase in FCR in OTA treated groups (0.5 and 1ppm) and in combined AFB1 and OTA treated groups i.e Groups 8 and 9, showing poor feed conversion efficiency in these groups. The reason for same may be attributed to reduced protein and energy utilization (Smith and Hamilton, 1970; Verma *et al.,* 2002), which impaired the nutrient absorption and reduced pancreatic digestive enzyme production (Osborne and Hamilton, 1981). In addition, reduced appetite during

mycotoxicoses could be due to impaired liver metabolism caused by the liver damage due to uptake of toxin contaminated feed and could be responsible for increased FCR in birds fed with AFB1 and OTA.

In agreement with findings of the present study, Verma *et al.* (2004) reported that AF at a dietary concentration of 1 mg/kg or more and OTA at 2 mg/kg or more, either alone or in combination, caused severe reduction in growth and immune response and combination of 2 mg/kg AF and 4 mg/kg OTA exerted the maximum adverse effect, reduced feed intake and feed efficiency, indicating a synergistic effect on performance. Increase in FCR due to feeding of OTA has been reported by several workers (Prior *et al.*, 1980; Elaroussi *et al.*, 2006; Wang *et al.*, 2009; Sakthivelan and Rao, 2010).

In the present study, only birds fed with high dose of (1ppm) AFB1 showed significant increase in FCR but no significant increase in FCR of was noticed in AFB1 groups fed with 0.5ppm. This variation may be attributed to strain, dose and duration of exposure (Smith, 2002). Several studies support the results of present study. Aflatoxin levels 0.5 mg/kg and 1 mg/kg or above diet decreased the feed intake and feed conversion (Carnaghan *et al.,* 1966; Bryden *et al.,* 1980; Zahid *et al.,* 2008; Denli *et al.,* 2009; Shabani *et al.*, 2010; Indresh *et al.*, 2013).

In the present study, DE binder counteracted the combined deleterious effect of AFB1 and OTA in coloured broilers. The results are in agreement with findings of Denli *et al.*(2008), who reported addition of OcraTox (DE, 1-5 g/kg) to the contaminated diet alleviated the negative effects resulting from OTA (2 mg/kg), there by improvement of FCR was noticed in Groups 10 and 11 which were treated with D.E as toxin binder.

5.1.3 Mortality

Highest mortality rate was noticed in birds exposed to combined AFB1 and OTA *i.e* Groups 8 and 9 (12.12 and 21.21 per cent, respectively). The present observations are in accordance with those of Bailey *et al.* (1989) and Kubena *et al*. (1994). Mortality due to ochratoxin or their nephrotoxic and hepatotoxic effects are generally observed if the feed is contaminated with high levels of ochratoxins. However, concurrent presence of other mycotoxins in the feed (Huff *et al*., 1981) can cause mortality in birds or can enhance the adverse effects leading to production losses. OTA being the most toxic during early life and could be the cause of mortality in young birds (Raju and Devegowda, 2000).

5.1.4 Relative organ weight

In the present study, per cent organ weight was altered due to dietary mycotoxins individually or in combination.

5.1.4.1 Liver and Kidney

The results of the present study indicated that dietary aflatoxin, ochratoxin individually or in combination significantly ($P<0.001$) increased the relative weight of liver and kidney. These findings are supported by various studies where similar findings of increase in relative weight of liver and kidney were found in both individual and combined aflatoxicosis and ochratoxicosis (Quezada *et al.,* 2000; Raju and Devegowda, 2000; Santin *et al.,* 2002; Verma *et al.,* 2004; Kalorey *et al.,* 2005; Sakhare *et al.,* 2007;Yang *et al.,* 2013).

Liver is the primary organ involved in mycotoxin detoxification, which is probably the reason why it was affected much more than the kidneys (Yang *et al.,* 2013). The increase in liver weight could be attributed to increased lipid deposition in liver due to impaired fat metabolism (Batina *et al.,* 2005). Renal enlargement might be related to a compensatory functional effect against the actions of the aflatoxins (Quezada *et al.,* 2000), also lipidaemia with subsequent fat deposition and inflammation leads to increase in kidney weight.

