

**PATHOMORPHOLOGICAL AND
IMMUNOSUPPRESSIVE STUDIES ON INDUCED
AFLATOXICOSIS AND ITS AMELIORATION
USING DIATOMACEOUS EARTH (DAE) IN
BROILER CHICKEN**

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SCIENCES UNIVERSITY, BIDAR
MAY, 2015**

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BROILER CHICKEN**

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*In partial fulfillment of the requirements
for the award of the degree of*

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in

VETERINARY PATHOLOGY

By

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SCIENCES UNIVERSITY, BIDAR
DEPARTMENT OF VETERINARY PATHOLOGY
VETERINARY COLLEGE, HEBBAL, BANGALORE
CERTIFICATE**

This is to certify that the thesis entitled “*PATHOMORPHOLOGICAL AND IMMUNOSUPPRESSIVE STUDIES ON INDUCED AFLATOXICOSIS AND ITS AMELIORATION USING DIATOMACEOUS EARTH (DAE) IN BROILER CHICKEN*” submitted by **Mr. AVINASH WARUNDEO LAKKAWAR, ID No. DVHK 1227** in partial fulfilment of the requirements for the award of degree of **DOCTOR OF PHILOSOPHY in VETERINARY PATHOLOGY of the Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar** is a bonafide record of research work carried out by him during the period of his study in this University, under my guidance and supervision and that no part of the thesis has not previously formed the basis for the award of any degree, diploma, associate ship, fellowship or other similar titles.

Bangalore
25th May, 2015

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Dedicated to

my father

Late Shri Warundeo Balaji Lakkawar,

the source of inspiration, my brother

Late Shri Anil Warundeo Lakkawar

And to my inlaws

Late Shri Sabulaji Jaget

Late Smt Rajkumari Jaget

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LIST OF ABBREVIATIONS

⁰ C	Degree Celsius
AF	Aflatoxin
AFB	Aflatoxin B
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
APC	Antigen Presenting Cell
AST	Aspartate aminotransferase
bp	Base pair
BSA	Bovine Serum Albumin
BUN	Blood urea nitrogen
BW	Body weight
CD	Cluster of differentiation
Con A	Concanavalin A
DAE	Diatomaceous earth
DCs	Dendritic cells
dL	Decilitre
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbant assay
F	Forward
FACS	Flourescence activated cell sorting
FAO	Food and Agriculture Organization
Fig.	Figure
FITC	Fluorescein isothiocyanate
g	Gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
h	hour
H & E	Haematoxylin and Eosin

HCl	Hydrochloric acid
HDL	High density lipoprotein
HI	Haemagglutination inhibition
HRP	Horseradish Peroxidase
IBDV	Infectious Bursal disease virus
IBM	International Business Machines
IFN	Interferon
Ig	Immunoglobulin
IL-2	Interleukin-2
IL-6	Interleukin-6
IL-8	Interleukin-8
IU	International unit
Kg	Kilogram
L	Litre
LD ₅₀	Lethal dose 50
LDL	Low density lipoprotein
mg	Miligram
MHC	Major Histocompatibility Complex
min	Minute (s)
ml	Mililitre
mm	Milimeter
MNC	Mononuclear cell
mRNA	messenger Ribonucleic acid
MTCC	Microbial Type Culture Collection
MTT	3 (4,5-dimethylthiazol-2, 5-diphenyltetrazolium bromide)
MW	Molecular weight
NDV	Newcastle disease virus
nm	Nanometer
OD	Optical Density
OIE	Office International des Epizooties
P	Phosphorous

PBS	Phosphate buffer saline
PBS	Phosphate Buffer Saline
PCR	Polymerase chain reaction
PDRC	Poultry Diagnostic Research Center
Pg	Picogram
ppb	Parts per billion
ppm	Parts per million
RPE	Red-Phycoerythrin
rpm	Rotation per minute
RT-PCR	Reverse transcription polymerase chain reaction
SPSS	Statistical Package for the Social Sciences
TCR	T-cell receptor
TG	Triglycerides
Th cell	T helper cell
TLC	Thin layer chromatography
TM	Tunisian Montmorillonite
TMB	3,3',5,5' tetramethyl-benzidine
TNF- α	Tumor Necrosis Factor-Alpha
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
U	Unit
U/L	Units per litre
UTR	Untranslated region
VHL	Venkateshwara Hatcheries Private Limited
VLDL	Very low density lipoprotein
μ	Micron
μ g	Microgram
μ L	Microlitre
μ m	Micrometer

Introduction



I. INTRODUCTION

Poultry production is one of the fastest growing sectors of Indian agriculture. India is emerging as World's 2nd largest poultry producer with an annual growth of more than 14 per cent, contributing 61 million tonnes or 3.6 percent of global egg production. The Indian poultry industry has been growing around 8-10 per cent annually over the last decade with the broiler production growing at more than 10 per cent, while table egg at 5-6 per cent driven by increased domestic consumption. 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 of poultry meat is approx 2.4 kg, which is much lower than the National Institute of Nutrition's recommendations of 11 kg (International Poultry and Livestock Expo, 2014). This enormous growth and spurt in the poultry production has put a tremendous pressure on proper feed production for poultry in order to sustain the poultry industry in India.

Contamination of poultry feeds with mycotoxins is one of the major problems associated with feeding of poultry (Mohanamba *et al.*, 2007). The contamination of mycotoxins is one of the major detractors of poultry production and also the product quality, hence, it becomes more relevant now than ever to control their impact in poultry farming which is critical (Oguz, 2011).

According to the Food and Agriculture Organization (FAO), approximately 25 per cent of world's grain supply is contaminated with mycotoxins (FAO, 2011). The greatest economic impact of mycotoxin contamination is felt by crop and poultry

producers, as well as food and feed producers. In India, the tropical climate with hot and humid condition coupled with poor harvesting practices of crops during monsoon season make feed stuff susceptible to fungal contamination resulting in greater economic losses to poultry industry through mycotoxicosis. Among mycotoxins, aflatoxins are predominant and are one of the major fungal toxins, which are attributed for potential problem in poultry feeds. The U.S. Food and Drug Administration (FDA) had established 20 ppb as the maximum permissible aflatoxin level in poultry feed ingredients (Richard, 2000). In India, the aflatoxin contamination of feed and feed stuffs has been reported to range from 10-1500 ppb in feed ingredients and 0.54- 204.7 ppb in mixed feed samples (Ramesh *et al*, 2013). These levels of aflatoxin content in feed and feed ingredients is considered to be detrimental to the health of the broilers and layer birds as well as to the consumers, since aflatoxin can enter the human food chain through eggs and poultry meat.

Aflatoxins (AF) are secondary metabolites and a class of mycotoxins produced predominantly by *Aspergillus flavus* and *A. parasiticus* (Huwig *et al.*, 2001). The toxin occurs worldwide in feeds and feed stuffs resulting into severe economic loss to poultry and livestock industries in many countries of the world (Miller, 1995). Aflatoxin contamination may cause the difference between profit and loss to the poultry industry (Hamilton, 1984). Studies have been related to negative effects of aflatoxin in broiler chickens including decrease in body weight gain, efficiency of feed utilization, liver damage, poor performance and immune responses. AF also caused pathologic alteration in important organs such as liver, kidneys and lymphoid organs (Kubena *et al.*, 1993; Dafalla *et al.*, 1987). The pathological alterations in broilers are characterized by hepatic

lesions such as enlargement, paleness, fatty change, bile duct hyperplasia and periportal fibrosis (Dafalla *et al.*, 1987; Ledoux *et al.*, 1999). Furthermore, the transmission of AFB₁ and its metabolites from feed to animal edible tissues and products, such as liver and eggs (Bintvihok, 2002), becomes particularly important as a potential hazard for human health.

Decontamination of AF is a major task in the poultry industry. Producers and researchers desire to develop an effective de-contamination technology dealing with the feed-borne toxin (Leeson *et al.*, 1995). Approaches used included physical, chemical and biological treatment of contaminated feed and feed stuffs. A successful detoxification process must be economical and must be capable of eliminating all traces of toxin without leaving harmful residues and also should not impair the nutritional quality of the commodities (Parlat *et al.*, 1999). In the last two decades several studies have been performed using adsorbent materials for detoxifying AF in contaminated feed and feed stuffs (Abo-Norag *et al.*, 1995).

Several approaches to avoid contamination such as decontamination or remediation of feed and feedstuffs have been proposed (Ledoux *et al.*, 1999). A variety of adsorbents such as bentonite (Rosa *et al.*, 2001), zeolite (Miazzo *et al.*, 2000), hydrated sodium calcium aluminosilicate (Scheideler, 1993), *Saccharomyces cerevisiae* (Celik *et al.*, 2001) and activated charcoal (Jindal *et al.*, 1994) have been successfully used in detoxifying aflatoxins in contaminated feeds (Ramos and Hernandez, 1997). Adsorbent compounds utilized to ameliorate aflatoxicosis in poultry diets includes Aluminosilicates, Bentonite, Silicas, Zeolite, Quartz, etc have been evaluated for their ability to remove or diminish the adverse effect of mycotoxins in animal feed. These compounds must not be

absorbed from the gastrointestinal tract and must have the ability to bind physically with chemical substances, precluding their adsorption (Santurio *et al.*, 1999; Miazzo *et al.*, 2000). The major advantages of adsorbents include cost effectiveness, safety and easy administration through animal feeds. However, no compound is 100 per cent effective in eliminating aflatoxin from feed.

Diatomite or Diatomaceous earth (DAE) is a kind of clay that consists of 90 per cent silicon dioxide. It is fine-grained, biogenic siliceous sediment and is available in large quantities at low cost (Kamigasa and Kato, 2000). DAE consists essentially of amorphous silica derived from opalescent frustules of diatoms resulting in an inert, light weight, highly porous, super-absorbent material, and has a fine porous structure with low density (Wajima *et al.*, 2006). DAE is an inert dust formed by milling of fossilized remains of phytoplanktons (diatoms), composed of silicon dioxide, effectively used for the control of insects infesting stored products (Alves *et al.*, 2006). Modirsanei *et al.* (2008) reported that diatomaceous earth increased the body weight gain, feed intake, and improved the feed conversion ratio as well as productive efficiency index. DAE also increased serum albumin levels in the birds that were subjected to AFB₁ supplementation.

Considering the above properties of DAE, the present study was undertaken with following objectives.

1. To study symptomatology, biochemical and pathomorphological changes in induced aflatoxicosis in broiler chicken.
2. To assess the immunosuppressive effect of aflatoxin in broiler chicken.
3. To assess amelioration of aflatoxin using Diatomaceous earth.

Review of Literature



II. REVIEW OF LITERATURE

Mycotoxins have become increasingly important in understanding of feed safety and feed poisoning in animals and poultry. The major problem with mycotoxicosis for the clinician is that few produce overt signs of poisoning, and therefore mycotoxicosis is very difficult to diagnose (Pitt *et al.*, 2000). The impact of fungal toxins upon animals extends beyond their obvious effect in producing death in a wide variety of animals that are likely to consume mycotoxin-contaminated grains or feeds. The economic impact of lowered productivity, reduced weight gain, reduced feed efficiency, subtle damage to vital body organs and interference with reproduction is many times greater than that of immediate morbidity and mortality. Potential threats of cancer induced by mycotoxins in feeds and human foods, along with the unknown subtle effects of these mycotoxins, are coupled to the universal concerns about health risks (Marquardt, 1996). Consumption of some mycotoxins, at levels that do not cause overt clinical mycotoxicosis, may suppress immune functions and decrease resistance to infectious disease. The sensitivity of the immune system to mycotoxin-induced immunosuppression arises from the vulnerability of the continually proliferating and differentiating cells that participate in immunomediated activities and regulate the complex communication network between cellular and humoral components (Corrier, 1991).

2.1 AFLATOXICOSIS

Aflatoxicosis is a serious fungal problem affecting wide range of species of animals, birds and human beings world over. The aflatoxins produce serious health hazards by affecting the growth, performance and produce deleterious effects on various

visceral and lymphoid organs. The effect of aflatoxin and its amelioration have been reviewed under appropriate headings.

2.2 AFLATOXIN

The name aflatoxin was derived by using first letter from *Aspergillus* and the first 3 letters from *Flavus* (Patterson, 1977). The aflatoxins are potent liver toxins and their effects vary with dose, length of exposure, species, breed and diet or nutritional status. These toxins may be lethal when consumed in large doses. Substantial doses produce an acute toxicity and chronic exposure to low levels can result in cancer (Marquardt, 1996).

The aflatoxin problem is long-standing and inextricable (Phillips, 1999). Aflatoxin is the most prevalent and economically significant mycotoxin which needs immediate attention. It is found in maize, peanuts, cottonseed, millet, sorghum and other feed grains. *Apergillus flavus* produces the majority of the toxin, which explains the origin of its name, but aflatoxin is also produced by *A. parasiticus*. The fungi are found widely in the environment, contain toxigenic and non-toxigenic strains, and produce aflatoxin in warm (30-35° C) and high humidity (water activity of 0.90-0.99) conditions. Aflatoxin contamination is thus more likely in grains grown or handled in the tropics or subtropics. Stressing host plants by insect damage, drought, poor nutrition or delayed harvest increases aflatoxin production (Brown, 1996). According to Moss (1996) the aflatoxin levels of foods for human consumption often exceed the upper limit of 30 µg/kg initially established by the Food and Agricultural Organization (FAO) and the World Health Organization (WHO). Meanwhile, many developed countries had set legislative or guideline levels that were more stringent than the limits set initially by the FAO/WHO.

Grains and oil seeds, adulterated with aflatoxins at concentrations higher than 20 µg/kg are condemned in the United States. However, in lesser-developed countries with limited supplies of food, it is not always feasible, nor practical, to condemn and destroy mouldy grains. Thus, these practices may result in enhanced aflatoxin exposure and a higher incidence of aflatoxin induced disease in humans and animals.

Like many microbial secondary metabolites, the aflatoxins are a family of closely related compounds (Moss, 1996). Naturally occurring aflatoxin consists of aflatoxins B₁ and B₂, but AFB₁ is usually in the highest concentration and is the most toxic. According to Moss (1996) AFB₁ is an acute toxin as well as being carcinogenic and immunosuppressive. Aflatoxin is stable once formed in grain, and is not degraded during normal milling and storage (Brown, 1996).

According to Lanza *et al.* (1980) chickens of different ages showed differential susceptibility to different aflatoxin doses. Quezada *et al.* (2000) found that one-week-old broilers were more susceptible to aflatoxin than 4-week-olds. There are also large species differences, with ducks being 10 times more sensitive than chickens, and turkeys intermediate between the two (Brown, 1996). Giambrone *et al.* (1985) found that aflatoxin is highly toxic to 2-week-old turkeys and mildly toxic to 2-week-old broilers when fed daily for 5 weeks at 0.4 mg/kg and 0.8 mg/kg feed. The day-old duckling and the adult dog are remarkably sensitive to the acute toxicity of AFB₁, with LD values of 0.35 and 0.5 mg respectively, while others, such as the adult rat and the mouse, are more resistant (LD₅₀ ca. 9mg/kg). Not all animals respond to the carcinogenic activity of aflatoxin, but for the rat aflatoxin is one of the most carcinogenic compounds known.

Acute toxicity of aflatoxin in humans was reported in India in 1974, when a large outbreak of poisoning occurred, involving nearly 1000 people, of whom nearly 100 died (Moss, 1996).

2.2.1 Occurrence

Agricultural and food commodities contaminated with aflatoxins include cereals (maize, sorghum, pearl millet, rice, barley, beans, and wheat), oilseeds (groundnut, soyabean, sunflower, and cotton), spices (chillies, black pepper, coriander, turmeric, and ginger), tree nuts (almonds, pistachio, walnuts, and coconut) cassava, sweet potato and milk. Aflatoxins are also found in fruits particularly apples, beer and wine due to usage of contaminated barley, cereals and grapes for their production. Mycotoxins also enter the human food chain via meat or other animal products such as eggs, milk and cheese as a result of livestock eating contaminated feed. The results of the contamination monitoring program for mycotoxins from 1976 to 1983 showed that, much of the monitored grain contained aflatoxin above 20.00 to 50.00 ppb, higher than the regulatory limits in feeds of most countries (Jelinek *et al.*, 1989).

Author and year	Ingredient	Level (ppb)
Shetty <i>et al.</i> (1987)	Mixed poultry feed	30-1610
Devegowda <i>et al.</i> (1990)	Groundnut, Maize, Bajra	48-900 32-1000 12-15
Devegowda and Arvind (1993)	Maize Ground nut cake Others	25-1002 45-1500 10-80
Dhavan and Choudary (1995)	Feed ingredients and mixed feeds	High concentrations
Chandrasekharan (2000)	Maize	21to 500

Pandey <i>et al.</i> (2001)	Maize	948
	Wheat	285
	Groundnut extraction	225
Chandrasekharan <i>et al.</i> (2002)	Different feed samples	0 – 50
Manafi <i>et al.</i> (2009)	Mash Poultry feeds	450
Manafi, (2010)	Pelleted Poultry feeds	470
Chandra <i>et al.</i> (2013)	Maize samples for Poultry feed	9-250
Sarathchandra and Muralimanohar (2013)	Poultry feed	20-50
Ramesh <i>et al.</i> (2013)	Different Feed samples	0.54- 204.7
Kannan <i>et al.</i> (2014)	Poultry feeds	upto 160.7

2.2.2 FAVOURABLE CONDITIONS FOR AFLATOXIN BIOSYNTHESIS AND PRODUCTION

The formation of aflatoxins is influenced by physical, chemical and biological factors. The physical factors include temperature and moisture. The chemical factors include the composition of the air and the nature of the substrate. Biological factors are those associated with the host species (Hesseltine, 1983).

Specific nutrients, such as minerals (especially zinc), vitamins, fatty acids, amino acids and energy source (preferably in the form of starch) are required for aflatoxins formation (Wyatt, 1991). Large yield of aflatoxins are associated with high carbohydrate concentrations, such as wheat rice and to a lesser extent in oilseeds such as cottonseed, soyabean and peanuts (Diener and Davis, 1968).

The limiting temperatures for the production of aflatoxins by *A. flavus* and *A. parasiticus* are reported as 12 to 41 °C, with optimum production occurring between 25 and 32°C (Lillehoj, 1983). Synthesis of aflatoxins in feeds are increased at

temperatures above 27°C, humidity levels greater than 62% and moisture levels in the feed above 14% (Royes and Yanong, 2002).

2.2.3 CAUSES OF AFLATOXIN

Crops grown under warm and moist weather in tropical or subtropical countries are especially more prone to aflatoxin contamination than those in temperate zones. Groundnuts and groundnut meal are by far the two agricultural commodities that seem to have the highest risk of aflatoxin contamination. Although, these commodities are important as substrates, fungal growth and aflatoxin contamination are the consequence of interactions among the fungus, the host and the environment. The appropriate combination of these factors determines the infestation and colonization of the substrate and the type and amount of aflatoxin produced. Water stress, high-temperature stress and insect damage of the host plant are major determining factors in mould infestation and toxin production. Similarly, specific crop growth stages, poor fertility, high crop densities and weed competition have been associated with increased mould growth and toxin production. The moisture content of the substrate and temperature are the main factors regulating the fungal growth and toxin formation. A moisture content of 18% for starchy cereal grains and 9-10% for oil-rich nuts and seeds has been established for maximum production of the toxin (WHO, 1979).

2.2.4 ABSORPTION

Aflatoxins are lipid soluble compounds and are therefore, readily absorbed from the site of exposure into the bloodstream. Absorption of ¹⁴C-labelled aflatoxin administered orally to laying hens occurred very rapidly from the gastrointestinal tract

(Sawhney *et al.*, 1973). Absorption of aflatoxin from the respiratory system has been reported in workers at feed mills, although there have been no studies to determine the quantitative importance of this route of absorption of aflatoxins in poultry (Leeson *et al.*, 1995).

2.2.5 METABOLISM

After absorption from the small intestine, AF readily binds to plasma albumin, which serves as the major transporter of aflatoxin in blood (Yatin and Sachan, 2001). Aflatoxin is metabolised in liver to produce various metabolites in different animals. AFB₁ is oxidized by microsomal mixed-function oxidase (cytochrome P450) to several water-soluble metabolites. It is generally believed that the formation of AFB-8,9-epoxide, an active metabolite and its subsequent covalent binding to DNA, RNA and proteins plays a critical role in both acute and chronic toxicity (Eaton and Gallagher, 1994). Animals, which fail to produce the epoxide are relatively resistant to both, but those animals which produce the epoxide, but do not effectively metabolize it further, may be at the highest risk to the carcinogenic activity of AFB₁, but relatively resistant to the acute toxicity. Those animals that not only produce the epoxide but also effectively remove it with a hydrolase enzyme, thus producing a very reactive hydroxyacetal, are the most sensitive species to the acute toxicity (Moss, 1996). In most animal models examined, glutathione S-transferases are probably the most critical AFB₁-detoxifying enzymes. The extreme sensitivity of animal species such as turkeys, to AF may be due to a combination of efficient activation by cytochrome P450 and deficient detoxification as a result of the absence of AFB₁-specific glutathione S-transferase activity in the liver (Klein *et al.*, 2000). The glutathione S-transferase isozyme M1a-1a appears to be the

most active in conjugating 8, 9-epoxide in humans (Johnson *et al.*, 1997). This isozyme is expressed as null phenotype in approximately 50% of the Caucasian population and individuals so expressing it may be at an increased risk of developing chemically induced cancers (Ryberg *et al.*, 1997).

The epoxide of aflatoxin is known to react with guanine residues in DNA, and can cause subsequent depurination while the hydroxyacetale derivate reacts with proteins through such residues as lysine. The parent molecule may thus be seen as a very effective delivery system, having the right properties for absorption from the gut and transmission to the liver as well as other organs of the body. It is, however, the manner in which it is subsequently metabolized *in-vivo* which determines the precise nature of the animal's response (Moss, 1996).

An alternative biotransformation of AFB₁ to aflatoxicol is mediated by a reductase and the product can be reconverted to AFB₁ by a dehydrogenase. AFB₁ reductase and aflatoxicol dehydrogenase are not well studied, but this mechanism is considered to be very important because aflatoxicol may play a role as a reservoir of AFB₁ in some organisms (Lee *et al.*, 2001).

[illegible]

2.2.6 ELIMINATION OF AFLATOXIN FROM BODY

Aflatoxin is rapidly excreted in the bile and urine and does not accumulate or persist in body tissues. This perhaps explains the rapid recovery of egg production and

hatchability after cessation of toxin ingestion (Brown, 1996). Trucksess *et al.* (1983) fed 18 laying hens on aflatoxin-contaminated diet (8 mg AFB₁/kg feed) for seven days after which half of the group was sacrificed, while the remaining were sacrificed after feeding an aflatoxin-free diet for seven more days. Aflatoxin and its metabolite aflatoxicol were detected in the eggs and edible tissues of all hens sacrificed seven days after exposure. Liver and eggs contained the highest levels of AFB₁ and aflatoxicol. Seven days after withdrawal, only trace amounts of aflatoxicol were detected in eggs and no aflatoxin residues were found in edible tissues.