The relative weight of liver due to combined toxicity (1 mg/kg AFB1 and 1 mg/kg OTA) was significantly different from those fed on individual AFB1 and OTA, this suggests that there is synergistic effect in causing hepatic lesions.

5.1.4.2 Lymphoid organs

In the present study, AFB1 and OTA caused lymphoid depletion in thymus and bursa of Fabricius in coloured broiler chicken. Presumably this may be the possible reason for decreased weight of these organs. In the present study, there was significant reduction in size of thymus noticed in individual OTA groups, combined AFB1 and OTA groups.

The decrease in relative weights of thymus, bursa of Fabricius and increase in relative weight of spleen in the present study are supported by similar findings of the many research workers (Raju and Devegowda, 2002; Santin *et al.,* 2002; Verma *et al.,* 2004; Kalorey *et al.,* 2005; Sakhare *et al.,* 2007; Indresh *et al.*,2013)

In the present study, although the bursa of birds exposed to AFB1 and OTA with or without DE had a decreased relative weight of bursa of Fabricius as compared to control birds, the difference was not significant. The present findings are in agreement with reports of Santin *et al.* (2002) who reported similar findings where broilers fed with OTA @ 2 ppm for 42 days. Ochratoxin, at all concentrations used in the present work, failed to cause regression of the bursa of Fabricius, which agrees with the work of Kubena *et al.* (1986).

5.2 Serum biochemistry

5.2.1 AST and ALT

In the present study, a significant increase in the AST and ALT activity were observed in both individual (Groups 4, 5, 6 and 7) and combined AFB1 and OTA groups (Groups 8 and 9).

The present findings are supported by many research workers where they found elevation in the activity of liver marker enzymes (AST and ALT) in birds fed with various levels of aflatoxin (Shi *et al.,* 2006; George *et al.,* 2007; Mohamed and Mohamed, 2009; Denli *et al.,* 2009; Umar *et al.,* 2012; Indresh *et al.,* 2013; Bintvihok and Kositcharoenkul, 2006) and ochratoxin (Bailey *et al.,* 1989; Huff *et al.,* 1988; Gentles *et al.,* 1999; Santin *et al.,* 2002; Denli *et al.,* 2008; Sawale *et al.,* 2009; Yang *et al.,* 2013).

A combined mycotoxins groups AFB1 and OTA (low and high dose, 0.5 and 1ppm each) have a synergism effect on liver damage and showed increased AST and

ALT activity and it was in agreement with similar findings of many workers with various dietary levels of aflatoxin and ochratoxin combinations (Kalorey *et al.,* 2005; Sakhare *et al.,* 2007; Sawarkar *et al.,* 2011).

Aflatoxins are hepatotoxic in all vertebrate species and induce fat infiltration, hepatocyte degeneration and necrosis and they alter the liver function (Riley and Pestka, 2005). In addition to nephrotoxic effect, ochratoxin also has hepatotoxic effect (Bozzo *et al.,* 2011) thereby affecting liver function.

Serum ALT and AST activities represent biomarkers for liver function and alterations in serum levels of ALT and AST are liver specific and have been considered as a tool for studying varying cell viability and changes in cell membrane permeability. Increased activities of AST and ALT in blood plasma could be a sign of hepatitis, liver cancer, inflammation of the gall bladder and bile duct course and toxic liver damage (Henry *et al.,* 2000; Nyblom *et al.,* 2004; Petar *et al.,* 2008).

5.2.2 Gamma-glutamyl transferase (GGT)

Significant rise in the serum GGT levels in combined mycotoxin, aflatoxin followed by ochratoxin groups were recorded in the present investigation.

Marked increase in the GGT activity was highly suggestive of bile duct injury. The present findings are supported by similar increase in GGT activity in aflatoxicosis of birds (Bintvihok and Kositcharoenkul, 2006; Shi *et al.,* 2006; Mohamed and Mohamed, 2009; Indresh *et al*., 2013) and ochratoxicosis (Santin *et al.,* 2002). Consequently, the activities of serum glutamate pyruvatate transaminase, serum gluatamate oxaloacetate

tranferase and γ-glutamyl transferase are increased, primarily indicating hepatic damage by aflatoxin and ochratoxin (Devegowda and Murthy, 2005).

5.2.3 Creatinine and uric acid

Significantly elevated serum creatinine and uric acid levels in combined mycotoxicoses, ochratoxin followed by aflatoxin groups were recorded in present investigation.

Present findings are in agreement with findings of Manning and Wyatt (1984), Ramadevi *et al.* (2000), Stoev *et al.* (2000) as well as Indresh and Umakantha, (2013) for ochratoxin report and aflatoxin-ochratoxin combination findings are supported by similar findings of Doerr and Huff (1980), Huff *et al.,* 1992, Kalorey *et al.,* 2005, Sakhare *et al.,* 2007 as well as Mohamed and Mohamed (2009), Sawarkar *et al*. (2011).

Also increase in uric acid level in aflatoxin group is in agreement with findings of Perozo and Rivera, (2003) and Batina *et al.,* 2005. Increase in serum creatinine and uric acid may be attributed to nephrotoxic effect of ochratoxin as evident in present study leading to renal dysfunctions.

Feeding of mycotoxins binder Diatomaceous earth (DE) to coloured broilers significantly prevented the rise in these values indicating its protective effect on kidney during combined mycotoxicoses.

5.2.4 Total protein

Significant decrease in the serum total protein levels in combined mycotoxicoses, aflatoxin followed by ochratoxin groups were recorded in present study, thereby showing the synergistic effect of aflatoxin and ochratoxin on hepatic and kidney injury.

The present results are in agreement with findings of many researchers who exposed the birds with different dietary levels of aflatoxin showed alternation in protein synthesis and decreased total protein concentration (Shi *et al.*, 2006; Arab *et al.,* 2007; Mohamed and Mohamed, 2009; Denli *et al.*, 2009; He *et al.*, 2013; Indresh *et al.,* 2013). Also many researchers who exposed the birds with different dietary levels of ochratoxin reported decrease in total protein concentration in ochratoxicosis (Manning and Wyatt, 1984; Sreemannarayana *et al.,* 1989; Ramadevi *et al.*, 2000; Stoev *et al.*, 2000; Santin *et al.*, 2002; Indresh and Umakantha, 2013).

Severe decrease in the total serum protein levels in combined mycotoxicoses are in agreement with findings of Doerr and Huff (1980), Huff *et al.* (1992), Kalorey *et al.*, 2005, Sakhare *et al.,* 2007, Sawarkar *et al.* (2011) and Umar *et al.* (2012) who reported that reduction in serum total protein due to synergistic action of dietary aflatoxin and ochratoxin in chicks.

Decrease in protein concentration was mainly due to severe binding of AF and ochratoxin to serum proteins, formation of adducts and severe damage to liver leads to hampering of synthesis of proteins in liver thereby decrease the serum level of proteins (Diaz *et al.,* 2010), also it has been reported that ochratoxin A inhibits bacterial, yeast and liver phenylalanyl-tRNA synthetase and thereby affect the protein synthesis followed by

an inhibition of RNA synthesis, which might affect proteins with a high turnover (Dirheimer and Creppy, 1991).

Total serum protein concentration significantly increased in mycotoxins binder Diatomaceous earth (DE) treated combined mycotoxicoses birds suggesting the protective role of toxin binder as far as protein synthesis is concerned.

5.3 Gross and histopathological examination

5.3.1 Liver and kidney

Grossly, the liver of AFB1 fed birds showed pale to yellowish, friable with fatty change, enlarged and mild hemorrhages in the later weeks of the experiment. Kidneys were pale, swollen in the first two weeks and in the later weeks they appeared hemorrhagic. These changes are in agreement with the findings of earlier workers (Mohamed and Mohamed, 2009; Magnoli *et al.,* 2011).

Microscopically, liver showed increased sinusoidal spaces, fatty degeneration and necrotic changes, congestion of parenchyma, vacualation of cytoplasma, bile duct hyperplasia, and presence of inflammatory cells in combined mycotoxicoses and aflatoxin groups followed by ochratoxin exposed birds.

Microscopically, kidney showed swollen tubular epithelium, presence of urate crystals and hyaline droplets, inter tubular haemorrhages were more pronounced in groups fed with ochratoxin and aflatoxin-ochratoxin. Whereas, aflatoxin groups showed presence of degenerative changes in the tubular epithelium, congestion and hemorrahage of parenchyma.