Comparative LD50 or lethal values for Aflatoxin B₁ (WHO, 1979)

Species	Oral LD50/Lethal dose (mg/Kg)
Chick embryo	0.025
Duckling	0.3
Turkey poultry	0.5
Chicken, New Hampshire	2.0
Chicken, Rhode Island	6.3
Sheep	5.0
Rat (male)	7.2
Rat (female)	17.9
Rabbit	0.3
Cat	0.6
Pig	0.6
Guinea pig	1.4
Hamster	10.2
Mouse	9.0
Baboon	2.0
Dog	6.3

2.3 TOXICITY OF AFLATOXIN

All animal and bird species are susceptible to aflatoxicosis, but outbreak occurs mostly in poultry, swine, sheep and cattle. Susceptibility of birds and animals to toxic effects of aflatoxin varies with several factors such as breed, strain, age, nutritional status, amount of toxin intake, duration of exposure and also the capacity of liver microsomal enzymes to detoxify AFB₁ (Eaton and Groopman, 1994; Smith, 2002).

Aflatoxicosis has been studied in numerous animal species including swine, cattle, goats, dogs, chickens, turkeys and laboratory animals (Dalvi, 1986). Patterson and Allcroft (1970) divided animal species into two groups (a) susceptible- calves, chicks, ducklings, guinea pigs and pigs and (b) relatively resistant- goats, sheep, rats and mice. Ducklings are the best model for the bioassay of aflatoxicosis. Signs of acute aflatoxicosis in ducklings are similar to those in chicks and turkey poult which included anorexia, poor growth rate, ataxia and death. The aflatoxin pathway is similar to any other toxin; the aflatoxin ingested through the contaminated diet accumulates in the blood and organs. The bio accumulated mass of the toxin at lethal levels leads to death of the animal whereas at sub lethal levels it leads to immunotoxicity, genotoxicity, carcinogenicity, teratogenicity and other functional disorders.

2.3.1 GROWTH PERFORMANCE IN BIRDS

Growth of birds is an important parameter that is particularly sensitive to aflatoxin. Retarded growth, reduction in weight or weight loss is reported commonly in aflatoxin fed birds. The main causes for the growth depression are reduced feed intake, altered protein metabolism, altered enzymatic activity and decreased nutrient utilization and absorption.

The growth performance of broiler birds was affected even at minimum levels of aflatoxin in the diet (Reddy *et al.*, 1982; Doerr *et al.*, 1983; Nandkumar Reddy *et al.*, 1984 and Misri, 1994).

Giambrone *et al.* (1985) reported that chicks fed with the diet containing 1.10 ppm AFB₁ from naturally contaminated mouldy wheat had produced poor feed conversion than those of control. Interestingly, no effect on feed conversion was noticed when pure AFB₁ was administered at 0.50 and 1.00 ppm to 2 and 5 week old broiler chickens.

Huff *et al.* (1986) observed that the body weights were significantly decreased when 2.5 µg of aflatoxin/g of feed administered to young broiler chicken. Further, the authors observed that the body weights of broilers were significantly decreased following feeding with 5.0 µg of aflatoxin/g of feed for six days and 2.5 µg of aflatoxin/g of feed for 17 days.

Dafalla *et al.* (1987) reported that 0.5 ppm dietary aflatoxin administered to seven-day old broilers for four weeks resulted in significant decrease in body weights and reduction in feed efficiency in broilers.

Ghosh *et al.* (1989) recorded reduced feed consumption when chicken were fed with 0.3 and 1.0 ppm of aflatoxin B₁ for six weeks. Dietary aflatoxin levels as low as 0.2 ppm was found to have depressing effects on body weight gain in broilers (Johri and Sadagopan, 1989).

Pearson *et al.* (1990) reported that addition of 2.5 and 5.0 mg of aflatoxin/kg of feed resulted in decreased body weight in broiler chicken. In a similar experiment, the

reduction in body weights of chicks were noted at the dose level of 0.3 to 1 ppm in the feed from day-old to six weeks of age (Ghosh *et al.*, 1990).

Subramanya (1993) observed a significant reduction in body weight gain in broilers and ducklings following feeding with 0.25 and 0.5 ppm of aflatoxin. Rajendra (1993) also recorded reduction in body weight gain in broilers fed with 0.5 to 1.0 ppm of aflatoxin. Kubena *et al.* (1993) reported reduction in body weight gain and increase in feed conversion ratio when broiler chickens were fed with 5 ppm of aflatoxin in the diet for four weeks.

Gayathri (1995) reported significant reduction in feed conversion efficiency in broilers following feeding with 500 ppb of aflatoxin B₁ for a period of 4 weeks. Significant reduction in feed conversion efficiency was noted in broilers fed with 0.1 to 0.5 ppm of aflatoxin (Sundaresan and Mani, 1996). Mishra *et al.* (1996) observed that aflatoxin at the dose ranging from 0.25 to 2.0 ppm produced significant reduction in feed intake and body weight gain in broiler birds.

Feed efficiency (upto 6 weeks) in broilers fed with 200 and 400 ppb aflatoxin was significantly lowered when compared to control birds (Swamy and Devegowda, 1998). Poor feed efficiency (upto 5 weeks) was observed in broilers when fed with diets containing 0.30 ppm AFB₁ (Raju and Devegowda, 2000). They further noted 21 per cent decrease in body weight at 35 days age in broilers fed with 0.3 mg AFB₁/kg diet.

Churchil *et al.* (2001) reported that the aflatoxin caused significant reduction in body weight gain in broiler chicken fed with 5 ppm of aflatoxin in the diet for four weeks. Anilkumar (2002) and Bhanuprakash (2002) reported significant reduction in

body weight gain in broiler chicken fed with 0.5 ppm of aflatoxin in the diet for five weeks and Santhosh Kumar (2003) also found reduction in feed intake, lowered body weight and increased FCR after feeding aflatoxin at 1.0 ppm level in the diet of broilers for 42 days. Dersjant-Li *et al.* (2003) and Devegouda and Murthy (2005), reported that the dietary aflatoxin reduced the weight gain, feed intake and increased the feed conversion ratio.

Aravind *et al.*, (2003) observed 2.30 per cent reduction in feed efficiency by feeding naturally contaminated diet containing 168.00 ppb of aflatoxin to broilers. Verma *et al.* (2004) also reported reduction in feed efficiency due to aflatoxicosis in broilers. Dersjant-Li *et al.* (2003) opined that each mg of AFB₁/kg diet would decrease the growth performance of broilers by 5 per cent.

Tedesco *et al.* (2004) noted only 10 per cent reduction in weight gain of broilers at 28 days of exposure to 0.8 mg AFB₁/kg diet. Miazzo *et al.* (2005) reported a significant increase of feed conversion ratio of broilers fed 2.50 ppm of aflatoxin while Gowda *et al.* (2008) reported no significant effect on growth parameters in broilers fed with 1.00 ppm of aflatoxin.

Zahid Hussain *et al.* (2008) reported a significant decrease in body weight of broiler chicks which were fed with diet containing 0, 1600, 3200 and 6400 µg/kg aflatoxin B₁ for 7 days and the lowest body weight was recorded in birds fed 6400 µg/kg aflatoxin B₁ in feed. Zhao *et al.* (2010) noted 10 per cent reduction in weight gain at levels of AFB₁ of 1mg/kg diet for 21 days exposure. Kana *et al.* (2010) reported that AFB₁ as low as 0.02mg/kg in the diet can produce significant decrease in weight gain of broilers by 5 per cent in a 3 weeks feeding trial.

Mohmand (2010) reported poor weight gain and increased FCR from 14th day onwards and continued till the end of experiment (35th day) in broilers with dietary exposure of 1ppm aflatoxin. Yunus *et al.* (2011) reported that the low levels of aflatoxin B₁ caused deterioration of the performance parameters in broilers.

Pourelmi (2013) in his study reported that the chicks receiving AF contaminated feed had significantly decreased body weight, feed consumption and efficiency in comparison to chicks fed control diet. Efficiency of feed utilization was decreased significantly at the levels of 0.5ppm aflatoxin inclusion. High mortality rate of 14.20 per cent was observed in the group fed with diet containing 0.5 ppm aflatoxin.

Hedayati *et al.* (2014a) observed reduction in body weight, feed intake and increased FCR in broilers fed with 600 ppb of aflatoxin for a period of 42 days. Yalagod (2014) reported reduction in body weight, decreased feed consumption and increased FCR in broilers fed with 0.25, 0.50 and 1 ppm of aflatoxin through diet for a period of 28 days.

Sharma *et al.* (2014) reported reduction in body weight, decreased feed consumption and increased FCR in broilers fed with 250 ppb of aflatoxin B₁ through diet for a period of 42 days.

Daneshyar *et al.* (2014) studied the effect of feeding of diet contaminated with 150 and 300 ppb of aflatoxin in broilers for 5 weeks and reported significant reduction in body weight and feed intake as well as increased FCR after 4th week in broilers fed with 300 ppb of aflatoxin. However, no significant change in body weight, feed intake and FCR of broilers fed with 150 ppb of aflatoxin in comparison to control group.

2.3.2 CLINICAL SIGNS

Aflatoxicosis does not usually induce mortality directly even at higher concentration levels (more than 10.0 mg aflatoxin/kg feed in chickens) may be lethal. The clinical manifestation of aflatoxicosis is due to the direct action of the toxin in multiple organ system in birds. Poultry manifesting aflatoxicosis may also have failure of normal pigmentation, increased bruising (0.5 mg aflatoxin/kg feed) and derangement in feather development (Brown, 1996).

Feeding of aflatoxin at different dose levels ranging from 0.25, 0.5 and 1ppm have produced variable clinical signs like anaemia, lethargy, dullness, dropped wings, inappetence, huddling, poor growth rate, polydypsia, ruffled feathers, rigidity of legs, oedema and diarrhoea in broiler chicken was reported by many workers (Mohanraju, 1990; Jayaprakash, 1991; Arshad *et al.*, 1992; Singh *et al.*, 1995; George, 1998; Prakash, 2001; Anil Kumar, 2002; Bhanuprakash, 2002; Santhosh Kumar, 2003; Shivappa, 2005; Pradeep Kumar, 2007 and Senthil Kumar, 2007).

Different clinical manifestations were noticed like reduced feed intake, loss of appetite, dehydration, poor pigmentation, regression of bursa of Fabricius, lowered egg production, listlessness, jaundice and immunosuppression in aflatoxicosis of birds. (Hamilton, 1971; Thaxton *et al.*, 1974; VenkataRam *et al.*, 1988).

Devegowda (1994) reported adverse effects of aflatoxicosis in birds such as lack of appetite and stunted growth, decreased egg production and egg weight in layers, increased leg problems, decreased nutrient utilization and suppression of immune system leading to increased susceptibility to disease in broilers.

Ortatatli *et al.* (2005) reported restlessness, anorexia with lowered growth rate, poor feed utilization, decreased weight gain, decreased egg weight and production, increased susceptibility to environmental and microbial stresses and mortality following feeding of low levels of aflatoxin at the rate of 50 ppb and 100 ppb to the broiler birds for 42 days.

Zahid Hussain *et al.* (2008) reported clinical signs such as depression, ruffled feathers, decreased interest in feed, increased water intake and soft to watery droppings when 7 days old broiler chicks were offered feed containing 0, 1600, 3200 and 6400 µg/kg aflatoxin B₁.

Nathanael and Viveka Vardhani (2011) observed dullness, depression, reluctant to consume feed and water, prolonged asthenia, lethargy and reduced appetite resulting in decreased body weight in birds fed with 0.01ng, 0.1n g, 0.25ng and 0.5ng of AFB₁ for 14 days. Wafaa *et al.* (2013) reported severe depression, anorexia, ruffling and stunted growth in broilers fed with 2.5 ppm of aflatoxin for a period of 35 days.

Yalagod (2014) reported that birds fed 0.5 and 1 ppm aflatoxin for a period of 28 days showed varying degree of anorexia, reduced body weight, ruffled feathering, feather malformation diarrhoea and depression. The abnormal feathering pattern was evident from 21 days of experiment. However, birds fed with 0.25 ppm of aflatoxin remained healthy with apparently normal appearance till the end of toxicity trial.

2.3.3 RELATIVE ORGAN WEIGHT

Aflatoxin is known to cause alteration in the relative organ weight of various visceral and lymphoid organs in all the affected species. The change in relative organ

weight in most of the studies is dependent on species involved, dosage and duration of exposure of aflatoxin (Brown, 1996).

Dixon *et al.* (1982) observed increased weights of bursa of Fabricius and liver in young chicks when fed with 0.43, 1.26 and 1.66 ppm of aflatoxin in the feed. Reddy *et al.* (1982) observed a dose dependent increase in relative weights of liver and kidney in broilers fed with the diets containing 0.25 to 4.00 ppm of aflatoxin for a period of 6 weeks.

Maurice *et al.* (1983) reported increased liver weight in broilers fed with an increase in dietary aflatoxin level and duration of exposure. Shen *et al.* (1986) recorded no change in relative weight of liver by feeding 400 ppb of aflatoxin for 4 weeks in chicken. Huff *et al.* (1986) in their study found that feeding broilers with 1.25, 2.50 and 5.0 ppm of aflatoxin had significant effect on spleen and kidney weights.

VenkataRam *et al.* (1988) stated that the birds fed with 4 ppm aflatoxin for 4 weeks and 1 ppm aflatoxin for 12 weeks revealed a dose dependent decrease in weights of thymus and bursa of Fabricius. Phillips *et al.* (1988) in their study fed broiler chickens with 7.5 ppm aflatoxin for a period of 3 weeks and found pale friable and enlarged liver.

Hilton *et al.* (1989) observed significant increase in relative weights of liver and kidney when chicken were fed with 1.25 ppm aflatoxin for a period of three weeks. Feeding broiler chicken with 0.25 and 0.50 ppm levels of dietary aflatoxin had no significant effects on weight of liver, thymus, spleen and bursa of Fabricius (Barmase *et al.*, 1990).

Kubena *et al.* (1990) reported that feeding leghorn chicks with 5 ppm of feed for 28 days resulted in increase of relative weights of liver, kidney, and decrease in relative

weights of bursa of Fabricius. Mohanraju (1990) reported an increase in relative weights of liver and kidneys at doses of 0.2, 0.3 and 0.5 ppm of aflatoxin in broilers.

Jayaprakash (1991) recorded increase in relative organ weights of liver and kidneys following feeding of 1 ppm of aflatoxin to the broilers and opined that inflammatory, degenerative and necrotic changes were responsible for alterations in relative organ weights.

Kubena *et al.* (1993) noticed significant increase in relative weights of liver and kidneys in broiler chicken which received 2 ppm of aflatoxin. Fernandez *et al.* (1994) reported increased liver and kidney weights in hens fed aflatoxin at 2.5 and 5 ppm in feed for 4, 8, 16 and 32 days.

Abo-Norag *et al.* (1995) found an increase in the relative weights of liver and kidney in broiler chicken fed with 3.5 mg aflatoxin / kg of diet and these alterations were attributed to degenerative and necrotic changes in liver and kidneys. Sundarsean and Mani (1996) reported an increase in the weight of liver and bursal regression when broilers were fed with 0.10, 0.20, 0.30, 0.40 and 0.50 ppm of aflatoxin for a period of 5 weeks.

Swamy and Devegowda (1998) fed aflatoxin at the rate of 0.10, 0.20 and 0.40 ppm to broiler chicken for a period of 6 weeks and found that there was increase in relative weight of liver in birds fed with 0.40 ppm of aflatoxin. Raju and Devegouda (2000) reported increase in relative weight of both liver and kidneys, when birds were fed with 0.30 ppm of aflatoxin for a period of 5 weeks.

Ortatatli and Oguz (2001) noticed increase in relative weights of liver and kidneys and reduction in bursa of Fabricius, spleen and thymus weights by feeding broiler

chicken with 2.5 mg of aflatoxin per kg of feed. Broiler chickens fed with 0.16ppm of aflatoxin for 5 weeks revealed only increase in the relative weight of liver (Aravind *et al.*, 2003).

Girish and Devegouda (2004) reported increase in weight of liver, kidney and spleen in the broiler chickens were fed with 2 ppm of aflatoxin for a period of 5 weeks. Ortatatli *et al.* (2005) found non-significant difference in relative weights of liver, kidney, thymus, spleen and bursa of Fabricius in broiler chickens fed with 50ppb and 100 ppb of AFB₁ for 42 days.

Gowda *et al.* (2008) noticed increase in relative weight of liver when broiler chickens were fed with 1.00 ppm of aflatoxin for a period of 3 weeks. Yunus *et al.* (2011) reported increase in the weight of liver and spleen, whereas decrease in the weight of thymus and bursa of Fabricius of broiler chickens fed with AFB₁ in the diet.

Shareef and Omar (2012) reported that thymus, spleen and bursa of Fabricius were negatively affected by feeding AFB₁ @ 2.5 ppm in broilers for 42 days. Thymus and bursal relative weights were significantly reduced in treated group when compared with control group.

Study carried out on dietary levels of aflatoxin at 0.5 ppm revealed significant increase in relative weights of liver (19.56%) and kidney (18.38%). However, decrease in relative thymus (38.99%) and bursal weights (31.36%) were observed at the end of 5th week of experiment (Indresh *et al.*, 2013).

Manafi *et al.* (2014a) reported increased weights of liver and bursa and decrease in weight of spleen and pancreas in broilers fed with 600 ppb of aflatoxin for 42 days. Valchev *et al.* (2014a) recorded increased relative spleen weight and decreased relative

weights of the thymus and bursa of Fabricius following feeding of 0.5 and 0.8 ppm of aflatoxin to the broilers from 7 to 42 days of age.

A study conducted on broiler chickens following feeding of 0.5 and 0.8 ppm AFB₁ for a period of 42 days revealed significant increase in the relative weight of heart in toxin fed birds (Valchev *et al.*, 2013b).

Fowler *et al.* (2014) conducted study in the broilers fed with Distiller's Dried Grains and Soluble (DDGS) as well as corn contaminated with 16, 228, 366 and 681 ppb aflatoxin for 21 days and reported increased relative liver weights by all aflatoxin levels and relative kidney weights increased in 366 ppb level of aflatoxin. However, no adverse change was observed in relative spleen and bursa weights in any of the aflatoxin levels in the feed ingredient (DDGS and Corn).

2.3.4 SERUM BIOCHEMISTRY

Serum chemistry and enzyme activities are valuable tools for monitoring the health and nutritional status of animals (Lumeij, 1997). The serum biochemistry profile quantifies constituents and circulating enzymes in the blood or serum to provide very general information about the status of organ (especially the liver, kidneys, and pancreas) health, function and organopathies. The concentration of enzymes in serum describes degree of cell damage in the particular organ. Aflatoxin adversely causes alteration in the various constituents and enzymes level in serum of affected species, which can be considered as indicator of damage to the respective organ.

2.3.4.1 SERUM ENZYME ACTIVITY OF ALANINE AMINOTRANSFERASE (ALT), ASPERTATE AMINOTRANSFERASE (AST) AND ALKALINE PHOSPHATASE (ALP)

The alterations in levels of serum enzymes ALT, AST and ALP recorded during aflatoxicosis can be interpreted as the sequelae of hepatocyte degeneration or damage to the cell membrane and subsequent leakage of enzyme into the circulation. However, Huff *et al.* (1988) could not find any alteration in AST activity in aflatoxin fed broilers. No significant difference in ALT activity was observed in broilers fed with aflatoxin at 0.3 and 0.6 ppm for 21 days as observed by Kalra *et al.* (1995) and also in birds fed 3.5 mg/kg of feed for the period of three weeks of age as observed by Kubena *et al.* (1997).

Rehman and Shakoori (2000) reported a significant increase in serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) within 24h to 48h of administration of aflatoxin B₁ as a single dose of 9.287 mg per chick and gradually decreased to normal levels over the next 2 weeks.

Raju and Devegowda (2000) reported an increased AST activity at the end of the experiment in broilers fed with 0 and 0.3 ppm aflatoxin. However, there was no alteration in ALT and ALP activity. Valdivia *et al.* (2001) noted decrease in serum levels of alanine amino transferase (ALT) and increase in serum aspartate amino transferase (AST) in broilers fed upto 3ppm of AFB₁ for 21 days.

Tedesco *et al.* (2004) observed decreased serum levels of ALT activity and no alteration in AST activity in broilers fed with AFB₁ at 0 and 0.8ppm for a period of 14-49 days. Sandhya and Vardhani (2007) reported that there was an increase in serum level of AST and ALT from day 1 to 11 in broilers in comparison with untreated broilers.

Similarly, in two week old broilers treated with AFB₁ @ 0.25 ng and 0.5 ng the activity of AST and ALT increased from day 1 to 8 of experimental period and there after decreased by day 11 of infection.

Denli *et al.* (2009) noted increased activity of ALP and no effect on AST activity in broilers were fed with AFB₁ @ 1 ppm for 42 days. Madhuri *et al.* (2009) observed increase in serum levels of both AST and ALT in two week old broilers fed with AFB₁ @ 0.01ng orally.

A study conducted by AnandaManegar *et al.* (2010) on feeding of aflatoxin @ levels of 200, 400 and 600 ppb to broilers in feed revealed a non-significant activity of ALT between control and aflatoxin fed birds. However, decrease in AST activity was observed in aflatoxin treated birds.

Zhao *et al.* (2010) observed decrease in serum levels of ALP and no effects on AST activity when broilers were fed with 0 and 2ppm of AFB₁ for 21 days. Nathanael and VivekaVardhani (2011) observed increased serum levels of AST and ALT in one week old broilers treated with 0.1 ng AFB₁ orally.

Sawarker *et al.* (2011) reported elevation in values of liver marker enzymes(ALT and AST) when broilers fed standard basal diet and fed with 100 ppb each of aflatoxin B₁ and ochratoxin A in diet upto 42 days. Hassan *et al.* (2012) reported increased activity of AST, ALT and ALP following feeding of aflatoxin @ level of 2 ppm to the broilers for 21days.

Yogeswari *et al.* (2012) reported that aflatoxin administration at 1ppm level resulted in increase in serum AST and ALT for 1-42 days. Singh and Mandal (2013) reported increased activity of AST and ALT following feeding of aflatoxin @ level of 1 ppm to the broilers for a period of 42 days. However, Indresh *et al.* (2013) reported no alteration in activity of serum ALT in broilers fed aflatoxin @ level of 0.5 ppm for 35 days.

Singh *et al.* (2013) reported increased levels of AST and ALT following administration of diet containing 1.0 ppm aflatoxin to the broilers upto 42 days of age. Dumari *et al.* (2014) recorded significant increase in activity of AST and ALT following feeding of aflatoxin B₁ contaminated diet @ the level of 250 and 500 ppb to the broilers for a period of 21 days.