Above findings are in agreement with the findings of Zahid *et al.* (2008) who noticed pallor discoloration of liver and enlargement of liver and kidneys, hemorrhages in different organs of the body in broilers (7 day old) which were fed with aflatoxin B1 (6400 µg/kg) for 7 days.

Microscopically, congestion of liver parenchyma, cytoplasmic vaculation/fatty change of hepatocytes, necrosis of hepatocytes, newly formed bile ducts, mononuclear and hetrophilic cell infiltrations were observed. Kidneys were enlarged and microscopically, reveal degeneration and necrosis of tubular epithelial cells, congestion and hemorrhages of the parenchyma.

Also, many research workers showed similar findings of gross and histopathological changes in liver and kidney of aflatoxin and ochratoxin affected birds (Dwivedi and Burns, 1984; Herenda and Franco, 1996; Biro *et al.,* 2002; Elaroussi *et al.,* 2008; Denli *et al.,* 2009; Mohamed and Mohamed, 2009; Sumit *et al.,* 2010; He *et al.,* 2013).

The lesions in liver can be attributed to the altered fat metabolism and transport by AF and its metabolites. Lesions in kidney can be attributed to the effect of AF metabolites that are excreted through urine. It can be concluded that atrophy of lymphoid organs are due to lymphocytolytic action of AF and its metabolites leading to immunosuppression.

Several researchers have reported that AFB_1 caused hepatic architecture enlargement, fatty degeneration, bile duct hyperplasia, periportal fibrosis, hepatocytic vacuolation and necrosis (Teleb *et al.,* 2004; Ortatatli *et al.,* 2005; Ellakany *et al.,* 2011).

The gross and histopathological changes in combined mycotoxicoses groups are supported by similar findings of Kalorey *et al.,* 2005, Sakhare *et al.,* 2007; Sawarkar *et al.* (2011) who found additive effect of aflatoxin and ochratoxin on liver and kidney damage.

5.3.2 Lymphoid organs

In the present study there was severe reduction in size of thymus, followed by reduced size of spleen and bursa of Fabricius. Presence of hemorrhages, congestion noticed in thymus and microscopically all these lymphoid organs showed depletion and lymphocytolysis, congestion, proliferation of fibrous tissue. These conditions were more pronounced in combined mycotoxicoses groups followed by ochratoxin and aflatoxin fed groups.

Above findings are supported by similar findings by earlier research workers (Kalorey *et al.,* 2005, Sakhare *et al.,* 2007; Mohamed and Mohamed, 2009; Sawarkar *et al.,* 2011).In the present study both doses of mycotoxin binder of Diatomaceous earth (0.5 and 1g of DE) are shown to have protective effect against both individual and combined effect of AFB1 and OTA in coloured broiler chicken. Where in there was negligible damage to liver and kidney as the binder has capacity to adsorb aflatoxin and ochratoxin in the GIT thereby reducing the bioavailabilty of mycotoxins.The present

findings are also supported by Denli *et al.,* 2008 who showed that OcraTox counteracted the deleterious effects caused by OTA.

5.4 Mycotoxin residual analysis

AFB1 and OTA in liver and kidney were found to be a good indicator and probably suitable bio-marker which could show the exposure of broilers to these particular mycotoxins.

5.4.1 Aflatoxin residues

In the present study AFB1 residues was analyzed in coloured broiler chicken tissues found that an AFB1 residue in liver, kidney and muscles increases with the increased in dose and duration of exposure of the toxin. The rate of accumulation of AFB1 was higher in younger birds until 3 weeks of age as compared to older birds. This observation suggested that birds develop a more efficient mechanism of metabolizing AFB1 with increase in age and had a decreased retention of AFB1 in tissue (Zahid *et al.,* 2010).

The present findings are in agreement with findings of Zahid *et al.,* 2010 who reported that exposure of broiler chickens of different age of 7, 14 and 28 days to 1600, 3200 and 6400 µg/kg of three different levels of AFB1 contaminated ration for 7 days and detected the highest AFB1 residues earlier in younger birds and those fed with high AFB1 dietary levels.

The mean residual concentration of AFB1 in the present study may vary between 0.42 to 4.12 ng/g in different tissues after feeding individual AFB1 contaminated diet (0.5

and 1 ppm) for 42 days. These findings are in agreement with findings of Chen *et al.*, 1984 who found mean values for the AFB1 was less than 3 µg/kg of tissue, where broiler chickens that had been fed a diet containing 2057 µg/kg of aflatoxin B1 for 35 days.

In the present study increased AFB1 residual concentration in coloured broiler chicken tissue was observed when fed with the combined AFB1 and OTA contaminated diet as compared to individual AFB1 contaminated diet. This might be due to synergism between AFB1 and OTA in pathogenesis of liver and kidney, thereby affecting the metabolism and excretion of AFB1 leading to maximum residual accumulation (Micco *et al*., 1987). The present findings of synergism and additive effect of AFB1 and OTA are supported by data of many researchers (Huff and Doerr, 1981; Sakhare *et al.,* 2007; Golli-Bennour *et al.,* 2010; Sawarkar *et al.,* 2011; Maja *et al.,* 2013).

The present findings of synergism and additive effect of combined AFB1 and OTA on AFB1 residues are also supported by Micco *et al.* (1988) who found higher content of AFB1 in broiler liver and kidney (0.15 versus 0.02 ppb and 0.40 versus 0.05 ppb respectively), where male broilers exposed to contaminated feed with OTA (50 ppb) and AFB1 (50 ppb) from 14 days old and contaminated feed withdrawn at 37 day.

However, Vilar *et al.* (2008) found that the amount of total aflatoxin and ochratoxin A found in the liver, kidneys and plasma (determined by ELISA) were directly related to the amounts added to the experimental diets and feed consumption, but in this experiment they had not compared the same dose of toxin with that of same dose of combined total aflatoxin and ochratoxin. There is scarce information describing the

effect of combined mycotoxicoses (AFB1 and OTA) and age of the birds upon retention of AFB1 and OTA in body tissues of broiler chicks.

The rate of distribution of AFB1 in the present study in coloured broiler chicken tissues were found in decreasing order, in liver, kidney and muscles, these findings are supported by various studies of residue analyses of aflatoxin after feeding chickens with various levels of toxin in the diet were reported by many researchers (Arulmozhi *et al.*, 2002; Bintvihok and Kositcharoenkul, 2006; Denli *et al.*, 2009; Magnoli *et al*., 2011; Zahid *et al.*, 2010; Yang *et al*., 2012; Fan *et al.*, 2013; Saqer *et al.*, 2014).

However, large individual variation exists in the amount of residues (Wolzak *et al.*, 1986; Magnoli *et al.*, 2011).

5.4.2 Ochratoxin residues

In the present study, OTA residues was analyzed in coloured broiler chicken tissues and found that the concentration of OTA residues in liver, kidney and muscles increases with the increase in dose and duration of ingestion of the toxin, but OTA concentrations in younger broiler chicken organs appeared to reach higher levels in the early stages of exposure to OTA until 3 weeks of age as compared to older birds, and subsequently rate of accumulation was not increased significantly, although the birds continued to be exposed to OTA, thereby suggesting that OTA is highly toxic in early stage of life.

These findings are in agreement with similar reports by (Bozzo *et al.*, 2011; Raju and Devegowda, 2000).These findings suggest that shortly after initial exposure to OTA,

the bird's detoxification pathways are less effective to clear the toxin metabolites than in the late phases of exposure. The OTA metabolic pathways are mediated by the CYP450 enzymes (Ringot *et al.,* 2006; Simarro *et al.,* 2004) and likely undergo mechanisms of enzymatic induction able to increase the physiological levels of biotransformation of OTA.

In the present study high levels of OTA was widely distributed in kidney, followed by liver and muscles, these findings are supported with the findings of some previous studies in birds (Harwig *et al.,* 1983; Merquardt and Frohlich, 1992; Micco *et al*., 1987; Biro *et al.,* 2002; Stoev *et al.,* 2004; Ringot *et al.,* 2006; Bozzo *et al.,* 2009; Nafeesa *et al.,* 2012; Yang *et al.,* 2013). The accumulation of OTA residues in tissues especially liver and kidney is probably due to enterohepatic recirculation and hepatobiliary excretion of OTA. These metabolic routes provoke a direct toxic effect of this toxin in these organs. Also renal proteins have been demonstrated to have a strong affinity for OTA (Daniel *et al.,* 2005).