Yalagod (2014) reported elevated levels of AST, ALT and ALP in broilers fed with 0.25, 0.5 and 1 ppm of aflatoxin from 1 to 28 days of age and attributed these alterations to leakage of enzymes following hepatic damage in aflatoxicosis.

Motawe *et al.* (2014) reported elevated levels of AST and ALT following incorporation of 0.5 and 1 ppm of aflatoxin in feed of broilers for a period of 42 days. Li *et al.* (2014) reported elevated level of ALP in broiler fed with aflatoxin @ 74 µg/kg feed for a period of 3 weeks.

2.3.4.2 TOTAL PROTEIN, ALBUMIN AND GLOBULIN

Among the serum constituents, total protein levels in the serum proved to be sensitive indicators of aflatoxicosis in broilers. Aflatoxin has been shown to cause inhibition of protein synthesis. Liver and kidneys are the major target organs for aflatoxin

and hepato-renal damage is associated with alterations in serum constituents in affected species. The reduction in total serum protein in aflatoxicosis is due to the impairment of amino acid transportation at mRNA transcription level and there by inhibiting protein synthesis (Thaxton *et al.*, 1974).

Adverse alteration in serum proteins, albumin and globulin levels following admixture or contamination of feed with different concentrations of aflatoxin has been reported earlier by several workers (Harvey *et al.*, 1993; Bailey *et al.* 1998; Ledoux *et al.*, 1999).

Raju and Devegowda (2000) reported decrease in serum total proteins, albumin and globulin in broiler following feeding of 0.3 mg AFB₁/kg feed for a period of 35 days. Oguz *et al.* (2002) studied effect of feeding of 50-100 µg aflatoxin in diet to broiler for 42 days and found decrease in serum total proteins and albumin to be associated with hepatic damage.

A feeding trial conducted for 21 days in broiler following incorporation of 2 mg total aflatoxin/ kg feed showed decrease in total serum proteins and albumin (Basmacioglu *et al.*, 2005).

Ghahri *et al.* (2010) reported decrease in serum total protein and albumin levels in broiler following feeding of diet naturally contaminated with aflatoxin for a period of 35 days. Feeding of aflatoxin B₁ @ level of 100 ppb to broiler for 42 days caused decrease in serum total proteins, globulin and albumin (Sawarker *et al.*, 2011).

A study conducted in broilers following feeding of aflatoxin @ the level of 2 ppm in feed for 21 days resulted in decrease in serum total proteins, albumin and globulin (Hassan *et al.*, 2012). Studies conducted in broilers following incorporation of 0.5 and 1 ppm of aflatoxin in feed reported decrease in serum total proteins, albumin and globulin levels (Indresh *et al.*, 2013; Kumar Dhanapal *et al.*, 2014).

A study carried out by Yalagod (2014) revealed reduction in levels of serum proteins and albumin in broilers fed with 0.25, 0.5 and 1 ppm of aflatoxin in feed and attributed the alterations in serum constituents to decreased protein synthesis due to liver damage .

Valchev *et al.* (2014a) reported decrease in total proteins, albumin and globulin in broilers fed with 0.5 and 0.8 ppm of aflatoxin in feed for the experimental period of 42 days. A study conducted by Li *et al.* (2014) revealed reduction in total serum proteins and albumin in broiler fed with aflatoxin @ 74 µg/kg feed.

2.3.4.3 SERUM CHOLESTEROL AND TRIGLYCERIDES

The liver constantly synthesizes cholesterol and triglycerides by utilizing as substrates free fatty acids and carbohydrates; these endogenous components are secreted into the circulation as components of chylomicrons and lipoproteins. In toxic liver damage, the ability of hepatocytes to synthesize cholesterol and triglycerides is reduced leading to lowered blood levels of these serum lipids.

Denli *et al.* (2005) observed increased serum cholesterol and triglycerides in broiler fed with aflatoxin B₁ @ 200 and 300 µg/kg feed from 1 to 42 days of age. Matur *et al.* (2010) reported non-significant decrease in levels of cholesterol and triglyceride

concentrations in 58-wk-old breeder hens fed with aflatoxin @ 100 µg of AF/kg of feed for a period of 3 weeks.

Shabani *et al.* (2010) reported significantly lower levels of serum cholesterol and triglyceride concentrations in broilers fed 500 ppb of aflatoxin for a period of 35 days. Sawarkar *et al.* (2011) reported significantly lower serum levels of cholesterol and triglyceride in broilers fed 100 ppb each of aflatoxin B₁ and ochratoxin A for a period of 42 days.

Yenice *et al.* (2014) reported non-significant decrease in levels of cholesterol in layer birds of 26 weeks of age following feeding of 120 ppb aflatoxin. Valchev *et al.* (2014a) conducted study in the broilers by feeding 0.5 and 0.8 ppm of aflatoxin for 42 days and reported decreased level of cholesterol and triglyceride from 21st day of experimental study.

Sadeghi *et al.* (2014) reported decrease in cholesterol and triglyceride levels following feeding of aflatoxin @ 0.5 mg/kg of feed in broilers from 8th to 42nd day of age. Sridhar *et al.* (2014) reported significantly lower levels of cholesterol and triglyceride concentrations in broilers fed 1 ppm of aflatoxin for a period of 42 days. Manafi *et al.* (2014a) reported increase in cholesterol, LDL and HDL levels in the broilers fed with 600 ppb aflatoxin upto 42 days of age.

2.3.4.4 BLOOD UREA NITROGEN (BUN) AND CREATININE

Serum/plasma BUN and creatinine are the indicators of the toxic damage to renal tissue in aflatoxin exposed chickens. Aflatoxins are known to cause alteration in BUN

and creatinine due to renal damage. Kubena *et al.* (1998) observed decrease in creatinine levels in broiler fed upto 3.5 ppm of aflatoxin for 1-21 days.

Matur *et al.* (2010) observed no significant alteration in creatinine and urea in 58-wk-old breeder hens fed with aflatoxin @ 100 µg of AF/kg of feed for a period of 3 weeks. Sawarkar *et al.* (2011) reported an increase in serum creatinine and uric acid in broilers fed with 100 ppb each of aflatoxin B₁ and ochratoxin A from 1-42 days of age.

Umar *et al.* (2012) observed increased concentration of creatinine and urea in broiler birds fed with 3.0mg/kg of AFB₁ from 7-42 days. El-Afifi *et al.* (2013) reported that the broiler chickens received aflatoxin contaminated feed showed significantly higher serum levels of creatinine and urea which was attributed to renal damage.

Amiridumari *et al.* (2013) reported elevated levels of urea and creatinine levels in broilers fed with 250 and 500 ppb of aflatoxin in feed for 21 days. Valchev *et al.* (2013a) reported significant reduction in creatinine and urea levels following feeding of 0.5 and 0.8 mg of aflatoxin B₁ to female mulard ducks for a period of 42 days and attributed these changes to deranged renal function following damage to the kidneys.

Li *et al.* (2014) reported non-significant increase of creatinine and urea in broilers fed with aflatoxin @ 74 µg/kg feed for a period of 3 weeks. Motawe *et al.* (2014) reported significantly higher urea and creatinine levels in broilers fed with aflatoxin @ 0.5 and 1 ppm in feed for a period of 42 days.

Study on effect of aflatoxin on renal functions conducted in the broilers fed with 0.5 and 0.8 ppm of AF for 42 days revealed increased level of urea and creatinine from

day 21st onwards of experimental study which could be correlated to the histopathological changes in renal parenchyma (Valchev *et al.*, 2014b).

2.3.5 IMMUNOTOXIC EFFECT OF AFLATOXIN

Avian immune system is complex in comparison to animals due to the presence of multiple lymphoid organs/tissues and absence of prominent lymphnodes. Several reports showed that the immune system in poultry is highly sensitive to mycotoxicosis (Giambrone *et al.*, 1985). Among all mycotoxins, aflatoxin is one of the major immunosuppressive agents in poultry feed/diet. Aflatoxins even in low levels are immunomodulatory (Paterson and Lima, 2010).

Aflatoxin-induced immunosuppression may be manifested as depressed cellular (Giambrone *et al.*, 1978) and humoral immune reactions. Although a definite explanation for the immune suppressive effect of aflatoxin has not yet been found, Carrier (1991) proposed that aflatoxin depresses protein synthesis via inhibition of RNA polymerase, which results in suppression of specific immunoglobulin synthesis (Giambrone *et al.*, 1985). In addition, nonspecific factors such as complement, interferon and nonspecific serum protein concentrations also decrease as a result of aflatoxin induced liver damage (Tung *et al.*, 1975; Stewart *et al.*, 1985).

2.3.5.1 CELL MEDIATED IMMUNITY (CMI)

Cell-mediated response are particularly sensitive, as reflected by decreased weight of thymus, lower peripheral T lymphocytic number and function, suppressed phagocytic and complement activity in chicken fed with aflatoxin (Kataria *et al.*, 2005; Paterson and Lima, 2010).

Peripheral blood lymphocytes are one of the critical as well as reactive components in immunological studies, which can be identified and quantified by using fluorescent antibodies to cell surface antigens. Typical markers most commonly used to assess the T cell population in relation to CMI are designated as CD4⁺ (Helper T cells) and CD8⁺ (Cytotoxic T cells). Quantification of CD4⁺ and CD8⁺ cells in peripheral blood is one of the specific tools to study the cell mediated response in chicken against any pathogen or toxicants.

Quist *et al.* (2000) conducted a study on turkey poult with AFB₁ in the diet at the rate of 0,100,200 and 400ug/kg feed for two weeks and found an increased percentage of CD8⁺ cells, and the groups fed with 100 and 200ug of aflatoxin/kg feed had a decreased percentage of CD4⁺ cells. The CD4/CD8 ratio was decreased in all poult fed with aflatoxin, as a result of increase in CD8⁺ cells.

Kamalavenkatesh *et al.* (2005) conducted an experiment in broiler chicken for 1-28 days to assess the immunopathological effect of cyclopiazonic acid (CPA) (10 ppm) and T-2 toxin (1 ppm) either individually or in combination. At the end of the trial, they recorded decrease in the thymic and splenic CD 4⁺ and CD8⁺ lymphocytes and decrease in the stimulation index of splenocytes to concavalin A in toxin fed groups. However, there was decrease in the thymic CD8⁺ lymphocytes in T-2 toxin fed groups compared to CPA group.

Total lymphocyte and T lymphocyte counts were significantly lower in chicks fed 0.3 and 1 mg AFB /kg feed (Ghosh *et al.*, 1990; Ghosh *et al.*, 1991) and 2.5 mg total aflatoxin/kg feed (Çelik *et al.*, 2000).

Study conducted in Turkey birds on feeding blends of grains naturally contaminated with *Fusarium* mycotoxins for 6 weeks significantly increased the percentage of CD4⁺ lymphocyte populations; however, there was no change in the percentage of CD8⁺ and B-lymphocyte populations (Girish *et al.*, 2008).

Effect of combination of ochratoxin A (0.25 ppm and 0.5 ppm) and T-2 toxin (0.5 ppm and 1 ppm) on the immune function was assessed in yellow-feathered broiler chickens (Wang *et al.*, 2009). They concluded that an impaired immune status as evidenced by a significant decrease in mitogenic response of peripheral blood lymphocytes and a decrease in CD4⁺/CD3⁺ and CD4⁺/CD8⁺ ratios.

Chen *et al.* (2014) conducted a study in broilers which were exposed to aflatoxin at the rate of 0.3mg/kg diet for 7-21 days. The percentage of CD3⁺, CD3⁺ CD4⁺ and CD3⁺ CD8⁺ T cells in the AFB₁ group were lower than those in the control group. The results indicated impaired immune function of spleen by AFB₁.

Yalagod (2014) reported decreased percentage of CD4⁺ and CD8⁺ cells in peripheral blood of broilers fed 1ppm of AFB₁ for a period of 28 days in comparison to control birds.

Study conducted to evaluate the effects of corn naturally contaminated with aflatoxin B₁ and aflatoxin B₂ revealed increased percentages of CD3⁺ CD8⁺ T cells and decreased ratios of CD3⁺CD4⁺ T cells to CD3⁺CD8⁺ T cells in broilers fed with 53.46 µg and 96.6 µg of aflatoxins at 21 days of age. At 42 days of age, increased percentage of CD3⁺CD8⁺ T cells in the birds fed with 96.6 µg of aflatoxins increased and ratios of CD3⁺CD4⁺ to CD3⁺CD8⁺ were found to be decreased (Peng *et al.*, 2014).

2.3.5.2 HUMORAL IMMUNITY

Aflatoxicosis affects the poultry by causing immunosuppression manifested as reduced/lowered antibody response to the vaccination against potential diseases. IBD and ND viruses cause high mortality in broilers and the birds need to be protected against these pathogens. Studies indicate the potential role of aflatoxin to interfere in the antibody response following vaccination against ND and IBD viruses in the broilers.

Ilgaz (1985) and Bakshi (1991) reported that the inadvertent feeding of aflatoxin contaminated feed may lead to breakdown of immunity. Thaxton and Hamilton (1971) fed the aflatoxin to 3 week old male chicks at the rate of 0.5 and 1 ppm in the diet and observed agglutinin titres against sheep erythrocytes. Thaxton *et al.* (1974) described a dose related suppression of primary immune response in chicken which were fed with low dose level of aflatoxin (@ 0.625µg/gm of feed).

Giambrone *et al.* (1978) recorded reduction in the serum immunoglobulins (IgG and IgA but not IgM) in chickens fed with aflatoxin at 2.5 ppm level. Mohiuddin *et al.* (1981), reported decrease in antibody titre to ND vaccination in birds fed aflatoxin at levels as low as 0.1 to 0.2 ppm. Similarly, Boulten *et al.* (1982) observed that birds vaccinated by a single initial ND vaccine and fed 0.5 ppm aflatoxin showed a significant decrease in HI titre when compared to birds that received inactivated aflatoxin and vaccinated with ND vaccine.

HI titres against NDV were found to be lowered following feeding with 0.3 ppm (Mangat *et al.*, 1988) and 1 and 4 ppm aflatoxin (Venkat Ram *et al.*, 1988) in broilers.

Virdi *et al.* (1989) observed a decrease in antibody titres against NDV from day 6th onwards of aflatoxin feeding with 0.5 and 1ppm to the chickens.

Bakshi (1991) reported lower HI titre in aflatoxin treated birds but a dose of 0.38 ppm aflatoxin did not adversely affect the humoral immune response. Mohiudin (1993) documented a marked decline in antibody titres and phagocytic activity representing an impairment of humoral and cell mediated immunity following feeding of aflatoxin as low as 0.2 ppm to the broiler birds.

Anjum (1994) reported a severe IBD outbreak with a mortality rate of up to 35.8% in grower pullets that were vaccinated with IBD vaccine, ascribable to immunosuppression owing to higher aflatoxin content in the poultry feed.

Devurkar *et al.* (1995) and Misri (1994) reported that the HI titre showed an inverse correlation to the dose of aflatoxin in the diet of the broiler chicken. Similarly, Bakshi *et al.*, (1997) recorded a significant decrease in serum globulin component by feeding aflatoxin level at 31 and 100 ppb in different feed samples and opined that high aflatoxin content in feed can impair the immune system, thereby resulting in vaccination failure. Shivachandra *et al.* (2003), observed reduction in the HI antibody titre following vaccination against NDV to the birds fed with aflatoxin at 1 ppm from 7 to 49 days of age.

Outbreak of infectious bursal disease in the birds vaccinated with intermediate strain (Georgia strain) of IBD virus at 18-20 days with a booster dose at 28-30-day intervals was reported in two adjoining organized poultry farms in Himachal Pradesh, India. The analysis of poultry feed from both the farms revealed 100 to 500 ppb of

aflatoxin which was found to be responsible for the vaccination failure (Mahajan *et al.* 2002).

Otim *et al.* (2005) reported decreased antibody titres against Newcastle disease (ND) and Infectious bursal disease (IBD) in indigenous chickens injected intramuscularly in the breast muscle with 0.250 mg aflatoxin B₁ per bird for a period of 42 days. Manegar *et al.*, (2010) confirmed that aflatoxin caused bursal regression and suppress primary immune response for ND and Gumboro disease as evident by decline in ELISA titres in broilers.

Mahmood *et al.* (2011) reported non-significant decrease in antibody titre (HA) against Newcastle disease (ND) in broilers fed with 70 ppb of aflatoxin for a period of 42 days. Indresh *et al.* (2013) reported decreased antibody titres against Newcastle disease (ND) and Infectious bursal disease (IBD) in broilers fed aflatoxin @ level of 0.5 ppm for 35 days.

Yalagod (2014) revealed lowered antibody titre against ND viruses in broilers fed with different levels of aflatoxin through feed for a period of 28 days. Valchev *et al.* (2014a) reported lower antibody titres against Newcastle disease virus in the broilers fed with aflatoxin @ 0.8 mg/kg of feed for a period of 42 day of age. Hedayati *et al.* (2014b) reported lower antibody titres against Newcastle disease virus in the broilers fed with 0.6 ppm aflatoxin B₁ for a period of 42 day of age.

2.3.6 CYTOKINE ESTIMATION AND mRNA EXPRESSION

Studies on cytokines in avian species have been finite due to the complexity of immune system. However, in recent years advances in avian immunology and genetics

has led to the discovery of a range of cytokines mainly in the chicken. Cytokines are small glycoproteins produced by a number of cells predominantly leucocytes, that regulate immunity, mediate and coordinate the inflammatory process through the activation and regulation of cell functions. All the cytokines act through receptor on the surface of target cells, which may lead to activation or down regulation of cellular activity (Khan, 2008). Cytokines have been classified into a number of groups based on their activity and the cells they are produced by or act upon. Most of the cytokines are proinflammatory and some are anti-inflammatory, depending on their functions and effect on immunocytes (Rothwell, 1999). The proinflammatory cytokines includes many cytokines belong to different families and the most important ones are IL-2, IL-6, IL-8, IFN γ and TNF- α . Aflatoxins, being immunotoxic are likely to cause imbalance in production and deregulation of cytokines (Hinton *et al.*, 2003; Tayal and Kalra, 2007).

Dugyala and Sharma (1996) used male mice to test the effects of AFB₁ (0.7 mg of AFB₁/kg) on the gene expression of IL-1 α and IL-2 and reported that AFB₁ significantly suppressed the levels of IL-1 α protein and mRNA expression and protein levels of IL-2.

Martin *et al.* (1996) studied effect of cytokine production EL-4 Thymoma cells following exposure different mycotoxins and reported that aflatoxin concentration upto 10 μ g/ml did not affect IL-2 production in thymoma cells. Study on murine peritoneal macrophages for cytokine production and expression revealed that aflatoxin B₁ inhibited production of tumour necrosis factor (TNF α), IL-1 and IL-6 production (Moon and Pyo, 2000).

Duck peritoneal macrophages (DPM) treated with different concentrations of AFB₁ (5, 10, 20, 50 or 100 μ g/ml) for 12 h showed dose dependent decreased secretion of

TNF-like substance indicating the suppressive effect on TNF (Cheng *et al.*, 2002). Hinton *et al.* (2003) investigated the effects of AFB₁ on IL-1, IL-2, and IL-6 production in male rats and found an increase in the production of IL-1 and IL-6 cytokines.

García *et al.* (2007) studied IL-8 production in a porcine intestinal epithelial cell line (IPEC-1) following exposure to aflatoxin B₁ and reported increased IL-8 secretion at low concentrations of AFB₁ (1.3 mM). However, higher concentrations (100 uM) of AFB₁ decreased IL-8 production.

Yarru (2008) reported down regulation of mRNA levels of IL-6 and increased expression of TNF in the liver of broilers fed with 2mg of aflatoxin/kg of feed for 21 days. Study conducted by Yarru *et al.* (2009) in broilers following feeding of 1ppm aflatoxin in feed for 3 weeks revealed no alteration in gene expression of IL-2 in hepatic tissue. However, an increased expression of IL-6 was observed in the same study.

Ahmad *et al.* (2011) estimated the serum level of TNF- α in mice following injection of aflatoxin @ 50ug/0.1ml/day for 3 weeks and reported highly significant reduction (15.5 ± 1.7 pg/ ml) of TNF- α in comparison to 26 ± 1.09 7pg/ ml in control group.

Chaytor *et al.* (2011) reported an increased serum TNF- α level of broiler chickens fed 180 μ g of aflatoxin (AF)/kg and 900 μ g of deoxynivalenol (DON)/kg, which could be attributed to an acute-phase response to inflammation induced by the toxic effects of AF and DON.

Wang *et al.* (2012) exposed the chicken splenic lymphocytes to different concentration (0.1, 0.4, 1.6, 6.25, and 25 µg/mL) of zearalenone (ZEN) for 48 h and reported consistently significant increase in the IL-2 levels of mRNA expression. However, the IL-6 levels were critically suppressed by ZEN.

Li *et al.* (2014) studied the effect of low level of aflatoxin B₁ (74µg/kg feed) in broilers for 3 weeks and reported increased mRNA expression of IL6, TNF-α and IFNγ. In addition, significant increase in TNF-α level in serum was also observed in the same study.

Mehrzaad *et al.* (2014) treated dendritic cells (DC) to aflatoxin at concentration of 10 ng/ml *in-vitro* for 24 h followed by collection of cell-free culture supernatant to determine the concentration of TNF-α, IL-1b, IL-6, IL-8 and IL-10 using ELISA kits. The study revealed increase in IL-1b and IL-6 secretion by DCs due to the presence of AFB₁ for 12 and 24 h, respectively. However, the IL-10, IL-8 and TNFα secretion levels were unaffected by AFB₁.

2.3.7 DNA FRAGMENTATION STUDIES

DNA fragmentation is the separation or breaking of DNA strands into pieces. Apoptotic DNA fragmentation is a key feature of apoptosis, a type of programmed cell death. Apoptosis is characterized by the activation of endogenous endonucleases with subsequent cleavage of chromatin DNA into internucleosomal fragments of roughly 180 base pairs (bp) and multiples thereof (360, 540 etc.). This effect can be used to detect apoptosis, for example via the DNA laddering assay or TUNEL assay or Nicoletti assay. Aflatoxin B₁ is one among the potent genotoxic agent, which causes chromosomal

aberration and DNA breakage in plant and animal cell. Aflatoxins induce DNA damage, negatively affect the amelioration of damage and alter the DNA base composition of genes (Paterson and Lima, 2010). The toxic and carcinogenic effects of AFB₁ are intimately linked with its biotransformation mechanism (Wu *et al.*, 2009). The active intermediate, AFB₁-exo-8,9-epoxide, can bind with DNA to form the predominant trans-8,9-dihydro-8-(N7-guanyl)-9-hydroxy-AFB₁ (AFB₁-N7-Gua) adduct which causes DNA lesions (Wild and Turner, 2002). There is a tendency for AFB₁ to convert into the epoxide and produce DNA adducts that in turn cause DNA strand breaks and point mutations (Eaton and Gallagher, 1994).