In the present study, increased OTA residual concentration in coloured broiler chicken tissue was observed when fed with the combined AFB1 and OTA contaminated diet as compared to individual OTA contaminated diet. This might be due to synergism between AFB1 and OTA in pathogenesis of liver and kidney, thereby affecting the metabolism and excretion of OTA. These metabolic routes provoke a direct toxic effect of this toxin in these organs leading to maximum residual accumulation.

The present findings of synergism and additive effect of AFB1 and OTA are supported by data of many researchers (Huff and Doerr, 1981; Sakhare *et al.,* 2007; Golli-Bennour *et al.,* 2010; Sawarkar *et al.,* 2011; Maja *et al.,* 2013).

The present findings of synergism and additive effect of combined AFB1 and OTA on OTA residue are also supported by Micco *et al.* (1988) who found higher content of OTA in broiler livers (40 versus 5.0 ppb) and, to a lesser extent, in kidneys and skin, where male broilers exposed to contaminated feed with OTA (50ppb) and AFB1 (50ppb) from 14 days old and contaminated feed withdrawn at 37 day.

5.4.3 Aflatoxin withdrawal period

In the present study, complete withdrawal of AFB1 was observed in liver and kidney varied between 10-13 days in both individual AFB1 and combined mycotoxicoses groups after removal of mycotoxins contaminated diet. Whereas, in muscles complete clearance of AFB1 was observed in both individual AFB1 and combined mycotoxicoses groups may vary between 7-10 days after withdrawal of contaminated diet.

A complete clearance of AFB1 was observed in liver and kidney after seven days and in muscle it was three days after removal of combined mycotoxins contaminated diet along with mycotoxins binder of Diatomaceous earth (0.5 and 1g of D.E) from coloured broilers.

The capacity of the liver and kidneys to concentrate aflatoxins is probably associated with their important role in the metabolism and elimination of xenobiotics.

(Chen *et al.,* 1984). However, large individual variation exists in the time required for their total clearance (Wolzak *et al.,* 1986; Magnoli *et al.,* 2011).

Some authors have reported elimination of aflatoxins from tissues after withdrawal of aflatoxin contaminated feed within four days where broiler chickens that had been fed a diet containing 2057 µg/kg of aflatoxin B1 for 35 days (Chen *et al.,* 1984), 7-8 days in layers (Trucksess *et al.,* 1983; Wolzak *et al.,* 1986).

According to reports of Zahid *et al.,* 2010 who showed that birds of age 28 day old given 1600 and 3200 ppb AFB1 for seven days had no detectable AFB1 levels in muscles and liver after 3 and 8 days of withdrawal of contaminated feed, whereas on same withdrawal periods AFB1 residues was still present young birds of age seven days fed with same concentration of AFB1 for seven days.

The variation in the complete clearance after withdrawal of mycotoxins contaminated feed from broiler chicken may be due of variation in the susceptibility of species, strain, age, sex, dose, duration of exposure, liver enzyme status, nutritional status and disease condition etc may affect the metabolism and excretion of mycotoxins (FDA, 1979).

5.4.4 Ochratoxin withdrawal period

In the present study, complete clearance of OTA in liver and kidney was more than 13-18days (2.5 weeks) in both individual OTA and combined mycotoxicoses group after removal of mycotoxins contaminated diet. Whereas, in muscles complete clearance

of OTA was observed in both individual OTA and combined mycotoxicoses group was 13 days after the withdrawal of contaminated diet.

A complete clearance of OTA was observed in liver and kidney were ten days and in muscle it was seven days after removal of combined mycotoxicoses contaminated diet along with mycotoxins binder of Diatomaceous earth (0.5 and 1g of DE) from coloured broilers

Above observations are supported by the findings of Wolzak *et al. (*1986) found four days after the withdrawal of ochratoxin from the feed and no residues could be detected in the edible tissues of poultry.