Cheng *et al.* (2002) exposed Duck peritoneal macrophages (DPM) to different concentrations of AFB₁ (5, 10, 20, 50 or 100 µg/ml) for 12 h in order to identify the possible cell apoptosis through the DNA fragmentation or laddering assay. The study indicated DNA fragmentation as ladder phenomenon in the cells treated with aflatoxin.

Abbès *et al.* (2008) reported fragmentation of Caco-2 cell DNA after treatment with 100 nM of AFB₁ and the fragment sizes varied between 750 and 2000 base pairs. Feeding 211.88 µg of aflatoxin/ kg feed to the broilers for 40 days resulted in increased percentage (42.40) of DNA fragmentation in liver cells in comparison to 10.40 per cent in control birds (Shebl *et al.*, 2010).

Williams *et al.* (2011) undertook single-cell gel electrophoresis (comet) assay to investigate the potential of AFB₁ to produce DNA damage in turkey and chicken fetal livers following exposure to 0.062 and 6.2 µg of aflatoxin. The results demonstrated that AFB₁ produced dose-related DNA damage in the fetal livers of both turkeys and chicken at 4 h, which was reduced by 4 day.

Wang *et al.* (2013) studied TUNNEL assay and reported increased apoptotic cells with fragmented DNA both in the red pulp and white pulp of the spleen from broilers fed with 0.3mg of aflatoxin/kg of feed. Eshak *et al.* (2013) reported higher rate of DNA fragmentation in the kidneys of the Japanese quail exposed to 1mg aflatoxin/Kg feed for 3 weeks.

2.3.8 GROSS PATHOLOGY

Mottled liver, pale kidneys, petechiae on the heart, splenomegaly and atrophy of the thymus and bursa of Fabricius were recorded in chicken fed with 1 ppm of aflatoxin B₁ in the feed (Ghosh *et al.*, 1989).

Arshad *et al.* (1992) studied experimental aflatoxicosis in day-old broiler chicks and observed hepatomegaly with mottling and ecchymotic haemorrhages. The authors also noticed enlarged and congested kidneys with urate deposits in the ureters at the dose levels of 637, 1274, 3720 and 5310 ppb of aflatoxin.

Bakshi *et al.* (1994) observed haemorrhagic spots along with infarction on the liver when broiler chickens were fed with aflatoxin at the dose levels of 0.38 ppm and 0.75 ppm for six weeks. At 1.5 and 3 ppm levels they observed enlargement, yellowish discolouration, petechial haemorrhages and fatty changes in liver.

Channakrishnappa *et al.* (1999) reported gross lesions of pale, enlarged, friable liver with round borders and petechial haemorrhages in birds fed with 200 ppb aflatoxin in the diet. Miazzo *et al.* (2000) reported enlarged, friable and yellowish livers in broiler chicks fed with 2.5 mg of aflatoxin per kg diet for 3-6 weeks.

Pale to yellow, enlarged and friable livers, pale or mottled and enlarged kidneys, regressed bursa of Fabricius, thymus and spleen were observed in broiler chicken fed with 2.5 mg of aflatoxin (Ortatatli and Oguz 2001).

Rosa *et al.* (2001) noted pale, enlarged and friable livers in broiler chicken that received aflatoxin at the levels of 5 mg/kg of diet.

Prakash (2001) reported pale, enlarged liver and kidneys, atrophy of lymphoid organs such as thymus and bursa of Fabricius and increase in size of spleen in broiler chicken fed with 1 ppm aflatoxin for 5 weeks.

Anilkumar (2002) and Bhanuprakash (2002) reported that the livers were enlarged, pale, friable and fatty with distended gall bladder. The kidneys were pale and swollen. Bursa of Fabricius and thymus were atrophied in broiler chicken fed with 0.5 ppm of aflatoxin for five weeks and 1 ppm aflatoxin in diet for 42 days (Santhosh Kumar, 2003).

Zahid Hussain *et al.* (2008) fed broiler chicks with feed containing 0, 1600, 3200 and 6400 $\mu\text{g} / \text{kg}$ AFB₁ for 7 days and reported pallor, discoloration and enlargement of liver and kidneys as well as hemorrhages in different organs. These gross pathological changes were related with dosage of aflatoxin, with severe gross changes noted at 6400 $\mu\text{g}/\text{kg}$ as compared to 1600 $\mu\text{g}/\text{kg}$ AFB₁ in feed.

Experimental mycotoxicoses was induced in broiler chickens by feeding 1 ppm aflatoxin for 28 days of age. The histological changes included enlargement, pallor or yellowish discoloration, occasional mottling of liver with distension of gall bladder. In

addition, the kidneys were enlarged, pale or congested with a few petechiae (Kumar and Balachandran, 2009).

Magnoli *et al.* (2011) reported no adverse change in liver of broilers following feeding of 100 µg of aflatoxin /kg feed from 6 to 33day of age.

Broilers fed mycotoxicated with 100ppb each of aflatoxin B₁ and ochratoxin A upto 42 days revealed enlarged, swollen and pale kidneys whereas the thymus, spleen and bursa of Fabricius appeared to be atrophied (Sawarker *et al.*, 2011).

Ramdas *et al.* (2013) reported enlarged, icteric, mottled soft and friable liver, congestion and enlargement of spleen and congestion of bursa of Fabricius following feeding of broilers with 100 and 150 ppb of AFB₁ upto 45 days of age.

A study conducted to determine the prevalence of aflatoxicosis in broiler chickens fed with contaminated feed with aflatoxin content ranging from 3.0 - 11.7 µg/kg revealed congested and oedematous carcass; hemorrhages in subcutaneous tissues and internal organs; friable, fatty, enlarged, hard, pale, liver with distended gall bladder, swollen kidneys, enlarged pancreas and spleen, hydropericardium and myocardial congestion and atrophy of thymus as well as bursa of Fabricius (Rashid *et al.*, 2013).

Experimental aflatoxicosis induced after feeding of 1.2 mg of AFB₁/kg of feed from 7 to 28 days of age to broilers showed pale and friable appearance of liver (Pizzolitto *et al.*, 2013).

Yalagod (2014) reported enlarged, swollen friable, yellowish pale livers from second week onwards in broilers fed AFB₁ @ 1ppm and from third week onwards in

birds fed AFB₁ @ 0.5 ppm. A mild enlargement was also observed in broilers fed with AFB₁ @ 0.25 ppm during fourth week of age. Kidneys of all toxin fed groups (AFB₁ @ 0.25, 0.5 and 1 ppm) were pale and moderately enlarged during third and fourth week of age. Intestine revealed catarrhal enteritis with mild mucosal congestion. A moderate atrophy of thymus with petechiae on lobes, a mild atrophy of spleen and bursa of Fabricius were also observed at 28th days of age.

Study conducted on individual and combined pathological effects of citrinin at 5 ppm and aflatoxin at 0.5 ppm in broiler chicken by feeding the mycotoxins from 1 to 42 days of age revealed swelling, congestion and petechial haemorrhages in kidneys. The liver showed enlargement, congestion, pallor or yellowish discolouration with distended gall bladder. Splenomegaly, atrophy of the bursa of Fabricius and catarrhal enteritis was also observed during the study period (Theophilus Anand Kumar and Balachandran, 2014).

2.3.9 HISTOPATHOLOGY

2.3.9.1 LIVER

Liver is the main target organ for toxic effects of aflatoxins. As a result, metabolism of proteins, carbohydrate and lipids in liver is seriously impaired causing accumulation of lipids and damage to the hepatocytes leading to fatty liver. Kiran *et al.* (1998) reported diffuse and severe hydropic degeneration of hepatocytes, bile duct hyperplasia and periportal fibrosis in the broilers fed with 2.5 mg of aflatoxin per kg of diet for a period of 21 days.

Study on feeding of 4 mg AFB₁/kg diet to broilers for 21 days revealed moderate to severe periportal cytoplasmic vacuolation of hepatocytes with moderate periportal heterophil infiltration and mild biliary hyperplasia (Ledoux *et al.*, 1999). Fatty change, acinar arrangement of hepatocytes surrounded by a thin stroma suggesting hepatocellular regeneration, periportal fibrosis, bile duct hyperplasia and nodular lymphoid cell accumulation were observed in broiler chicken fed with 2.5 mg aflatoxin in feed (Ortatatli and Oguz, 2001).

Moderate to severe fatty changes were observed in broiler chicken that received aflatoxin at the levels of 5 mg/kg of feed (Rosa *et al.*, 2001). Prakash (2001) recorded congestion, haemorrhages and degeneration of hepatocytes with loss of architecture, moderate biliary hyperplasia and periportal lymphoid cell infiltration in broiler chicken that received 1 ppm aflatoxin in the diet.

Anilkumar (2002) and Bhanuprakash (2002) reported congestion, haemorrhages, fatty change, hydropic degeneration and biliary hyperplasia along with periportal infiltration of heterophils and lymphoid cells in liver of broiler chicken that received 1.0 ppm of aflatoxin in the diet for 35 days. Similar changes were also reported by Santhosh Kumar (2003) in broiler chicken following feeding of 1 ppm of aflatoxin in the diet for 42 days.

Ortatatli *et al.* (2005) reported that, liver in chickens fed diet containing 100 ppb AF showed lesions such as slight to moderate hydropic degeneration and small fatty vacuoles in hepatocytes at centrilobular and midzonal areas, and bile duct proliferation in the portal areas along with periportal fibrosis. Zahid Hussain *et al.* (2008) reported, congestion of liver parenchyma, cytoplasmic vacuolation and heterophilic cell infiltration

in aflatoxin fed broiler chicks. Severe lesions were noted at 6400 µg/kg as compared to 1600µg/kg AFB₁ in feed.

A study conducted in broilers following feeding 1 ppm aflatoxin for 28 days revealed vacuolar degeneration, fatty changes, formation of fatty cysts and ballooning of the hepatocytes. The architecture of the liver was completely altered and the regenerating hepatocytes showed arrangement in acinar or ductular patterns. The acinar hepatocytes also showed fatty degeneration and fatty cysts in some areas. Congestion, bile duct hyperplasia, focal infiltration of heterophils and mononuclear cells, perivascular infiltration of mononuclear cells and heterophils and fibrosis were also noticed in the livers (Kumar and Balachandran, 2009).

Ahmed *et al.* (2009) reported moderate to severe fatty changes, loss of architecture, mild to severe aggregation of lymphoid cells and heterophils in periportal and parenchymatous areas. In addition, multifocal necrotic foci in liver were also noticed in broilers fed with 1ppm of aflatoxin for 1- 42 days.

Liver of the White Leghorn layer breeder hens of 30 weeks of age fed with diet containing 100, 500, 2,500, 5,000 and 10,000 µg of aflatoxin B₁/Kg feed showed moderate to severe fatty change, hepatocellular necrosis, congestion and dilatation of sinusoids and inflammatory changes around triads and blood vessels (Khan *et al.*, 2010).

The liver of broilers fed with 100 µg AFB₁/kg feed from day 6th to 33rd day of age revealed multifocal and multiple varying sized cytoplasmic vacuolation with perilobular location (Magnoli *et al.*, 2011). Yogeswari *et al.* (2012) in their histopathological studies of liver from aflatoxin fed group recorded macrovesicular changes, replacement

of necrosed hepatocytes by infiltrating mononuclear cells, bile duct hyperplasia and central venous congestion in broilers fed with 1ppm of aflatoxin for 1-42 days of age.

Pizzolitto *et al.* (2013) observed multifocal and varied cytoplasmic vacuolation with perilobular location in liver of broilers fed with 1.2 mg of AFB₁/kg feed from 7-28 days of age. Ramdas *et al.* (2013) reported, vacuolar and fatty degeneration, lymphoid aggregation with degeneration of hepatocytes. The acinar hepatocytes also showed fatty changes and fatty cysts in some areas. Focal infiltration of heterophils and mononuclear cells, bile duct hyperplasia and fibrosis were noticed in liver of broilers fed with 100 and 150 ppb of AFB₁ from 1-45 days of age.

Li *et al.* (2014) studied effect of diet containing 74 µg/kg AFB₁ for 3 weeks in broilers and reported mild vacuolar degeneration in hepatocytes, interlobular bile duct hyperplasia and lymphocytic infiltration in periportal and periductal areas. Motawe *et al.* (2014) observed focal aggregations of inflammatory cells, mild perivascular coagulative necrosis of hepatic parenchyma in the broilers fed with 0.5 ppm of aflatoxin and liver of birds fed with 1 ppm aflatoxin showed hepatocyte degeneration, vascular degradation thickening in the wall of arteries and veins with edematous changes and distended hyperplastic bile ductules.

A study conducted on feeding of naturally contaminated groundnut with aflatoxin concentration of 36 and 60 ppb mixed in feed from 22nd to 49th day of age revealed varying degree of vascular and sinusoidal congestion focal to diffuse necrosis of hepatocytes, fatty degeneration and steatosis (Kana *et al.*, 2014).

A study conducted on feeding of 0.5ppm of AF/kg of feed to broilers for 42 days of age revealed congestion, venous dilatation and extensive vacuolar degeneration of hepatocytes. In addition, ballooning degeneration and fatty cyst formation within the hepatocytes were also observed. The regenerating hepatocytes were found to be arranged in acinar pattern. In addition, microgranulomas, periductular mononuclear cell infiltration, bile duct hyperplasia, periportal fibrosis were also observed (Theophilus Anand Kumar and Balachandran, 2014).

2.2.9.2 KIDNEYS

The kidneys are susceptible to many toxic agents due to the high amount of blood it receives with the large amount of circulating toxicants to filter and reabsorb salt and water. Different parts of nephrons are exposed to aflatoxins as well as its metabolites leading to nephrotoxicity and cause damage to glomerular and tubular components manifested as deranged biochemical parameters of renal functions.

Feeding of broiler with 2.5 mg of aflatoxin/kg of diet for a period of 21 days produced degeneration and/or necrosis of renal tubular epithelium. The degenerating tubular epithelium was swollen and showed cytoplasmic granularity as well as pyknotic nuclei (Kiran *et al.*, 1998).

Ledoux *et al.* (1999) studied experimental aflatoxicosis following feeding of 4 mg AFB₁/kg diet to broilers for 21 days and observed lesions in kidneys characterised by thickening of glomerular capillary basement membrane and mild infiltration in heterophils in glomeruli.

Thickening of glomerular basement membrane, increase in mesangial cells, adhesions between glomeruli and Bowman's capsule, hydropic degeneration and focal mononuclear cell infiltration were the lesions observed in chicken fed with 2.5 mg of aflatoxin (Ortatatli and Oguz, 2001).

Prakash (2001) observed congestion of vessels, focal areas of heterophil infiltration and distended tubules with presence of eosinophilic proteinaceous material and membranous proliferation of glomeruli in broilers fed with 1 ppm of aflatoxin for five weeks.

Broiler chicks fed with 0.5 ppm of aflatoxin showed distended tubules with presence of eosinophilic proteinaceous material and focal areas of heterophil infiltration in the kidneys (Anilkumar, 2002 and Bhanuprakash, 2002). Santhosh Kumar (2003) recorded moderate degree of congestion and multifocal areas of haemorrhages in kidneys of birds fed with 1 ppm of aflatoxin in the diet for 42 days of age and attributed these changes to aflatoxin induced nephrotoxicity.

Ortatatli *et al.* (2005) observed mild tubular degeneration in kidneys of broiler fed with 100 ppb of AFB₁ for 42 days. Zahid Hussain *et al.* (2008) reported degeneration and necrosis of tubular epithelial cells, congestion and hemorrhages in kidneys of birds which received 6400 µg/kg of AFB₁ in feed for 7 days.

Ahmed *et al.* (2009) observed severe fatty changes with considerable loss of architecture of kidney due to accumulation of large fat droplets within the cells. In addition, mild infiltration of lymphoid cells in interstitial spaces of kidney was also reported along with vacuolar degeneration of renal tubular cells of broilers fed with 1ppm

of aflatoxin for 1-42 days. Kumar and Balachandran (2009) reported congestion, focal haemorrhages and degeneration of renal tubular epithelium and occasional thickening of basement membrane in the broilers fed with 1 ppm aflatoxin for 28 days of age.

A study conducted on feeding of 0.5, 1.0, and 1.5 mg of AF/kg of feed to White Leghorn hens from 14 to 56 week of age revealed necrosis of renal tubular epithelial cells. Cellular necrosis characterised by an increase in cytoplasmic acidophilia, pyknosis, karyorrhexis, karyolysis, intracellular vacuolation. In addition, glomerulonephritis with thickening of the glomerular basement membrane, cellular agglomeration, and lymphocytic infiltration were also observed (Martínez-de-Anda *et al.*, 2010).

Ramdas *et al.* (2013) observed focal haemorrhages, increased glomerular cellularity, vacuolar degeneration of tubular epithelium in fed broilers fed with 150 ppb of AFB₁ upto 42 days of age. Broiler fed with 0.5 and 1 ppm of aflatoxin produced lesions in kidney characterised by severe congestion and hemorrhage, desquamation and degeneration of tubular epithelium with cellular infiltrations. In addition, mild degree of infiltration of lymphoid cells in interstitial spaces of kidney and vacular degeneration of renal tubular cells were also noticed at 1ppm level of aflatoxin. Mild degree of renal congestion was noticed only during fourth week in the birds fed with 0.25 ppm AFB₁ in diet (Yalagod, 2014).

A study conducted by Theophilus Anand Kumar and Balachandran (2014) following feeding of 0.5 ppm of aflatoxin to broilers for 42 days revealed renal lesions including mild thickening of glomerular basement membrane, vacuolar degeneration, necrosis of tubular epithelial cells, atrophic glomeruli and calcification during sixth week of age.

Broilers fed with 0.5 and 1 ppm aflatoxin from 1 to 42 days of age revealed vascular congestion, haemorrhages, vacuolar degeneration and necrosis of renal tubular epithelial cells (Motawe *et al.*, 2014). Valchev *et al.* (2014a) observed generalised congestion of renal parenchyma and the tubular epithelial cells revealed varying degree of cloudy swelling, cytoplasmic vacuolation, and degeneration and desquamated cells from the basement membrane in the broiler fed with 0.5 mg AFB₁ in diet. Birds treated with the higher dose (0.8 mg/kg AFB₁) in the same study revealed haemorrhages, desquamation of tubular epithelial cells within the lumens and multiple necrotic foci within the renal parenchyma.

2.3.9.3 HEART

Kiran *et al.* (1998) reported heterophilic and mononuclear cellular infiltrations between the myofibrils of heart in the broilers fed with 2.5 mg of aflatoxin per kg of diet for a period of 21 days. Ramdas *et al.* (2013) reported granular degeneration of sarcoplasm, necrosis and collection of mononuclear cells in the heart following feeding of 100 and 150 ppb of aflatoxin to broilers upto 45 days of age.

Broilers fed with 0.5 and 1 ppm of aflatoxin showed intermyocardial haemorrhage and degeneration, perivascular edema, few heterophils in between myocardial muscle fibres (Motawe *et al.*, 2014). A study conducted by Theophilus Anand Kumar and Balachandran (2014) revealed myocardial degeneration following feeding 0.5 ppm of aflatoxin to the broilers for a period of 42 days.

2.3.9.4 INTESTINES

The gastrointestinal tract (GIT) is the main route of entry of aflatoxins as a result of consumption of mycotoxin contaminated feed. GIT is also the main route of excretion of aflatoxin metabolites from the bile. The aflatoxin, its metabolites and AF-8, 9-epoxides have been reported to cause acute effects on GIT by damaging the integrity of intestinal morphology and interfere in sialic acid production as well as digestible energy in the affected birds (Coulombe Jr, 1994; Applegate *et al.*, 2009).

Ledoux *et al.* (1999) reported no histological changes in duodenum, jejunum, cecum and ileum following feeding of 4 mg AFB₁/kg diet to broilers for 21 days of age. Experimental study on broilers following feeding of aflatoxin contaminated ration for 4 weeks at the rate of 1 mg AFB₁/kg diet revealed catarrhal enteritis with lymphocytic or mononuclear cellular infiltrations in the mucosa of intestines (Kumar and Balchandran, 2009).

Applegate *et al.* (2009) recorded a linear increase in the crypt depth in the distal jejunum with increasing levels of AFB₁ in the diet at the rate of 0, 0.6, 1.2 and 2.5mg/kg feed to the broilers. However, no effect on the villus height and number of goblet cells was reported in the same study.

Kana *et al.* (2010) observed decrease in the density of the whole intestine (weight/length) after 3 weeks of dietary exposure of broilers to 0.02mg of AFB₁/kg feed. Yunus *et al.* (2011) reported decrease in the density of whole intestine (weight/length) after 3 weeks of dietary exposure to AFB₁ at 0.7mg/kg.

Nathanael and VivekaVardhani (2011) noticed damage of duodenal epithelium characterised by mild hemorrhages in the duodenal glands, desquamation of villus epithelium, congestion of jejunum and oedema in ileum in broilers fed AFB₁ orally.

Study conducted on broilers fed with corn naturally contaminated with 134 µg AFB₁ and 23.6 µg AFB₂ demonstrated significant decrease in villus height and ratio of villus height to crypt depth of duodenum, jejunum and ileum (Yang *et al.*, 2012).

Fanimakki *et al.* (2013) reported lower intestine length (135.25 cm) in comparison to the control group (151.35 cm) in the broilers fed with 500 ppb of AFB₁ for 42 days of age.

TheophilusAnandKumar and Balachandran, (2014) observed increased goblet cell activity, necrosis of intestinal mucosal epithelium and moderate to severe lymphoid depletion both in the nodular and diffuse lymphoid tissues of the caecal tonsils in the third and sixth weeks following incorporation of 0.5 ppm aflatoxin in feed of broilers from 0 to 6 weeks of age.

2.3.9.5 PANCREAS

Simşek *et al.* (2007) demonstrated mild mononuclear cell infiltration of exocrine tissue and vacuolisation of acinar cells of pancreas in the Japanese quails (*Coturnix coturnix japonica*) fed with 2.5 ppm of aflatoxin for 21 days of age.

Study conducted on broilers following feeding of 1ppm aflatoxin for 28 days revealed vascular congestion, decrease in the volume of the zymogen granules,

degeneration and necrosis of acinar cells and mononuclear cellular infiltration in the pancreas (Kumar and Balachandran, 2009).

Valchev *et al.* (2014a) studied the effects of 0.5 and 0.8mg of aflatoxin/kg feed to mulards ducks for 42 days. At 0.5 mg of aflatoxin level the changes in pancreas were dilatation of capillaries, interlobular swelling, disintegration, disorganisation, degeneration and necrosis of pancreatic acinar cells. The dystrophic pancreatic changes in ducks treated with 0.8mg/kg AFB were much more intensive characterised by distension and congestion of capillaries and extensive haemorrhages. The interlobular swelling appeared severe with complete disorganisation of glandular acini, necrotic foci and infiltration with mononuclear cells.