The variation in the complete clearance after withdrawal of mycotoxin contaminated feed from broiler chicken may be due of variation in the susceptibility of species, strain, age, sex, dose, duration of exposure, liver enzyme status, nutritional status and disease condition which will affect the metabolism and excretion of mycotoxins (FDA, 1979).

In the present study, both doses of mycotoxins binder of Diatomaceous earth (0.5 and 1g of DE) were significantly reduced the AFB1 and OTA residues in coloured broiler chicken tissues when exposed to combined AFB1 and OTA at level of (0.5 and 1ppm each). The present findings are supported by Denli *et al.*, 2008 who showed that OcraTox counteracted the deleterious effects caused by OTA.

Summary

VI. SUMMARY

In the present study, impact of induced aflatoxicosis, ochratoxicosis and combined mycotoxicoses on various parameters of coloured broiler chickens and tissue distribution of mycotoxins have been studied. An attempt was also made to evaluate the protective role of Diatomaceous earth (DE) in combined mycotoxicoses in coloured broilers, also established the pre-slaughter withdrawal period in coloured broiler chickens following dietary exposure of aflatoxin and ochratoxin contaminated feed for 42 days.

In the present study, coloured broiler chickens were procured from Department of Poultry Science, Veterinary College, Bangalore and assigned to 10 experimental groups (Groups 2-11) and the negative control group (Group 1) each group containing 66 birds with three replicates of 16-17 birds each exposed to dietary treatment for six weeks. Groups 2 and 3 served as D.E binder control (0.5 and 1g respectively), Groups 4 and 5 served as aflatoxin control (0.5 and 1ppm respectively), Groups 6 and 7 served as ochratoxin control (0.5 and 1ppm respectively), Groups 8 and 9 served as combined aflatoxin and ochratoxin control (0.5ppm+0.5ppm and 1ppm+1ppm respectively), and Groups 10 and 11 served as combined aflatoxin and ochratoxin treated with D.E mycotoxins binder (0.5ppm+0.5ppm+0.5g and 1ppm+1ppm+1g respectively) and these groups have been exposed these dietary treatment for a period of 42 days.

Birds were sacrificed at weekly interval six birds from each group were selected randomly and sacrificed by cervical dislocation (exsanguinations), blood samples were collected for serum biochemical analysis. Birds were subjected to detailed postmortem examination for any pathological lesions and organs were collected for histopathological

examination. For mycotoxins residual analysis liver, kidney, breast muscle and leg muscle samples were collected. The tissue depletion from the aforesaid edible tissues were determined by sacrificing the remaining birds (n=6 each) at on day 3, 7, 10, 13 and 18 following withdrawal of aflatoxin and ochratoxin contaminated feed for 42 days.

Coloured broilers showed good pattern of aflatoxicosis, ochratoxicosis and combined mycotoxicoses. There was increase in the mortality rate, greater feed conversion and relative weight of liver, kidney and spleen, decrease in body weight, also reduced relative weight of lymphoid organs mainly bursa of Fabricius and thymus, morbidity and immunosuppression were noticed in combined mycotoxicoses, followed by ochratoxicosis and aflatoxicosis groups. Marked changes in serum biochemical parameters, gross and histopathological changes were noticed in combined mycotoxicoses, followed by aflatoxin as well as ochratoxin. Addition of diatomaceous earth (DE) mycotoxin binder to contaminated feed showed protective role against above said parameters.

A simple, economical method was developed with less usage of solvents, tissue samples were subjected to SPE for extraction of target analyte (AFB1 and OTA) , 20 µl of the extract was injected into LC- MS/MS (Agilent Technologies, Waldbron, Germany) system equipped with reverse phase C18 column (Thermo Scientific BDS Hypersil C18 RP, 100x4.6 mm, 5 µm) with a flow rate of 700 µl. Mobile phase consisted of acetonitrile, 0.1 % formic acid water , v/v (70:30) with gradient elution and short run time (3.5 min) was used in the present study.

Mean per cent recoveries for AFB1, AFB2 and OTA (75.95, 66.87 and 79.05 % respectively) were optimized with achieving the criteria of USFDA guidelines.