Feeding of broilers with aflatoxin @ 0.5 ppm for 6 weeks of age showed reduced zymogen granules in the third and sixth week of the study (TheophilusAnandKumar and Balachandran, 2014).

2.3.9.6 LYMPHOID ORGANS

The avian immune system mainly consists of lymphoid tissue and lymphatic vessels. The thymus, bursa of Fabricius and bone marrow are primary avian lymphoid organs, whereas the spleen, mucosal associated lymphoid tissues (MALT), germinal centers and diffuse lymphoid tissues are secondary lymphoid organs. The lymphoid organs of chicken are very sensitive and vulnerable to the toxic effects of aflatoxins. Atrophy of immunological organs such as the thymus, spleen and bursa of Fabricius due to destruction of lymphoid cells causes lowered cell as well as humoral mediated immunity.

Kiran *et al.* (1998) studied effect of feeding 2.5 mg of aflatoxin per kg of diet for a period of 21 days to broilers and reported marked lymphocytolysis and depletion of lymphoid cells in the thymus, spleen and bursa of Fabricius. Follicular necrosis, depletion of lymphoid cells in the spleen, thinning of cortex with haemorrhages in medulla and cortex of thymus and bursa of Fabricius were the lesions observed in broiler chicken fed with 2.5 mg/kg of aflatoxin (Ortatatli and Oguz, 2001).

Prakash (2001) observed congestion, haemorrhages, lymphocytolytic activity and presence of histiocytes containing cellular debris in thymus, spleen and bursa of Fabricius in the broilers fed with aflatoxin. In addition to the above, the author also observed proliferation of fibrous connective tissue and follicular cysts in bursa of Fabricius in birds fed 1 ppm aflatoxin in the diet for five weeks.

Lymphoid organs such as thymus, spleen and bursa of Fabricius revealed congestion, haemorrhages, sparse cellularity, lymphocytic activity and presence of histiocytes following feeding of 0.5 ppm of aflatoxin (Anilkumar, 2002; Bhanuprakash, 2002) and 1.0 ppm of aflatoxin to the broiler chicks in diet for 42 days of age (Santhosh Kumar, 2003).

Shivachandra *et al.* (2003) observed mild depletion of lymphocytes in thymus, severe depletion and degeneration of lymphocytes in bursa of Fabricius followed by increased interfollicular space along with moderate proliferation of connective tissue in birds fed with AFB₁@ 1ppm from 7 to 49 days of age.

A study conducted in broilers following feeding 1 ppm aflatoxin for 28 days revealed lesions in spleen characterised by lymphoid depletion, an increase in the number

of germinal centres and reticulum cell hyperplasia. In the bursa of Fabricius there was lack of cortico-medullary differentiation, generalized lymphoid depletion and heterophilic infiltration. In addition, glandular transformation of isolated follicles and plial epithelial hyperplasia with cystic changes were also observed (Kumar and Balachandran, 2009).

Mohmand (2010) conducted study following feeding of 1 ppm aflatoxin to broilers and reported cellular sparsity, multiple secondary follicles as well as increased number of histiocytes in bursa of Fabricius. Mild congestion with multi focal haemorrhages involving the medulla and cortex and increase in the number of histiocytes in cortex were the findings in thymus. Mild degree of congestion of red pulp, sparse cellularity in the periarteriolar sheath with mild histiocytosis was seen in spleen on days 14th and 35th of experimental study. Shareef and Omar (2012) observed lymphoid cellular depletion in the medulla of the bursal follicles in the broilers fed with AFB₁@100ppb for a period of 42 days.

Ramdas *et al.* (2013) observed lymphoid depletion, an increase in the number of germinal centres and reticulum cell hyperplasia in spleen. The bursa revealed a lack of cortico-medullary differentiation, generalised lymphoid depletion and heterophilic infiltration in broilers fed with 100 and 150 ppb of AFB₁ upto 45 days of age.

A study conducted on broilers fed with aflatoxin @ 0.8 mg/kg of feed for a period of 42 day of age revealed moderate to severe reduction on lymphoid cell population in thymus, spleen and bursa of Fabricius. In addition, the thymus exhibited degenerative changes, cellular rarefaction of lymphatic tissue in the cortical layer and the cortex as well as medulla appeared undifferentiated. The bursa of Fabricius showed follicular

dystrophy, karyopyknosis and lymphocytic karyorrhexis. The splenic parenchyma showed karyolysis, karyopyknosis and karyorrhexis with reduction of the lymphoid cells and reduction in number and size of germinal centres (Valchev *et al.*, 2014a).

Motawe *et al.* (2014) recorded proliferation of capillary sheathes and haemorrhages, depletion of lymphocytes with proliferation of reticulo-endothelial cells in the spleen, moderate degree of hyperplasia of epithelial lining in bursa of Fabricius in broilers fed with 0.5 and 1 ppm of aflatoxin from 1 to 42 days of age.

Feeding of one day old broilers with 8 ppb of aflatoxin upto 40 days of age showed progressive changes in bursa of Fabricius characterised by slight to moderate depletion of medullary lymphocyte within bursal follicles, vacuolations in epithelium and fibroblastic tissue proliferation. Spleen revealed varying degree of depletion of lymphocyte population, dilatation and congestion of blood vessels and hyperplasia of vascular endothelial cells (Ali, 2014).

Study on feeding of 0.3 mg of aflatoxin/kg feed to the broilers for 21 days of age revealed depletion of lymphoid cells, vacuolations in the lymphatic nodules and periarterial sheath as well as congestion in the red pulp of the spleen (Chen *et al.*, 2014). On feeding of aflatoxin @ 0.5 ppm to broilers for 6 weeks, the bursa of Fabricius showed medullary lymphoid depletion and lymphocytolysis with starry sky appearance, mild oedema, atrophic changes with increase in interfollicular fibrous tissue and variable sized follicles. The spleen showed moderate lymphoid depletion, reticulum cell hyperplasia and necrosis of lymphoid cells. Thymus revealed mild degree of lymphoid depletion (Theophilus Anand Kumar and Balachandran, 2014).

2.4 AMELIORATION OF AFLATOXICOSIS USING DIATOMACEOUS EARTH

Mycotoxins have become an important issue for the grain industry and animal producers with a growing interest in the decontamination and remediation of highly contaminated feed stuffs. A recent approach to detoxifying mycotoxin contaminated grain is the use of non-nutritive adsorbents, which bind the toxin and thereby reduce their absorption from the gastrointestinal tract. Therefore this approach is seen as prevention rather than therapy.

Lately, several approaches to avoid contamination such as decontamination or remediation of feed and feedstuffs have been proposed (Bailey *et al.*, 1998; Ledoux *et al.*, 1999). Various sorbents have been tested such as hydrated sodium calcium aluminosilicate, zeolites, bentonites, clays of different origin, and activated carbons. One strategy is to use clay minerals to bind aflatoxin in the animal's digestive tract and thus reduce toxin bioavailability. The aflatoxin-clay complex is then excreted in the feces/droppings (Abdel-Wahhab *et al.*, 2002; Bintvihok, 2002). Clay products, such as bentonites, zeolites and HSCAS, are the most common feed additives which are effective in binding/adsorbing aflatoxins *in vitro* and *in-vivo* (Diaz and Smith, 2005; Jaynes *et al.*, 2007; Phillips *et al.*, 2008). Calcium montmorillonite clay) has been shown to act as an enterosorbent that tightly and selectively binds aflatoxins in the GI tract of animals thereby decreasing their bioavailability and associated toxicity. The high affinity of certain adsorbents for AF has been interpreted as the formation of a complex by β -carbonyl system of the aflatoxin molecule with aluminium ions (Phillips *et al.*, 1990). Adsorption studies demonstrated that the interlamellar region of the clay is the primary site of binding with aflatoxins. In addition, an electron donor acceptor mechanism has

been suggested for the affinity associated binding. This involves sharing electrons from the negative surface of the clay with the adsorbed molecule. The carbons comprising the dicarbonyl system in aflatoxins are partially positive and have been shown to be essential for the adsorption process. Other potential mechanisms might involve chelation or interaction of AFB₁ with various edge-site metals or interlayer cations, especially calcium ions (Phillips, 1999; Phillips *et al.*, 2008).

Diatomaceous earth (a type of tectosilicate) is a kind of clay that consists of ca. 90 per cent silicon dioxide. It is fine-grained, biogenic siliceous sediment and is available in large quantities at low cost (Kamigasa and Kato, 2000). Diatomite or Diatomaceous earth (DAE) consists essentially of amorphous silica derived from opalescent frustules of diatoms resulting in an inert, lightweight, highly porous, super-absorbent material and has a fine porous structure with low density (Wajima *et al.*, 2006). Clays, composed of mainly montmorillonite, and is produced by in situ diversification of volcanic ash. It may also contain feldspar, biotite, kaolinite, illite, cristobalite, pyroxene, zircon and crystalline quartz (Parker and McGraw-Hill, 1988). DAE has the unique characteristic of swelling to several times its original volume when placed in water and of forming thixotropic gels with water even though the amount of clay is relatively less. Though there is paucity of information on ameliorating efficacy of DAE against aflatoxicosis, a few relevant studies conducted earlier on this aspect are reviewed here under.

Manafi *et al.* (2009) conducted in-vitro efficacy of binding of diatomaceous earth to various mycotoxins and reported the highest percentage binding for aflatoxin (90.68%) whereas the lowest binding ability was recorded for ochratoxin (61.73%) at pH 4.5. At pH 6.5, the highest binding percentage was recorded for aflatoxin (94.71%) while for

ochratoxin it was only 63.13%. Natour and Yousef, (1998) reported significantly higher *in-vitro* adsorption ability of DAE to aflatoxin, which is directly proportional to the number of diatom valves. *In-vitro* study showed that DAE has high (94.71 %) ability to adsorb AF from the feed at pH 6.5 (Soleimani *et al.*, 2011).

Denli and Okan (2006) studied the effect of addition 2.5 g diatomite/kg to AFB₁ (40 µg AFB₁/kg) mixed feed to broilers for 42 days of age and concluded that DAE is not effective in reducing the detrimental effects of AFB₁ in broiler diets. Shi *et al.* (2006) reported that reduced growth rate and serum biochemical changes associated with AFB₁ contamination (0.1 mg/kg) could be ameliorated by the supplementation of a modified montmorillonite nanocomposite (compound similar to DAE) at doses of 3 g/kg feed.

Bailey *et al.*, (2006) reported that incorporation of montmorillonite clay (@ 5 g/kg feed) could ameliorate the adverse alterations in growth performance, serum biochemistry and the relative organ weights following feeding of 4 ppm of AFB₁ to the broiler birds. Modirsanei *et al.* (2008) reported that following supplementation of diatomaceous earth @ 30g/kg feed significantly improved body weight gain, feed intake, and improved feed conversion ratio as well as productive efficiency index and increased serum albumin in the birds fed with 1 ppm of aflatoxin.

Study conducted on broiler birds following supplementation of AflaDetox (natural product obtained from diatomaceous earth) @ level of 1, 2, 5 g/kg of feed containing 1 ppm of AFB₁ showed significant improvement in growth performance, counteracted the serum biochemical and histopathological changes in liver, reduced the relative weight of liver, and also improved the relative spleen weight (Denli *et al.*, 2009).

Similar studies conducted earlier reported no protective effects against aflatoxin on the biochemical parameters and histopathological changes in liver sections when sodium bentonite (compound rich in silicate like DAE) was included in the diet at levels of 5 and 3 g/kg of feed (Santurio *et al.*, 1999; Rosa *et al.*, 2001). Silambarasan *et al.* (2013) evaluated the comparative efficacy of diatomaceous earth, sodium bentonite and zeolites alone or in combination in broilers following feeding 300 ppb of aflatoxin and reported least binding capacity of DAE in ameliorating the adverse effect of aflatoxin.

Study conducted recently on immunotoxicological effects following addition of Tunisian Montmorillonite (TM) clay (a compound similar to DAE) to the aflatoxin-contaminated diet at a level of 5 g/kg feed indicated a significant improvement in lymphocytes of the total white blood cells, immunoglobulin profile (Ig G and Ig A), T-cells sub-types (CD₄⁺ and CD₈⁺ cells), NK and pro-inflammatory cytokines (TNF α and IL-1 β) levels. The ameliorative effect can be attributed to a strong aflatoxin-binding/adsorption capacity of TM resulting in a decrease in the bioavailability of aflatoxin in the gastrointestinal tract and subsequent reduction in the distribution to different organs and systems (Abbès *et al.*, 2010).

Materials and Methods



III MATERIALS AND METHODS

The present study was carried out in the Department of Pathology, Veterinary College, Hebbal, Bangalore, Karnataka with an objective of assessing the performance, biochemical, immunological, pathomorphological alterations in broiler chicken fed with aflatoxin and also to evaluate the ameliorating effects of diatomaceous earth. The experimental procedures followed are described as here under.

3.1 MATERIALS

3.1.1 SOURCE OF FUNGAL CULTURE

Aspergillus parasiticus (MTCC-2796) culture was procured from IMTECH, Chandigarh, India and sub-cultured on potato dextrose agar.

3.1.2 PRODUCTION AND QUANTIFICATION OF AFLATOXIN

Aflatoxin (AF) was produced on the rice using *Aspergillus parasiticus* (MTCC-2796) as per the method of Shotwell *et al.* (1966) with some modifications. Polished rice (50 g) was soaked overnight in water measuring approximately 40 per cent of the weight and was autoclaved (15 psi at 121°C) for 20 minutes and then cooled. It was then inoculated with spores of 7 days old culture of *Aspergillus parasiticus* (MTCC 2796), sub-cultured on potato dextrose agar (PDA), using inoculation loop. The inoculated rice flasks were incubated at the room temperature in dark place for 14 days with vigorous shaking three times a day to break the mycelial mass. On 15th day, the inoculated rice flasks were autoclaved (15 psi at 121°C for 20 minutes) to stop fungal growth. The fermented rice was dried in hot air oven (70°C overnight), powdered and stored in dark

place until further use. Aflatoxin was quantified using thin layer chromatography (AOAC, 1995).

3.1.3 EXPERIMENTAL BIRDS

Day-old healthy unsexed broiler chicks were procured from a reputed commercial hatchery in Bangalore, India and the chicks were housed on battery brooder fitted cages. Optimum conditions of brooding and management was provided to the birds throughout the period of experimentation (Plate 1). All the birds were checked daily for the health and husbandry conditions. All the sanitary and hygienic precautions were strictly followed throughout the experiment. Prior permission of the Institute Animal Ethical Committee (IAEC) was obtained before the conduct of experiment.

3.1.4 EXPERIMENTAL FEED

Mycotoxin and Diatomaceous Earth (DAE) free Starter and finisher broiler feed was procured from Department of Poultry Science, Veterinary College, Bangalore, India

3.1.5 TOXIN FEED

Required quantities of cultured aflatoxin material were added to make the final concentration of aflatoxin in feed to be 0.5 ppm and 1ppm.

3.1.6 MYCOTOXIN AMELIORATING AGENT

Diatomaceous Earth (DAE) obtained from M/S Agripower, Australia was incorporated in feed at the rate of 400 mg and 800 mg/Kg feed respectively.

3.1.7 EXPERIMENTAL DESIGN

360 day old broiler chicks were procured from a reputed hatchery and reared in cages fitted with wire floor under optimal condition of management and nutrition. The birds were divided into 9 groups comprising of different treatments as given below. Starting from day one, birds were fed different levels of aflatoxin individually and in combination with DAE upto 35th day of age as per the experimental design.

GROUPS	TREATMENT	NUMBER OF BIRDS
I	CONTROL (Toxin free & DAE free feed)	40
II	DAE @ 400 mg/Kg of feed	40
III	DAE @ 800 mg/Kg of feed	40
IV	AFLATOXIN @ 1 ppm	40
V	AFLATOXIN @ 0.5 ppm	40
VI	AFLATOXIN @ 1 ppm + DAE @ 400 mg/Kg of feed	40
VII	AFLATOXIN @ 1 ppm + DAE 800 mg/Kg of feed	40
VIII	AFLATOXIN @ 0.5 ppm + DAE @ 400 mg/Kg of feed	40
IX	AFLATOXIN @ 0.5 ppm + DAE 800 mg/Kg of feed	40

Before the day old chicks were separated into groups, blood samples were collected and maternal antibody titres against NDV and IBDV were measured by the hemagglutination-inhibition test (HI) and ELISA, respectively.

In each experiment, six birds were tested for IBD antibodies at 14 days of age before vaccination. Antibodies titres were considered negative in accordance with ELISA test guidelines.

All the chicks were vaccinated through occulo-nasal route against Newcastle disease on day 7th by using F1 strain (Ventribiological, Pune and Veterinary Biologicals, Bangalore) and against Infectious bursal disease virus on day 14th of the experiment.

The birds were observed daily for clinical signs and mortality (if any). A complete record of the daily mortality (if any) was also maintained. Six birds from each group were sacrificed on day 7, 14, 21, 28 and 35th day. During each sacrifice, blood samples and tissue specimens were collected for serum biochemical, histopathological and immunological studies.

3.1.8 GLASSWARE AND PLASTICWARES

All the glassware and plastic wares were washed and sterilised prior to use. The list of glassware and plastic wares used in the present study are presented in Appendices.

3.1.9 CHEMICAL, REAGENTS AND BUFFERS

All the chemicals of molecular biology or analytical grade were procured either from Sigma Chemical Co., (USA), Hi-media (India) or Mercks (India) Ltd. The composition of buffers and solutions used in the study are given in Appendices.

3.2 METHODS

3.2.1. BODY WEIGHT

Body weights (g) of chicks were recorded at weekly intervals from day one of experiment to 35th day of the experiment and body weight gain was calculated accordingly.

3.2.2. FEED CONVERSION RATIO

Weekly feed conversion ratio was calculated as follows,

$$\text{Feed conversion ratio} = \frac{\text{Average feed consumed/bird during the week (g)}}{\text{Average weight gain/bird during the week (g)}}$$

3.2.3 RELATIVE ORGAN WEIGHT

During each screening, liver, kidneys, heart, pancreas, thymus, spleen and bursa of Fabricius were collected in a petridish, blotted using filter paper and weighed using an electronic weighing balance. The organ weights were converted into relative organ weights and expressed in percentage.

$$\text{Relative weight of organ} = \frac{\text{Weight of the organ (grams)}}{\text{Body Weight (grams)}} \times 100$$

3.2.4 BIOCHEMICAL PARAMETERS

Blood was collected from the sacrificed birds and sera was separated by standard procedures and stored under -20°C until further use.

The individual serum samples were analysed for Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Alkaline phosphatase (ALP), Creatinine, Blood Urea Nitrogen, Total proteins, Albumin, Total cholesterol and Triglycerides by Semi-automatic analyser (Semi-Automatic Biochemistry Analyser, STATFAX 2000+, CPC Diagnostics Pvt. Ltd., India). The methodology and the set of reagents used in respect of each parameter were as per the recommendations of the manufacturer of the analyzer system.

3.2.5 IMMUNOLOGICAL PARAMETERS

3.2.5.1. HUMORAL IMMUNITY

Blood samples were collected in non-heparinized tubes from six birds of each group on 28th day of the experiment. Serum was separated as per the standard procedures

and was stored at -20°C for subsequent analysis. Haemagglutination inhibition test was performed for determining the antibody titres against Newcastle disease virus as described by Allan and Gough (1974).

Blood samples were collected individually from six birds each from all the groups on 28th day of experiment via wing vein puncture. Serum was separated by centrifugation at 1000 rpm for 15 min and stored at -20°C until tested. IBD antibody titres were determined using commercial ELISA kits (PDRC, VHL, Pune, India). The kits included both negative and positive control samples. An automated IBM computerized ELISA reader was used in the study. Two readings/samples were obtained for each serum dilution and mean antibody titres $\log^{10}$ were calculated according to the equation provided with the kit:

$$\text{Log}^{10} \text{ titres} = 1.09 (\log^{10} \text{ sample mean} / \text{positive mean}) + 3.36$$

$$\text{S/P ratio} = \text{Sample mean} - \text{negative control} / \text{positive control} - \text{negative control}$$

The mean antibody titres of the groups were compared with the control group.

3.2.5.2 CELL-MEDIATED IMMUNITY BY QUANTIFICATION OF CD4^{+} AND CD8^{+} CELLS FROM THE PERIPHERAL BLOOD.

Peripheral blood was collected from six birds of each group at the end of 35th day of age and lymphocytes were separated for quantification of CD4^{+} and CD8^{+} cells. All the reagents, chemicals, glasswares and antibodies used in the study are presented in Annexures.

3.2.5.2.1 OPTIMIZATION OF ANTI-CHICKEN CD4 AND CD8 ANTIBODIES

An end point titration was carried out to determine the optimum concentration of anti-chicken CD4⁺ and CD8⁺ monoclonal antibodies to be used to bind lymphocytes. It was performed by using different concentrations of 2.5, 5, 7.5 and 10 µl of the anti-chicken CD4⁺ and CD8⁺ monoclonal antibodies (1µg/µl) to bind to the lymphocytes.

3.2.5.2.2 PROCEDURE FOR MEASUREMENT OF CD4 AND CD8 CELLS BY FLOW CYTOMETRY

The procedure for processing of whole blood which was used for measurement of CD4⁺ and CD8⁺ cells by flow cytometry is described below.

- a. 2.5 ml of whole blood was collected in EDTA vacutainers, mixed and carried on ice to the laboratory.
- b. The blood was diluted in 2.5 ml of PBS- azide-BSA.
- c. 1.5 ml Lymphocyte separating medium (Histopaque) was taken in sterilized tube.
- d. The diluted blood was overlaid on lymphocyte separating medium and centrifuged at 1500 rpm for 30 min at room temperature.
- e. After removing the supernatant, leukocyte layer was carefully collected into another tube.
- f. The cells were washed twice in PBS.
- g. The cell pellet was dissolved in 20µl PBS.
- h. 9 µl of cell suspension was diluted in 2.7ml of PBS (1:300) dilution).
- i. 100µl of diluted cell suspension was taken in 5 ml round bottom FACS tubes.

- j. Mouse anti chicken CD4: FITC and mouse anti chicken CD8a: RPE were added (7.5 μ l at concentration of 1 μ g/ μ l) and incubated on ice for 30 min with intermittent mixing.
- k. Cells were washed with 2 ml of PBS-azide-1% BSA; the supernatant was discarded after centrifugation at 3000 rpm for 10 min.
- l. Cells were resuspended by adding 0.5ml of 2% PFA per tube.
- m. Finally, the tubes were taken to FACS machine (FACS Calibur® Instrument, BD Bioscience) for cell differentiation or acquisition at Centre for Cellular and Molecular Platforms (C-CAMP), National Centre for Biological Sciences (NCBS), Bangalore, India.

3.2.6 STUDIES ON CYTOKINE ASSAY

The chicken IL-2, IL-6 and TNF α , cytokine concentrations in the serum samples were determined using commercially available ELISA kits procured from Cusabio, United Kingdom (IL-2-: Catalog No. CSB-E06755Ch, IL-6: Catalog No. CSB-E08549Ch and TNF α : CSB-E112312Ch). Instructions from the manufacturer were followed for the assay.

3.2.6.1 CHICKEN INTERLEUKIN 2 (IL-2) CYTOKINE ESTIMATION:

The detection range, list of material in the kit and the procedure for the preparation of reagents is presented in Appendices.

3.2.6.1.1 PRINCIPLE OF THE ASSAY

The microtiter plate provided in this kit has been pre-coated with an antibody specific to IL-2. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated antibody preparation specific for IL-2 and Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB (3,3',5,5' tetramethyl-benzidine) substrate solution is added to each well. Only those wells that contain IL-2, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of $450 \text{ nm} \pm 2 \text{ nm}$. The concentration of IL-2 in the samples is then determined by comparing the O.D. of the samples to the standard curve.

3.2.6.1.3 ASSAY PROCEDURE

All reagents and samples were thawed to room temperature before use. All samples, standards, and controls were assayed in duplicate. All the reagents were added directly to the liquid level in the well.

1. 100 μ l of Standard, Blank, or Sample was added per well and covered with the adhesive strip. All were incubated for 2 hours at 37°C. The liquid was removed from each well after 2 hours.
2. 100 μ l of Biotin-antibody working solution was added to each well and incubated for 1 hour at 37°C. The Biotin-antibody working solution appeared cloudy. On warming up to room temperature and gently mixing, the solution appeared uniform.

3. The contents from each well was aspirated and washed. The process was repeated for three times for a total of three washes. For washing, each well was filled with wash buffer (200µl) and allowed to stand for 2 minutes and then the liquid was removed by flicking the plate over a sink. The remaining drops were removed by patting the plate on a paper towel. Complete removal of liquid at each step was ensured for good performance.
4. 100µl of HRP-avidin working solution was added to each well, and then the microtiter plate was covered with a new adhesive strip and incubated for 1 hour at 37°C.
5. Aspiration and washing was repeated for five times as per the step no 4.
6. 90µl of TMB Substrate was added to each well and incubated for 10-30 minutes at 37°C. The plate was kept away from drafts and other temperature fluctuations in the dark.
7. 50µl of stop solution was added to each well when the first four wells containing the highest concentration of standards developed blue color. Gentle taping of the plate was performed to ensure thorough mixing of all the reagents for proper colour development.
8. The optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm.

3.2.6.1.4 CALCULATION OF RESULTS

Professional soft "Curve Exert 1.3" was used to make a standard curve for determination of results. Average of the duplicate readings for each standard, control and sample was calculated and the average zero standard optical density was subtracted. A

standard curve was created by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, a standard curve was also constructed by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and a best fit curve was drawn through the points on the graph. The data was linearized by plotting the log of the IL-2 concentrations versus the log of the O.D. and the best fit line was determined by regression analysis.

3.2.6.2 CHICKEN INTERLEUKIN 6 (IL-6) ESTIMATION

The detection range, list of material in the kit and the procedure for the preparation of reagents is presented in Appendices.

3.2.6.2.1 PRINCIPLE OF THE ASSAY

The microtiter plate provided in the kit is pre-coated with an antibody specific to IL-6. Standards or samples are then added to the appropriate microtiter plate wells with biotin-conjugated antibody preparation specific for IL-6 and Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB (3,3',5,5'-tetramethyl-benzidine) substrate solution is added to each well. Only those wells that contain IL-6, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of $450 \text{ nm} \pm 2 \text{ nm}$. The concentration of IL-6 in the samples is then determined by comparing the O.D. of the samples to the standard curve.

3.2.6.2.2 ASSAY PROCEDURE

All reagents and samples were thawed to room temperature before use. All samples, standards, and controls were assayed in duplicate. All the reagents were added directly to the liquid level in the well.

1. 100µl of Standard, Blank, or Sample was added to each well, covered with the adhesive strip and incubated for 2 hours at 37°C. All the wells were emptied after 2 hours.
2. Biotin-antibody working solution was added to each well and incubated for 1 hour at 37°C. Warming up to room temperature and gentle mixing of solution was done till it appeared uniform.
3. Each well was aspirated and washed by repeating the process three times for a total of three washes. Each well was filled with wash buffer (200µl) and allowed to stand for 2 minutes and then the liquid was removed by flicking the plate over a sink. The remaining drops were removed by patting the plate on a paper towel. Complete removal of liquid at each step was ensured for good performance.
4. 100µl of HRP-avidin working solution was added to each well; the microtiter plate was covered with a new adhesive strip and incubated for 1 hour at 37°C.
5. The process of aspiration was repeated and washed for five times as per step no 4.
6. 90µl of TMB Substrate was added to each well and incubated for 10-30 minutes at 37°C. The plate was kept away from drafts and other temperature fluctuations in the dark.
7. 50µl of Stop Solution was added to each well when the first four wells containing the highest concentration of standards developed obvious blue color. Gentle tapping of the

plate was performed to ensure thorough mixing of all the reagents for proper colour development.

8. The optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm using a microplate reader.

3.2.6.2.3 CALCULATION OF RESULTS

Professional soft "Curve Exert 1.3" was used to make a standard curve. The duplicate readings were averaged for each standard, control and sample and average zero standard optical density was subtract. A standard curve was created by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, a standard curve was also constructed by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and a best fit curve was drawn through the points on the graph. The data was linearized by plotting the log of the IL-6 concentrations versus the log of the O.D. and the best fit line was determined by regression analysis.

3.2.6.3 CHICKEN TUMOR NECROSIS FACTOR-Alpha (TNF- α)

The detection range, list of material in the kit and the procedure for the preparation of reagents is presented in Appendices.

3.2.6.3.1 PRINCIPLE OF THE ASSAY

This assay employed the competitive inhibition enzyme immunoassay technique. The microtiter plate provided in the kit is pre-coated with TNF- α . Standards or samples are added to the appropriate microtiter plate wells with an antibody specific for TNF- α and Horseradish Peroxidase (HRP) conjugated TNF- α , then incubated. The competitive

reaction is launched between with pre-coated TNF- α and TNF- α in sample. Then substrate solutions are added to the wells, respectively. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of $450 \text{ nm} \pm 2 \text{ nm}$. The concentration of TNF- α in the samples is then determined by comparing the O.D. of the samples to the standard curve. The OD of the sample is inversely proportional to the amount of TNF- α in sample. The higher the OD, the lower is the amount of TNF- α in sample.

3.2.6.3.2 ASSAY PROCEDURE

All reagents and samples were brought to room temperature before use. All the samples, standards and controls were assayed in duplicate. All the reagents were added directly to the liquid level in the well.

1. Blank well was set without any solution. 50 μ l of standard or sample was added per well. Standard was tested in duplicate.
2. 50 μ l of HRP-conjugate was added to each well (not to blank well), followed by 50 μ l antibody to each well, then mixed properly and incubated for 2 hour at 37°C.
3. Each well was filled with wash buffer (about 250 μ l), allowed to stand for 10 seconds and spinning was done. The process was repeated for a total of three washes. Complete removal of liquid at each step was ensured for good performance. After the last wash, remaining wash buffer was removed by aspirating or decanting. The plate was inverted and blotted against clean paper towels.
4. 50 μ l of Substrate A and Substrate B was added to each well, mixed well and incubated for 15 minutes at 37°C. The plate was kept away from drafts and other temperature fluctuations in the dark.

5. 50µl of stop solution was added to each well when the first four wells containing the highest concentration of standards developed obvious blue color.
6. The optical density of each well was determined within 10 minutes, using a microplate reader set to 450 nm.

3.2.6.3.3 CALCULATION OF RESULTS

The duplicate readings for each standard, blank, and sample and subtract the optical density of blank were averaged. A standard curve was created by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, a standard curve was also constructed by plotting the mean absorbance for each standard on the x-axis against the concentration on the y-axis and drawn a best fit curve through the points on the graph. The data was linearized by plotting the log of the TNF- α concentration versus the log of the O.D. and the best fit line was determined by regression analysis. This procedure produced an adequate but less precise fit of the data.

3.2.7 CYTOKINE GENE EXPRESSION OF IL-8 IN mRNA IN LYMPHOCYTES

The lists of material, PCR components and conditions as well as primers used for the expression of IL-8 are presented in Appendices.

3.2.7.1 SAMPLE PREPARATION

Blood samples from birds of all the groups were collected on 35th day of experiment and lymphocytes were separated by adding equal quantity of Histopaque to the unclotted blood followed by centrifugation at 1500 rpm for 15 min. The separated lymphocytes was gently collected and further processed for extraction of RNA.

3.2.7.2 RNA EXTRACTION

Total RNA was extracted from the cells using RNAiso Plus (Takara Bio, Japan) as described by the manufacturer's instruction. Briefly, the cells were homogenized by adding 1ml of RNAiso Plus. The homogenate was kept at room temperature for 5 minutes followed by centrifugation at 12,000x g for 5 minutes at 4°C. The supernatant was transferred to a new centrifuge tube and chloroform was added equal to 0.2 volume of RNAiso Plus. Vortexing was done vigorously at room temperature for 5 minutes and centrifuged at 12,000x g for 15 minutes at 4°C. The upper layer was transferred to a new centrifuge tube and isopropanol equal to 0.5-1.0 volume of RNAiso Plus was added. The entire mixture was kept at room temperature for 10 minutes and centrifuged at 12,000x g for 10 minutes at 4°C. The RNA was washed with equal amount of 75 per cent ethanol and then centrifuged at 7500x g for 5 minutes at 4°C followed by air drying to dry precipitate. The dried sample comprising of RNA was dissolved with 20µl of DEPC-treated water, the supernatant was discarded and the precipitate was retained. To remove the chromosomal DNA, the samples were incubated with DNase I (0.5 U/10 mg of RNA, Merck, Germany) at 37° C for 5 min. The integrity of RNA was confirmed by denaturing agarose gel electrophoresis and the concentration was quantified by measuring the optical density (OD) at 260 nm in a UV-VIS spectrophotometer (Vivaspec Biophotometer, Germany).

3.2.7.3 cDNA SYNTHESIS

RNA was reverse transcribed using PrimeScript (Takara Bio, Japan) according to the manufacturer's recommendations. Briefly, 5.0 µg of RNA was combined with 10X first strand buffer, 2.0 µl of oligo (dT) primer (10pM), 2.0 µl of dNTP mix (25 mM of

each dNTP) and RNase-free water to total volume 19 µl. The mixture was incubated at 65° C for 5 min, cooled to room temperature and 50 units of PrimeScript reverse transcriptase 1.0 µl were added. The mixture was incubated at 42° C for 1 h and the reaction stopped by heating at 70° C for 5 min.

3.2.7.4 QUANTITATIVE RT-PCR ANALYSIS

Oligonucleotide primers for bird cytokines (IL8) and GAPDH control were designed based upon sequences available from public data base. Real-time PCR was performed using a StepOne, Applied Biosystems USA. Each PCR was performed in a total volume of 20 µl containing 10 µl of 2X FastStart DNA Master SYBR Green , 0.5µM each of HPLC purified forward and reverse primers, and 1µl of template DNA. Primers for quantification were selected using Primer Express 1.0 from ABI (Foster City, USA.) Real-time PCR was carried out with an initial incubation of 10 min at 95° C followed by 45 cycles consisting of denaturing at 95° C for 10 sec; annealing 60° C for 5 sec followed by amplification at 72° C for 30 sec. Amplification and detection were carried out in optical-grade 48 well plates in an ABI Prism Sequence Detection System. External cDNA standards for each gene under study were constructed by using the corresponding RT-PCR product. The concentration (mg/ml) of the standard was determined by measuring the OD at 260 nm and the copy number of product per ml (CN) was calculated with the following formula:

$$\text{Number of copies} = (A * 6.022 \times 10^{23}) / (N * 1 \times 10^9 * 650).$$

Where:

A = amount of amplicon (ng)

N = length of dsDNA amplicon

660 g/mole = average mass of 1 bp dsDNA

After amplification, a melting curve analysis was performed to determine the specificity of the PCR products. For melting curve analysis PCR products were incubated for 15 sec at 5° C below the annealing temperature for the respective primers and the temperature was increased to 95° C with a ramp rate of 0.1/s. Quantification analysis was performed using StepOne® software.

3.2.7.5 PCR data analysis

At the end of the PCR, the StepOne® software saved the results, allowing the data for further analysis. Some initial cycles were considered as a baseline (BL) or background, in which no changes in fluorescence intensity occurred, and the level above this, at which increments in fluorescence became detectable, is termed the threshold (Th). The software also determined the cycle number when a reaction reached the Th. This value, termed the 'cycle threshold' (Ct), appeared during the exponential phase of the PCR and was inversely proportional to the initial number of template molecules in the sample. The software only determined the number of templates present in the reactions; if the amounts of cDNA used in the RT-PCR are considered, the number of specific molecules of mRNA per mg in the samples can be calculated. Results were expressed as absolute copy number per mg of RNA or as relative expression. The number of cytokines mRNAs calculated was normalized with the GAPDH housekeeping gene.

3.2.8 STUDIES ON DNA FRAGMENTATION

3.2.8.1 EXTRACTION OF TISSUE DNA

The tissue DNA from liver, kidneys, heart, pancreas, thymus, spleen and bursa of Fabricius of birds belonging to all the groups was extracted as per the standard protocol, using the DNeasy® Blood and Tissue kit procured from Qiagen, Inc., (USA).

3.2.8.2 PROTOCOL FOR DNA EXTRACTION

- a. The tissues (≤ 10 mg spleen or ≤ 25 mg other tissue) were cut into small pieces, and placed in 1.5 ml microcentrifuge tube. 180 μ l buffer ATL and 20 μ l proteinase K were added and mixed by vortexing, and incubated at 56° C until completely lysed. Vortexing was done occasionally during incubation.
- b. 200 μ l of buffer AL was added and mixed thoroughly by vortexing.
- c. Two hundred microlitre of absolute ethanol (99%) was added to the sample and mixed by pulse-vortexing for 15 sec.
- d. After mixing, the microcentrifuge tube was briefly centrifuged to remove the drops from inside the lid.
- e. The mixture from the above step (including the precipitate) was carefully applied to the QIAamp mini spin column along with two ml collection tube without wetting the rim. The cap was closed and centrifuged at $\geq 6000 \times g$ for 1 min. Spin column was placed in a new clean 2 ml collection tube and collection tube containing the filtrate was discarded.
- f. Five hundred microlitre of buffer AW1 was carefully added without wetting the rim of the spin column.
- g. The cap was closed and centrifuged at $\geq 6000 \times g$ for one minute and the collection tube was discarded.
- h. The spin columns were carefully opened and 500 μ l of buffer AW2 was added without wetting the rim of the spin column.
- i. The cap was closed and centrifuged at 20,000 $\times g$ for three minute.

- j. QIAamp mini spin column was placed in a clean 1.5 ml microcentrifuge tube and 200 μ l buffer AE or distilled water was carefully added and incubated at room temperature for one minute. Finally, the DNA was eluted at $\geq 6000 \times g$ for two minute.

3.2.8.3 DETERMINATION OF PURITY AND YIELD OF THE DNA SAMPLES

The purity and concentration of the extracted tissue DNA was estimated by UV spectrophotometry. An aliquot of 20 μ l of DNA sample was dissolved in 0.98 ml of sterile distilled water. The diluted DNA was transferred into 1ml microcuvette and the optical density (OD) was checked at 260 nm and 280 nm in a UV spectrophotometer. Sterile distilled water was used as blank.

The ratio of OD at 260/280 nm was calculated. A ratio of 1.7 to 1.9 was considered pure. The concentration of the DNA was estimated by the equation: 1 OD 260 nm = 50 μ g/ml of DNA. Further, the purity of the DNA sample was checked by electrophoresis on 1.8 per cent agarose gel.

3.2.8.4 PROTOCOL FOR DNA CONFIRMATION AND FRAGMENTATION STUDY BY AGAROSE GEL ELECTROPHORESIS

For electrophoresis, 1.2 % agarose was used and it was carried out as follows.

- a. The edges of a clean, dry, gel casting tray was sealed at both the ends using adhesive tape. An appropriate comb was placed to form a sample slot in the gel.
- b. Agarose solution was prepared by dissolving required quantity of agarose in proportionate volume of 1X TBE buffer and melted in a microwave oven for one min.

- c. Once the molten gel cooled, 0.5 μg of ethidium bromide was added and mixed thoroughly by gentle swirling.
- d. Warm agarose solution was then poured into the gel casting tray avoiding formation of air bubbles and allowed to solidify.
- e. Once agar gel solidified, a small amount of electrophoresis buffer was poured on the top of the gel to remove the comb. Then the buffer was poured off and the tape was removed.
- f. The gel casting tray was mounted in the electrophoresis tank and the electrophoresis buffer was added just enough to cover the gel to a depth of one mm.
- g. 5 μl of DNA was mixed with 1/6th volume of 6X gel loading dye and slowly loaded into the slots of submerged gel using a micropipette.
- h. The gel tank was closed with the lid and electrical leads were attached so that the DNA would migrate towards the anode.
- i. The electrophoresis was carried out at 5V / cm at RT until the bromophenol blue dye migrated appropriate distance through the gel.
- j. Following electrophoresis, DNA bands were visualized at 300 nm wavelengths using a UV transilluminator and the images were captured by using Gel Doc XR (Bio Rad, USA) for further interpretation.

3.2.9 GROSS PATHOLOGY

Six birds from each group were randomly selected and sacrificed at weekly intervals and after exsanguination, the carcasses were subjected to detailed post-mortem examination and gross lesions if any were recorded accordingly.

3.2.10 HISTOPATHOLOGY

Representative tissue samples from liver, kidneys, heart, pancreas, intestines, thymus, spleen and bursa of Fabricius were immediately collected and fixed in 10 per cent buffered formalin for histological analyses. Samples from tissue were dehydrated in ascending grades of ethanol, cleared in xylene and embedded in paraffin. Sections of 3-5 μm were obtained and stained with haematoxylin/eosin (H&E) as per Luna (1968) for histopathological examination. The histological lesions observed in sections of various organs were systematically recorded and photomicrographs were taken wherever necessary using Zeiss AxioCam ERc5s microscope.

3.2.11 STATISTICAL ANALYSIS

Data were analysed using the General Linear Models (GLM) procedure by adopting one way ANOVA (Duncan's test) for multiple comparisons. The statements of statistical significance are based on $P < 0.05$ (SPSS 16, 2010).

Results



IV RESULTS

The results of the present study on the effects of feeding aflatoxin and diatomaceous earth individually as well as their combinations to broiler chicken under the headings of performance, biochemistry, pathology and immunological studies are presented in this chapter.

In the present study, aflatoxin was produced under laboratory condition to study the effect of aflatoxin in diet of experimental chicks. The steps involved in production of aflatoxin are presented as here under.

4.1 PRODUCTION AND QUANTIFICATION OF AFLATOXIN

4.1.1 SUB CULTURE OF *ASPERGILLUS PARASITICUS*

The physical characteristics of *Aspergillus parasiticus* (MTCC-2796) sub-cultured on potato dextrose agar were in conformity with those of pure culture. After two weeks of incubation in a dark place at room temperature, the mycelial mat of *A. parasiticus* appeared as dark green growth on the agar surface.

The culture of *Aspergillus parasiticus* (MTCC-2796) was maintained on potato dextrose agar. At the end of the incubation, the color of the mycelial mats of *A. parasiticus* on the agar surface was dark green (Plate 2).

4.1.2 RICE CULTURE

The broken rice was inoculated with spores of *A. parasiticus* incubated for the production of aflatoxin at room temperature. It was regularly observed for the growth of

the fungus and the consequential changes in its physical characteristics. The broken rice showed white mycelial growth with moisture condensation on the inner walls of the flask by third day. The color turned to greenish yellow by fourth day and gradually to yellowish brown to dull brown by seventh day (Plate 3).

4.1.3. MYCOTOXIN QUANTIFICATION

The harvested culture of *A. parasiticus* seeded on broken rice yielded 25 ppm of toxin which was incorporated into the standard poultry ration to give a final concentration of 0.5 and 1 ppm of aflatoxin. In the combined treatment groups, DAE powder (Plate 4) was added @ 400 and 800 mg/kg feed to the aflatoxin mixed diet.

4.2 TOXICITY STUDY

4.2.1 CLINICAL SIGNS

The birds of Group I (Control) and Group II and III (DAE mixed in control feed) were healthy with lustrous plumage (Plate 5) and did not show any clinical signs throughout the period of experiment.

Birds that received diet containing aflatoxin (Group IV and V) exhibited clinical signs such as dullness, poor growth (Plate 6), inappetence and diarrhoea from Day 7th of the experiment, which persisted till the end of the experiment (35th day). Feather malformation was less evident in birds fed with 0.5 ppm whereas in birds fed with 1 ppm of aflatoxin the feather abnormalities were more pronounced.

The birds fed with diet containing aflatoxin and supplemented with DAE (Group VI to IX) showed almost similar clinical signs to those recorded for the toxin alone fed

birds (Group IV and V). However, they were of reduced severity and the clinical manifestation and were observed after 10-12 days of experiment. The feather formation was evidently good and lustrous in birds of co-treatment groups (VI to IX) supplemented with AF and DAE (Plate 7).

4.2.2 GROWTH PERFORMANCE

4.2.2.1 BODY WEIGHT

The weekly mean body weights of different groups have been presented in Table 1 and the same has been graphically depicted in Fig. 1. The mean body weights were 1513 ± 4.38 , 1504 ± 4.25 , 1507 ± 5.69 , 724.17 ± 30.99 , 862.83 ± 29.13 , 784.33 ± 34.04 , 812.67 ± 22.63 , 930.5 ± 29.49 and 955.5 ± 30.32 g in Group I to IX, respectively at the end of the experiment.

The mean body weights among the birds of Group I, II, and III showed non-significant difference ($P \leq 0.05$) throughout the experimental period. However, significant reduction in the body weight was observed in toxin fed birds (Group IV) from 7th day onwards and continued till the end of the experiment (Plate 8, 9). In Group V significant reduction was seen from 28th day and continued till the end of the experiment (Plate 10).