Residual accumulation or tissue distribution was more for OTA in low (52.80, 53.63 and 7.16 ng/g) and high (71.85, 72.68 and 15.06 ng/g) dose of combined mycotoxicoses than AFB1 residues in the low (3.28, 3.19 and 1.23 ng/g) and high dose of (4.28, 3.87 and 2.64 ng/g) combined mycotoxicoses in liver, kidney and muscle respectively, followed by individual low (38.55, 39.35 and 5.18 ng/g) and high dose (51.65, 52.76 and 7.23 ng/g) of OTA than individual low (3.16, 3.09 and 0.42 ng/g) and high dose (4.12, 3.20 and 1.25 ng/g) of AFB1.

Use of D.E binder showed significantly reduced the AFB1 residual concentration in low (1.26, 1.22 and 0.80 ng/g) and high (1.33, 1.28 and 0.88 ng/g) dose of combined mycotoxicoses treated with binder of low (0.5 g) and high dose (1 g), also reduced OTA residual concentration was observed in low (17.15, 21.18 and 1.72 ng/g) and high (17.45, 21.51 and 1.86 ng/g) dose of OTA in combined mycotoxicoses treated with binder of low (0.5 g) and high dose (1 g) in liver, kidney and muscle respectively.

The pre-slaughter period for both individual and combined mycotoxicoses for AFB1 residues was in between 10-13 days and for both individual and combined mycotoxicoses for OTA residues was more than 18 days.

The effects of ochratoxin A appear to be long lasting than those of aflatoxin and highly toxic to younger birds, has more hepatotoxic and nephrotoxic effect. The rate of

OTA residues distribution was in decreasing order of kidney>liver> muscles and for AFB1 residues distribution was decreasing order of liver > kidney > muscles.

Combined mycotoxicoses of AFB1 and OTA showed synergism effect on reduced growth performance, increased hepatic functional enzyme markers (AST, ALT and GGT), creatinine, uric acid, total protein, gross and histopathological changes and bioaccumulation of AFB1 and OTA in liver, kidney and muscle.

In the present study, irrespective of aflatoxin and ochratoxin exposure levels, the rate of clearance of AFB1 and OTA from edible tissues was in the order of muscles > kidney $\geq$liver. Keeping in view of the minimum performance limit of the analytical system developed and in the absence of any standard regulatory limits for AFB1 and OTA residues or tolerance level it is suggested to adhere to 13 days and 18 days pre-slaughter withdrawal period for AFB1 and OTA exposure, respectively in broiler chickens.

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Abstract

VIII. ABSTRACT

The experiment was conducted to study the impact of induced aflatoxicosis, ochratoxicosis and combined mycotoxicosis on growth performance, serum biochemical, pathological changes and tissue distribution of AFB1 and OTA, also to establish pre-slaughter withdrawal period in coloured broiler chickens following dietary exposure of aflatoxin B1 and ochratoxin A contaminated diet for 42 days. An attempt was also made to adjudge the protective role of diatomaceous earth (D.E) on combined mycotoxicosis. A total of 10 experimental groups (Groups 2-11) and the negative control group (Group1) each group containing 66 birds with three replicates of 16-17 birds each exposed to dietary treatment for 42 days. Group 2 and 3 as D.E binder control (0.5 and 1g), Groups 4 and 5 as aflatoxin control (0.5 and 1ppm), Groups 6 and 7 as ochratoxin control (0.5 and 1ppm), Groups 8 and 9 as combined aflatoxin and ochratoxin control (0.5ppm+0.5ppm and 1ppm+1ppm), and Groups 10 and 11 served as combined aflatoxin and ochratoxin treated with D.E mycotoxins binder (0.5ppm+0.5ppm+0.5g and 1ppm+1ppm+1g, respectively).There was significant reduction in body weight, relative weight of lymphoid organs, total protein and poor FCR, increase in relative weight of liver and kidney, AST, ALT,GGT, creatinine and uric acid, also increase in AFB1 and OTA residues in combined followed by individual mycotoxicosis was observed. Supplementation of D.E showed protective role on all these parameters against combined mycotoxicosis. In general, pre-slaughter withdrawal period was established for combined mycotoxicosis for AFB1 and OTA were in between 10-13 days and 13-18 days respectively.

Keywords: Coloured broiler; Aflatoxin B1; Ochratoxin A; Residue; Withdrawal period