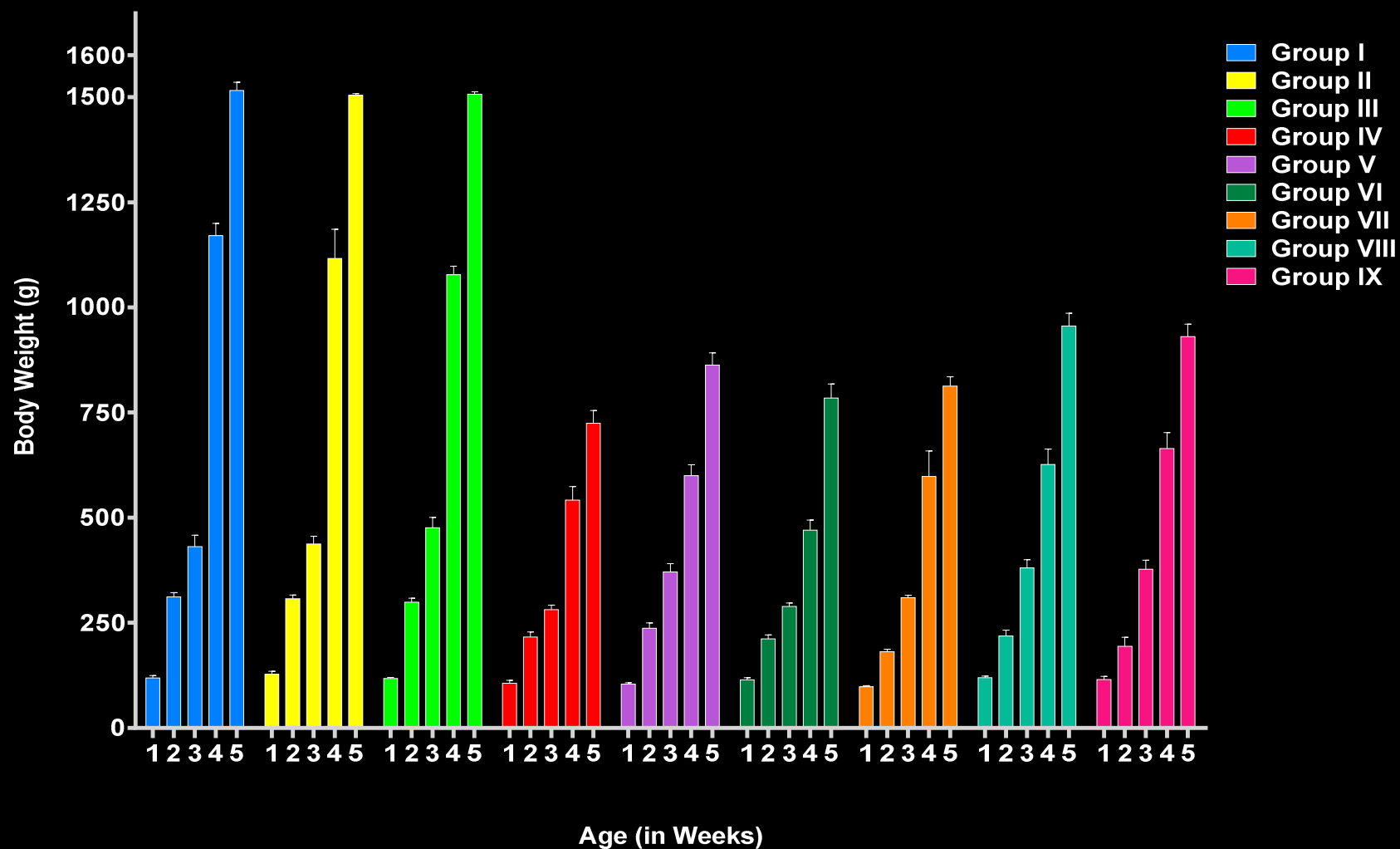
The birds supplemented with DAE along with toxin (Group VI to IX) showed numerical increase in their body weight throughout the period of experiment, however significant increase was observed from 28th day to the end of experiment as compared to only toxin fed groups (Group IV and V). Significant increase in the body weight of the birds in the DAE supplemented in AF mixed feed was seen on 35th day of experiment as compared to only toxin fed Group IV and Group V (Plate 11, 12).

Table 1: Mean ($\pm$ SE) weekly body weight (g) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination

Groups	Aflatoxin (ppm)	DAE (mg/kg feed)	Age in weeks				
			1	2	3	4	5
I	0	0	118.83 $\pm$ 5.96 ^{bc}	311.33 $\pm$ 10.52 ^c	430.83 $\pm$ 27.65 ^{cd}	1170.5 $\pm$ 29.54 ^c	1513 $\pm$ 4.38 ^e
II	0	400	127.33 $\pm$ 6.98 ^c	306.83 $\pm$ 9.3 ^c	437.17 $\pm$ 18.95 ^d	1116.17 $\pm$ 69.84 ^c	1504 $\pm$ 4.25 ^e
III	0	800	117.67 $\pm$ 2.67 ^{bc}	298.67 $\pm$ 9.73 ^c	475.67 $\pm$ 25.3 ^d	1078.33 $\pm$ 20.03 ^c	1507 $\pm$ 5.69 ^e
IV	1	0	106.17 $\pm$ 7.1 ^{ab}	216.33 $\pm$ 12.03 ^{ab}	281.5 $\pm$ 10.68 ^a	470.5 $\pm$ 23.5 ^a	724.17 $\pm$ 30.99 ^a
V	0.5	0	104 $\pm$ 3.48 ^{ab}	236.83 $\pm$ 12.85 ^b	371.17 $\pm$ 19.27 ^b	600 $\pm$ 25.6 ^b	862.83 $\pm$ 29.13 ^{bc}
VI	1	400	114 $\pm$ 5.55 ^{abc}	211.67 $\pm$ 9.29 ^{ab}	288.5 $\pm$ 8.22 ^a	541.83 $\pm$ 32.07 ^{ab}	784.33 $\pm$ 34.04 ^{ab}
VII	1	800	97.5 $\pm$ 3.23 ^a	181.17 $\pm$ 5.99 ^a	309.83 $\pm$ 5.95 ^a	598.33 $\pm$ 60.42 ^b	812.67 $\pm$ 22.63 ^{ab}
VIII	0.5	400	119.17 $\pm$ 3.95 ^{bc}	218.83 $\pm$ 13.95 ^{ab}	380.33 $\pm$ 19.8 ^{bc}	626.33 $\pm$ 37.09 ^b	930.5 $\pm$ 29.49 ^{cd}
IX	0.5	800	115.17 $\pm$ 7.55 ^{bc}	194.33 $\pm$ 21.15 ^a	377.33 $\pm$ 21.21 ^{bc}	664.5 $\pm$ 38.02 ^b	955.5 $\pm$ 30.32 ^d

Mean values bearing at least one common superscript within columns did not differ significantly ($P \geq 0.05$)

Fig. 1: Mean ($\pm$ SE) weekly body weight (g) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination



There was 52.14 and 42.97 per cent reduction in the body weights of birds fed with only toxin (Group IV and V) at the end of the fifth week as compared to control (Group I) birds.

In the co-treatment birds (Group VI and VIII), there was increase of 10.5 and 12.5 per cent body weight gain, respectively was observed at 35th days of age as compared to the only toxin fed birds (Group IV and V). However, 18.93 and 20.64 per cent body weight gain was observed in birds of Group VII and IX at 35th days as compared to the toxin fed birds (Group IV and V).

4.2.2.2 FEED CONVERSION RATIO (FCR)

The mean values of FCR with different dietary treatments have been presented in Table 2 and graphically represented in Fig. 2. The mean FCR in Group I to IX were 1.87 ± 0.012 , 1.79 ± 0.012 , 1.88 ± 0.016 , 2.93 ± 0.02 , 2.73 ± 0.01 , 2.86 ± 0.02 , 2.72 ± 0.02 , 2.68 ± 0.02 and 2.6 ± 0.02 , respectively at the end of the experiment.

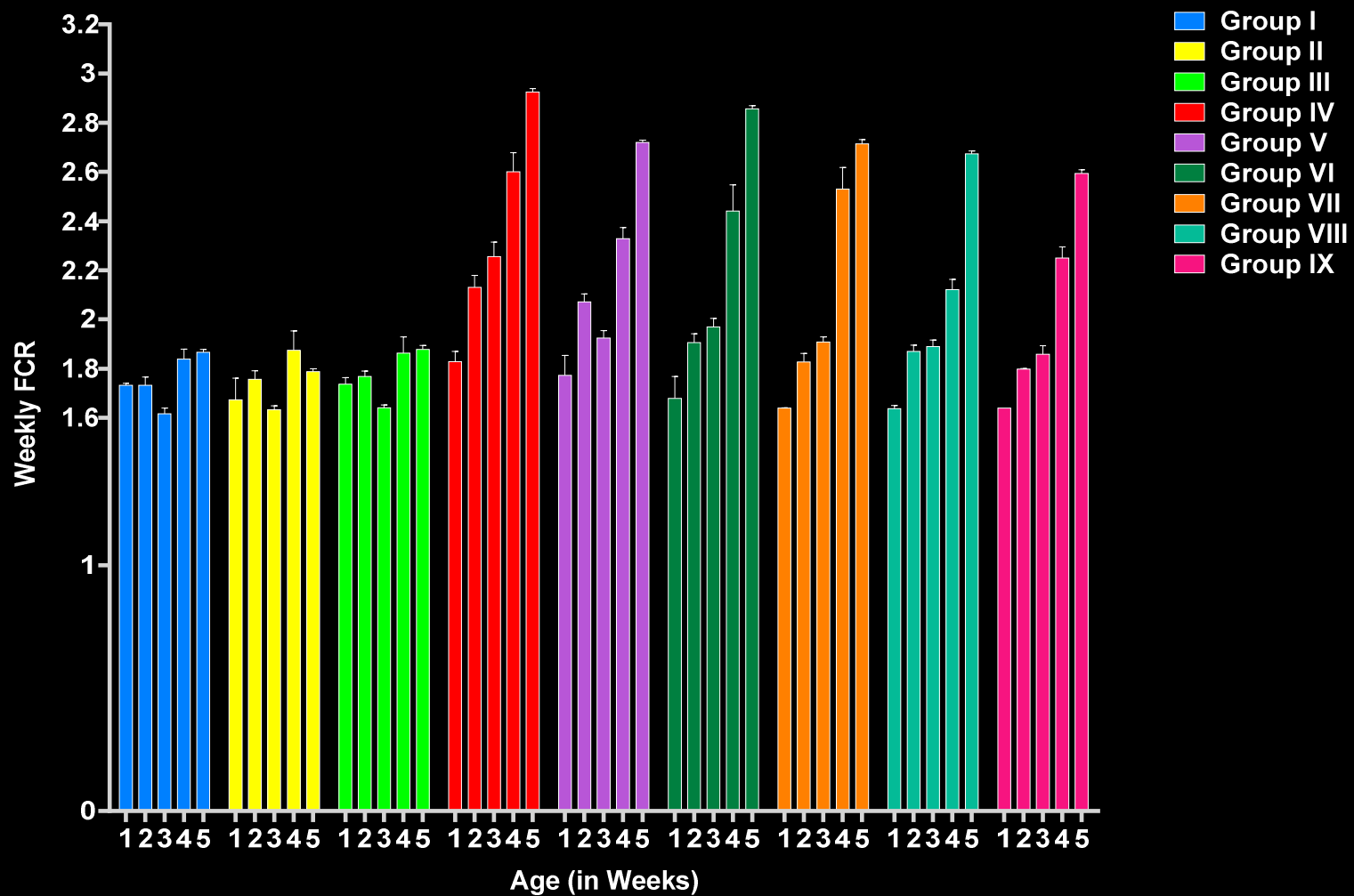
The FCR values were almost similar in Group I, II and III till fourth week of age. However, birds supplemented with diatomaceous earth to toxin mixed feed (Group VI to IX) showed increased FCR value in comparison to the control group (Group I) on day 35th of experiment. The FCR was increased in toxin fed birds (Group IV and V) from second week onwards in comparison to control groups (Group I, II and III). The toxin fed birds had FCR value of 2.93 and 2.6 (Group IV and V) against birds supplemented with diatomaceous earth and toxin (Group VI, VII, VIII and IX) were 2.86 ± 0.02 , 2.72 ± 0.02 , 2.68 ± 0.02 and 2.6 ± 0.02 , respectively at the end of the fifth week. The FCR was highest in Group IV (2.93) and it was least in Group I (1.87) on 35th day of the observation.

Table 2: Mean ($\pm$ SE) weekly FCR of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination

Groups	Aflatoxin (ppm)	DAE (mg/kg feed)	Age in weeks				
			1	2	3	4	5
I	0	0	1.74 $\pm$ 0.01 ^{ab}	1.74 $\pm$ 0.04 ^a	1.62 $\pm$ 0.03 ^a	1.84 $\pm$ 0.05 ^a	1.87 $\pm$ 0.012 ^a
II	0	400	1.68 $\pm$ 0.09 ^{ab}	1.76 $\pm$ 0.04 ^a	1.64 $\pm$ 0.02 ^a	1.88 $\pm$ 0.08 ^a	1.79 $\pm$ 0.012 ^a
III	0	800	1.74 $\pm$ 0.03 ^{ab}	1.77 $\pm$ 0.03 ^a	1.65 $\pm$ 0.02 ^a	1.87 $\pm$ 0.07 ^a	1.88 $\pm$ 0.016 ^a
IV	1	0	1.83 $\pm$ 0.05 ^b	2.14 $\pm$ 0.05 ^d	2.26 $\pm$ 0.06 ^d	2.61 $\pm$ 0.08 ^e	2.93 $\pm$ 0.02 ^f
V	0.5	0	1.78 $\pm$ 0.09 ^{ab}	2.08 $\pm$ 0.04 ^d	1.93 $\pm$ 0.04 ^{bc}	2.33 $\pm$ 0.05 ^{cd}	2.73 $\pm$ 0.01 ^d
VI	1	400	1.68 $\pm$ 0.09 ^{ab}	1.91 $\pm$ 0.04 ^c	1.97 $\pm$ 0.04 ^c	2.45 $\pm$ 0.11 ^{cde}	2.86 $\pm$ 0.02 ^e
VII	1	800	1.64 $\pm$ 0.01 ^a	1.83 $\pm$ 0.04 ^{abc}	1.91 $\pm$ 0.03 ^{bc}	2.54 $\pm$ 0.09 ^{de}	2.72 $\pm$ 0.02 ^d
VIII	0.5	400	1.64 $\pm$ 0.02 ^a	1.87 $\pm$ 0.03 ^{bc}	1.89 $\pm$ 0.03 ^{bc}	2.13 $\pm$ 0.05 ^b	2.68 $\pm$ 0.02 ^c
IX	0.5	800	1.64 $\pm$ 0.01 ^a	1.8 $\pm$ 0.01 ^{ab}	1.86 $\pm$ 0.04 ^b	2.25 $\pm$ 0.05 ^{bc}	2.6 $\pm$ 0.02 ^b

Mean values bearing at least one common superscript within columns did not differ significantly ($P \geq 0.05$)

Fig. 2: Mean ($\pm$ SE) weekly FCR of broiler chicks fed with aflatoxin (AF) , diatomaceous earth (DAE) and their combination



4.2.3 RELATIVE ORGAN WEIGHT

4.2.3.1 LIVER

The data pertaining to the mean relative weights of liver in birds of Group I to IX have been furnished in Table 3 and Fig. 3.

The mean relative weights of liver for Group I to IX were 2.74 ± 0.14 , 2.82 ± 0.13 , 2.91 ± 0.09 , 4.57 ± 0.42 , 4.5 ± 0.41 , 4.36 ± 0.12 , 4.09 ± 0.03 , 4.28 ± 0.17 and 3.79 ± 0.11 g/100g of body weight, respectively at 35th days of age.

The mean relative liver weights of birds in Group I, II and III did not alter significantly till 35th day of observations. The relative liver weight of toxin fed birds (Group IV and V) showed significant ($P \leq 0.05$) increase from 7th day onwards and continued to increase till the end of experiment in comparison to control birds.

The birds supplemented with DAE and toxin (Group VI, VII, VIII and IX), showed significant ($P \leq 0.05$) decrease in liver weights as compared to only toxin fed birds (Group IV and V) respectively at the end of experimental period. In the co-treatment (AF + DAE) groups, the birds supplemented with 800 mg/kg feed (Group VII and IX) to the aflatoxin mixed feed recorded significantly lower relative liver weight in comparison to Group VI and VIII fed with 400 mg DAE /kg feed along with toxin.

4.2.3.2 KIDNEYS

The weekly mean relative weights of kidneys in different groups have been presented in Table 4 and graphically depicted in Fig 4.

Table 3: Mean ($\pm$ SE) weekly relative weight of liver (g%) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.

Groups	Aflatoxin (ppm)	DAE (mg/kg feed)	Age in weeks				
			1	2	3	4	5
I	0	0	4.24 $\pm$ 0.43 ^{ab}	3.94 $\pm$ 0.29 ^{ab}	3.88 $\pm$ 0.2 ^a	3 $\pm$ 0.11 ^a	2.74 $\pm$ 0.14 ^a
II	0	400	4.55 $\pm$ 0.77 ^b	3.72 $\pm$ 0.13 ^{ab}	3.89 $\pm$ 0.54 ^{ab}	3.08 $\pm$ 0.48 ^{ab}	2.82 $\pm$ 0.13 ^a
III	0	800	3.5 $\pm$ 0.17 ^{ab}	3.47 $\pm$ 0.21 ^a	3.73 $\pm$ 0.31 ^{ab}	3.17 $\pm$ 0.14 ^{ab}	2.91 $\pm$ 0.09 ^a
IV	1	0	3.76 $\pm$ 0.39 ^{ab}	6.15 $\pm$ 0.55 ^{bc}	4.83 $\pm$ 0.19 ^c	5.37 $\pm$ 0.61 ^c	4.57 $\pm$ 0.42 ^c
V	0.5	0	3.16 $\pm$ 0.2 ^a	3.96 $\pm$ 0.46 ^{abc}	4.67 $\pm$ 0.53 ^c	5.09 $\pm$ 0.44 ^c	4.5 $\pm$ 0.41 ^c
VI	1	400	3.12 $\pm$ 0.34 ^a	4.85 $\pm$ 0.38 ^{bc}	4.82 $\pm$ 0.4 ^c	4.79 $\pm$ 0.52 ^c	4.36 $\pm$ 0.12 ^{bc}
VII	1	800	3.04 $\pm$ 0.16 ^a	4.63 $\pm$ 0.74 ^{bc}	4.89 $\pm$ 0.23 ^{bc}	4.34 $\pm$ 0.51 ^{bc}	4.09 $\pm$ 0.03 ^{bc}
VIII	0.5	400	3.48 $\pm$ 0.39 ^{ab}	4.83 $\pm$ 0.28 ^c	5.68 $\pm$ 0.25 ^c	4.71 $\pm$ 0.2 ^c	4.28 $\pm$ 0.17 ^{bc}
IX	0.5	800	4.04 $\pm$ 0.22 ^{ab}	5.37 $\pm$ 0.53 ^{abc}	4.71 $\pm$ 0.17 ^{bc}	4.32 $\pm$ 0.42 ^{bc}	3.79 $\pm$ 0.11 ^b

Mean values bearing at least one common superscript within columns did not differ significantly ($P \geq 0.05$)

Fig. 3: Mean ($\pm$ SE) weekly relative weight of liver (g %) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.

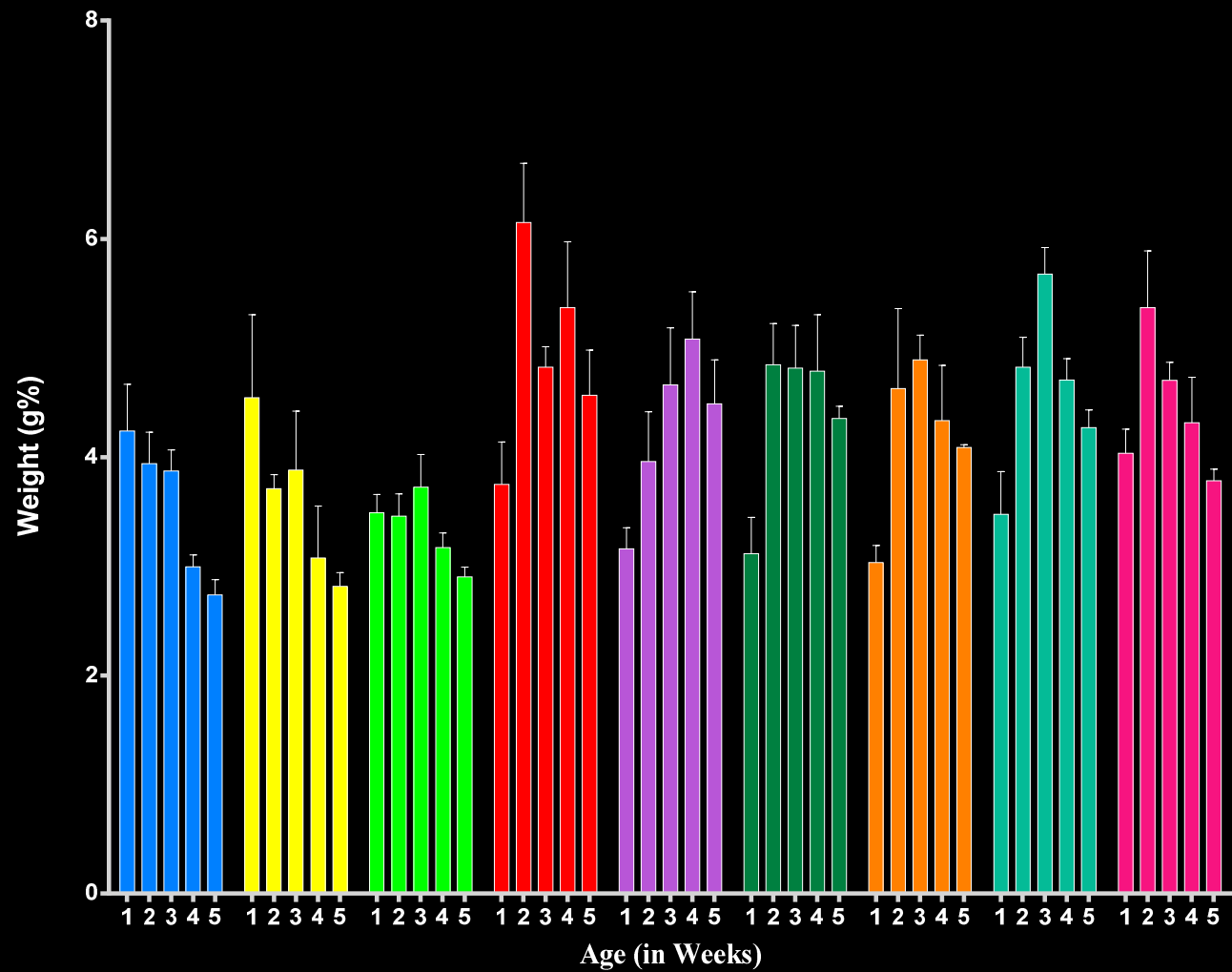
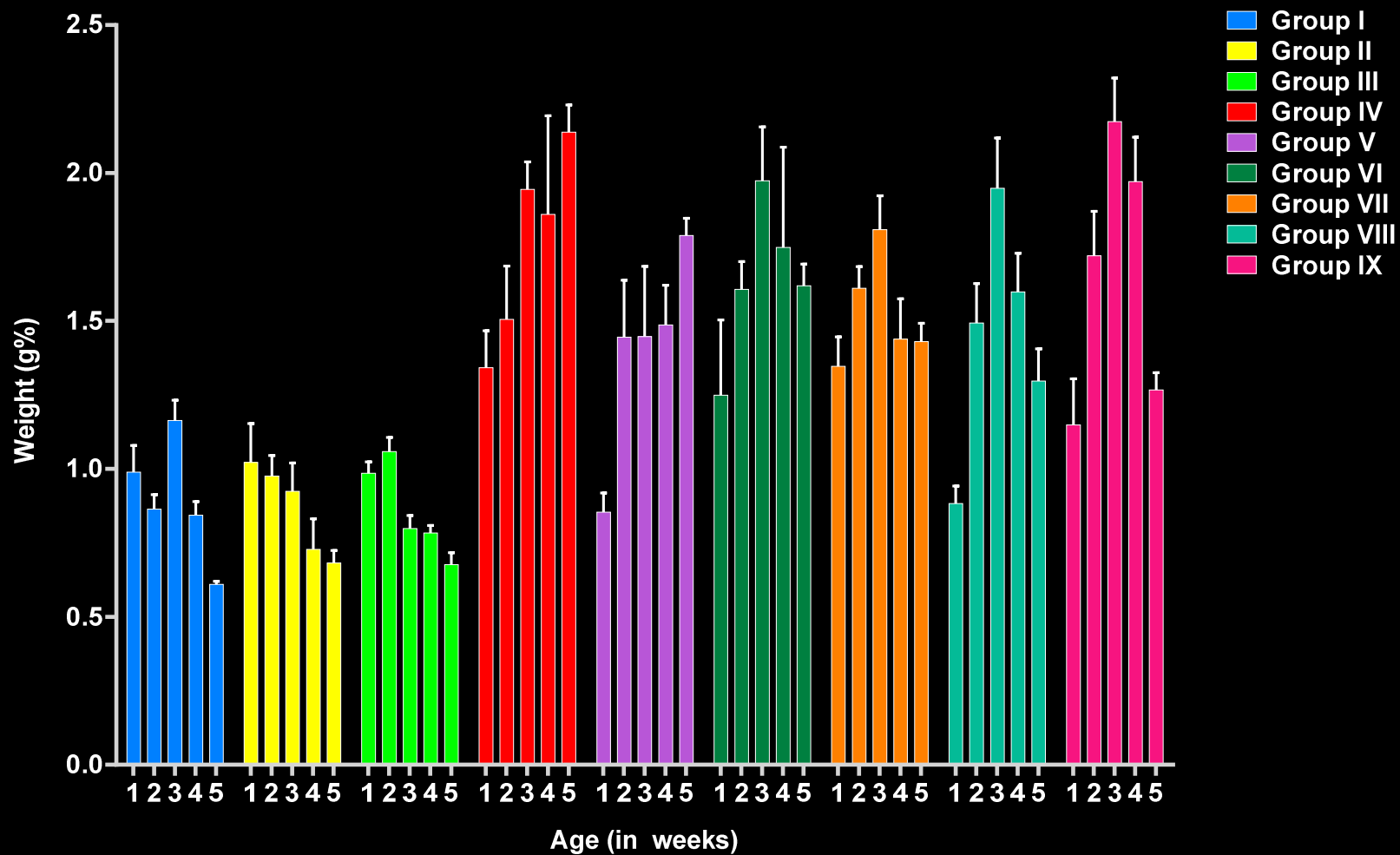


Table 4: Mean ($\pm$ SE) weekly relative weight of kidney (g %) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.

Groups	Aflatoxin (ppm)	DAE(mg/kg feed)	Age in weeks				
			1	2	3	4	5
I	0	0	0.99 $\pm$ 0.09 ^{ab}	0.87 $\pm$ 0.05 ^a	1.17 $\pm$ 0.07 ^{ab}	0.85 $\pm$ 0.05 ^a	0.62 $\pm$ 0.02 ^a
II	0	400	1.02 $\pm$ 0.14 ^{ab}	0.98 $\pm$ 0.08 ^a	0.93 $\pm$ 0.1 ^a	0.73 $\pm$ 0.11 ^a	0.69 $\pm$ 0.05 ^a
III	0	800	0.99 $\pm$ 0.04 ^{ab}	1.06 $\pm$ 0.05 ^a	0.8 $\pm$ 0.05 ^a	0.79 $\pm$ 0.03 ^a	0.68 $\pm$ 0.04 ^a
IV	1	0	1.35 $\pm$ 0.13 ^b	1.51 $\pm$ 0.19 ^b	1.95 $\pm$ 0.1 ^d	1.86 $\pm$ 0.34 ^b	2.14 $\pm$ 0.1 ^d
V	0.5	0	0.86 $\pm$ 0.07 ^a	1.45 $\pm$ 0.2 ^b	1.45 $\pm$ 0.24 ^{bc}	1.49 $\pm$ 0.14 ^b	1.79 $\pm$ 0.06 ^c
VI	1	400	1.25 $\pm$ 0.26 ^{ab}	1.61 $\pm$ 0.1 ^b	1.98 $\pm$ 0.19 ^d	1.75 $\pm$ 0.34 ^b	1.62 $\pm$ 0.08 ^c
VII	1	800	1.35 $\pm$ 0.1 ^b	1.62 $\pm$ 0.08 ^b	1.81 $\pm$ 0.12 ^{cd}	1.44 $\pm$ 0.14 ^b	1.43 $\pm$ 0.07 ^b
VIII	0.5	400	0.89 $\pm$ 0.06 ^a	1.5 $\pm$ 0.14 ^{bc}	1.96 $\pm$ 0.17 ^d	1.6 $\pm$ 0.14 ^b	1.3 $\pm$ 0.11 ^b
IX	0.5	800	1.15 $\pm$ 0.16 ^{ab}	1.73 $\pm$ 0.16 ^b	2.18 $\pm$ 0.15 ^d	1.97 $\pm$ 0.16 ^b	1.27 $\pm$ 0.06 ^b

Mean values bearing at least one common superscript within columns did not differ significantly ($P \geq 0.05$)

Fig. 4: Mean ($\pm$ SE) weekly relative weight of kidney (g %) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.



The relative weights of kidney for Group I to IX were 0.62 ± 0.02 , 0.69 ± 0.05 , 0.68 ± 0.04 , 2.14 ± 0.1 , 1.79 ± 0.06 , 1.62 ± 0.08 , 1.43 ± 0.07 , 1.3 ± 0.11 and 1.27 ± 0.06 g/100g of body weight, respectively at 35th days of experiment.

The mean relative weights of kidney of birds in Group I to III did not alter significantly during entire period of the experiment. A significant ($P \leq 0.05$) increase in the mean relative kidney weight in toxin fed birds (Group IV and V) was observed as compared with control birds (Group I) from 14th day onwards and continued till the end of the experiment. The birds supplemented with DAE to the toxin mixed feed (Group VI to IX) showed significant ($P \leq 0.05$) decrease in relative kidney weights as compared to that of toxin fed birds (Group IV and V) at the end of experiment. In the combined treatment groups (AF + DAE), the birds supplemented with 800 mg/ kg feed (Group VII and IX) to the aflatoxin mixed feed showed significantly lower relative kidney weight in comparison to Groups VI and VIII fed with 400 mg of DAE/kg feed along with toxin.

4.2.3.3 HEART

The weekly mean relative weights of heart have been presented in Table 5 and graphically represented in Fig 5.

The mean relative weights of heart of Group I to IX were 0.55 ± 0.04 , 0.51 ± 0.03 , 0.6 ± 0.04 , 1.04 ± 0.04 , 0.85 ± 0.03 , 0.7 ± 0.03 , 0.73 ± 0.05 , 0.7 ± 0.04 and 0.61 ± 0.04 g/100g of body weight, respectively at the end of experiment.

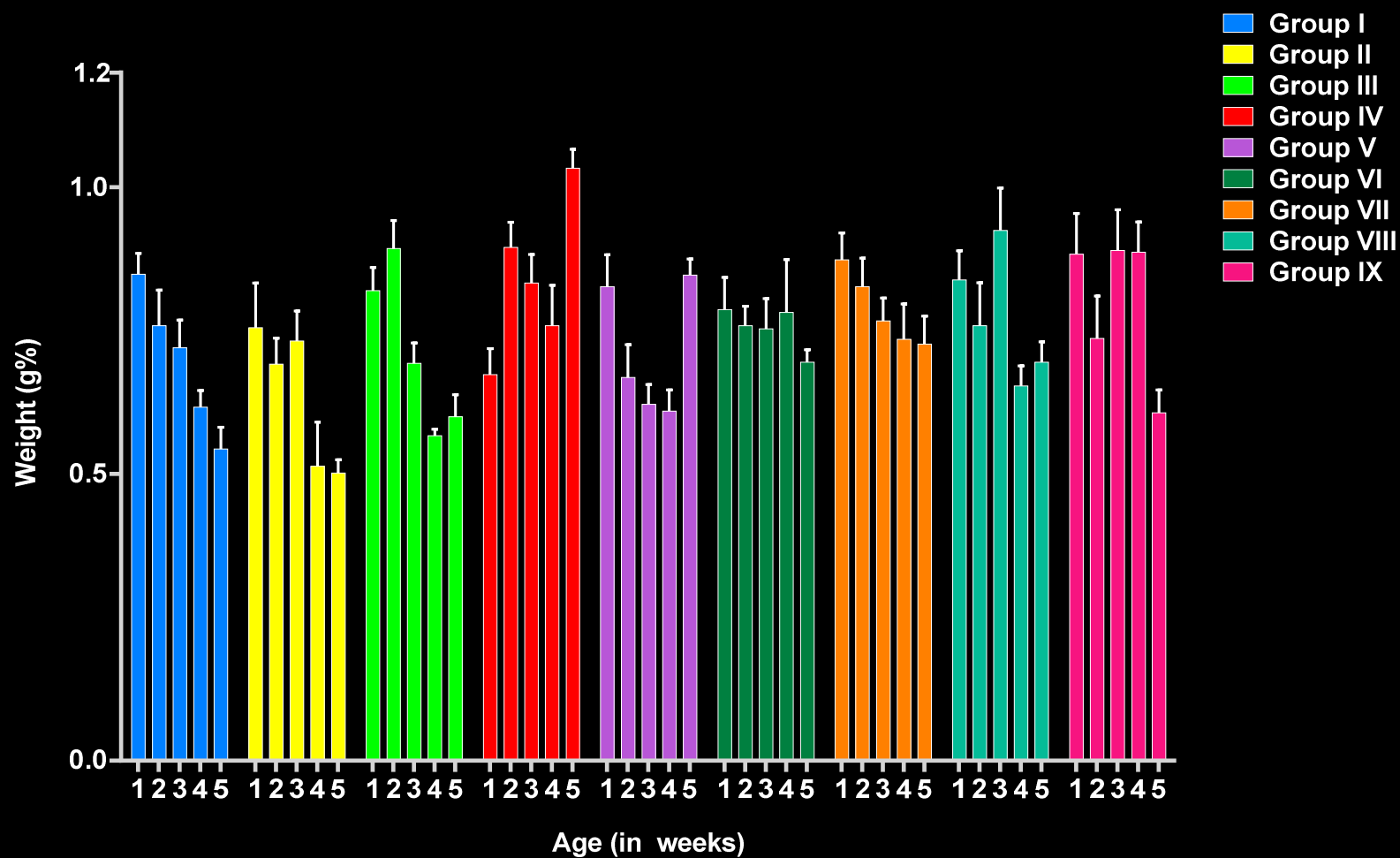
The mean relative weights of heart of Group I to III were almost similar till 35th day of observation. There was significant increase in relative weight of heart in Group IV and V (toxin fed groups) as compared to control groups. However, there was significant

Table 5: Mean ($\pm$ SE) weekly relative weight of heart (g%) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.

Groups	Aflatoxin (ppm)	DAE (mg/kg feed)	Age in weeks				
			1	2	3	4	5
I	0	0	0.85 $\pm$ 0.04 ^{ab}	0.76 $\pm$ 0.07 ^{ab}	0.72 $\pm$ 0.05 ^{ab}	0.62 $\pm$ 0.03 ^{abc}	0.55 $\pm$ 0.04 ^a
II	0	400	0.76 $\pm$ 0.08 ^{ab}	0.7 $\pm$ 0.05 ^a	0.74 $\pm$ 0.06 ^{abc}	0.52 $\pm$ 0.08 ^a	0.51 $\pm$ 0.03 ^a
III	0	800	0.83 $\pm$ 0.04 ^{ab}	0.9 $\pm$ 0.05 ^b	0.7 $\pm$ 0.04 ^{ab}	0.57 $\pm$ 0.02 ^{ab}	0.6 $\pm$ 0.04 ^{ab}
IV	1	0	0.68 $\pm$ 0.05 ^a	0.9 $\pm$ 0.05 ^b	0.84 $\pm$ 0.05 ^{bcd}	0.76 $\pm$ 0.08 ^{cd}	1.04 $\pm$ 0.04 ^c
V	0.5	0	0.83 $\pm$ 0.06 ^{ab}	0.67 $\pm$ 0.06 ^a	0.63 $\pm$ 0.04 ^a	0.61 $\pm$ 0.04 ^{abc}	0.85 $\pm$ 0.03 ^d
VI	1	400	0.79 $\pm$ 0.06 ^{ab}	0.76 $\pm$ 0.04 ^{ab}	0.76 $\pm$ 0.06 ^{abc}	0.79 $\pm$ 0.1 ^{cd}	0.7 $\pm$ 0.03 ^{bc}
VII	1	800	0.88 $\pm$ 0.05 ^b	0.83 $\pm$ 0.05 ^{ab}	0.77 $\pm$ 0.04 ^{abcd}	0.74 $\pm$ 0.07 ^{bcd}	0.73 $\pm$ 0.05 ^c
VIII	0.5	400	0.84 $\pm$ 0.06 ^{ab}	0.76 $\pm$ 0.08 ^{ab}	0.93 $\pm$ 0.08 ^d	0.66 $\pm$ 0.04 ^{abc}	0.7 $\pm$ 0.04 ^{bc}
IX	0.5	800	0.89 $\pm$ 0.08 ^b	0.74 $\pm$ 0.08 ^{ab}	0.89 $\pm$ 0.08 ^{cd}	0.89 $\pm$ 0.06 ^d	0.61 $\pm$ 0.04 ^{ab}

Mean values bearing at least one common superscript within columns did not differ significantly ($P \geq 0.05$)

Fig. 5: Mean ($\pm$ SE) weekly relative weight of heart (g %) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.



decrease in relative heart weight following co-treatment with DAE and aflatoxin (Groups VI to IX) at the end of experiment.

4.2.3.4 PANCREAS

The weekly mean relative weights of pancreas have been presented in Table 6 and graphically represented in Fig. 6.

The mean relative weights of pancreas of Group I to IX were 0.29 ± 0.02 , 0.26 ± 0.02 , 0.26 ± 0.01 , 0.52 ± 0.02 , 0.46 ± 0.06 , 0.43 ± 0.03 , 0.41 ± 0.03 , 0.41 ± 0.05 and 0.39 ± 0.02 g/100g of body weight, respectively on 35th day of age.

The mean relative pancreas weights of control birds (Group I, II and III) were almost similar till 35th day of observation. However, toxin fed birds (Group IV and V) showed significant ($P<0.05$) increase in mean relative weight of pancreas in comparison to control groups on 35th day of observation.

Birds supplemented with DAE in the toxin mixed feed (Groups VI to IX) showed significant ($P<0.05$) decrease in relative pancreas weight in comparison to toxin fed birds (Group IV and V).

4.2.3.5 THYMUS

The weekly mean relative thymus weights of different groups have been presented in Table 7 and expressed graphically in Fig 7. The mean values were 0.78 ± 0.06 , 0.82 ± 0.06 , 0.80 ± 0.08 , 0.26 ± 0.02 , 0.41 ± 0.03 , 0.31 ± 0.02 , 0.41 ± 0.03 , 0.51 ± 0.03 and 0.61 ± 0.03 g/100g of body weight, respectively at the end of the experiment.

Table 6: Mean ($\pm$ SE) weekly relative weight of pancreas (g %) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.

Groups	Aflatoxin (ppm)	DAE (mg/kg feed)	Age in weeks				
			1	2	3	4	5
I	0	0	0.54 $\pm$ 0.07 ^b	0.52 $\pm$ 0.08 ^{ab}	0.53 $\pm$ 0.05 ^{ab}	0.37 $\pm$ 0.02 ^a	0.29 $\pm$ 0.02 ^a
II	0	400	0.66 $\pm$ 0.09 ^b	0.45 $\pm$ 0.03 ^a	0.47 $\pm$ 0.05 ^a	0.39 $\pm$ 0.03 ^{ab}	0.26 $\pm$ 0.02 ^a
III	0	800	0.51 $\pm$ 0.04 ^{ab}	0.54 $\pm$ 0.03 ^{ab}	0.55 $\pm$ 0.06 ^{ab}	0.39 $\pm$ 0.01 ^{ab}	0.26 $\pm$ 0.01 ^a
IV	1	0	0.47 $\pm$ 0.08 ^{ab}	0.79 $\pm$ 0.12 ^c	0.63 $\pm$ 0.08 ^{bc}	0.59 $\pm$ 0.05 ^d	0.52 $\pm$ 0.02 ^c
V	0.5	0	0.33 $\pm$ 0.07 ^a	0.62 $\pm$ 0.07 ^{abc}	0.62 $\pm$ 0.04 ^{abc}	0.50 $\pm$ 0.05 ^{abc}	0.46 $\pm$ 0.06 ^{bc}
VI	1	400	0.57 $\pm$ 0.06 ^b	0.7 $\pm$ 0.04 ^{bc}	0.58 $\pm$ 0.05 ^{ab}	0.48 $\pm$ 0.03 ^{bc}	0.43 $\pm$ 0.03 ^{bc}
VII	1	800	0.49 $\pm$ 0.05 ^a	0.74 $\pm$ 0.09 ^{bc}	0.57 $\pm$ 0.05 ^c	0.5 $\pm$ 0.04 ^c	0.41 $\pm$ 0.03 ^b
VIII	0.5	400	0.47 $\pm$ 0.08 ^{ab}	0.86 $\pm$ 0.13 ^c	0.54 $\pm$ 0.06 ^{bc}	0.46 $\pm$ 0.04 ^{bc}	0.41 $\pm$ 0.05 ^b
IX	0.5	800	0.54 $\pm$ 0.07 ^b	0.73 $\pm$ 0.07 ^{bc}	0.52 $\pm$ 0.05 ^{bc}	0.42 $\pm$ 0.03 ^{bc}	0.39 $\pm$ 0.02 ^b

Mean values bearing at least one common superscript within columns did not differ significantly ($P \geq 0.05$)

Fig. 6: Mean ($\pm$ SE) weekly relative weight of pancreas (g %) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.

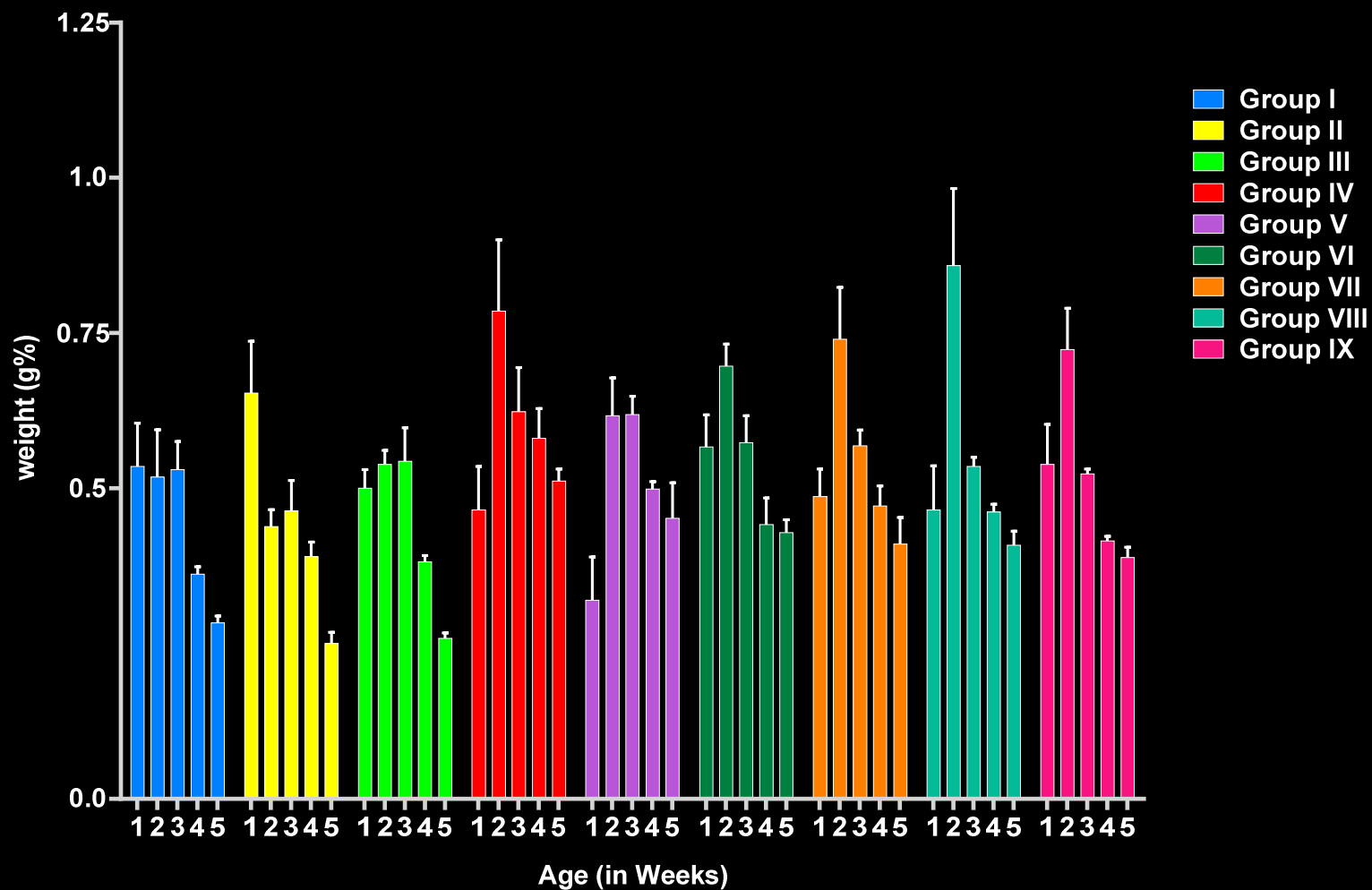
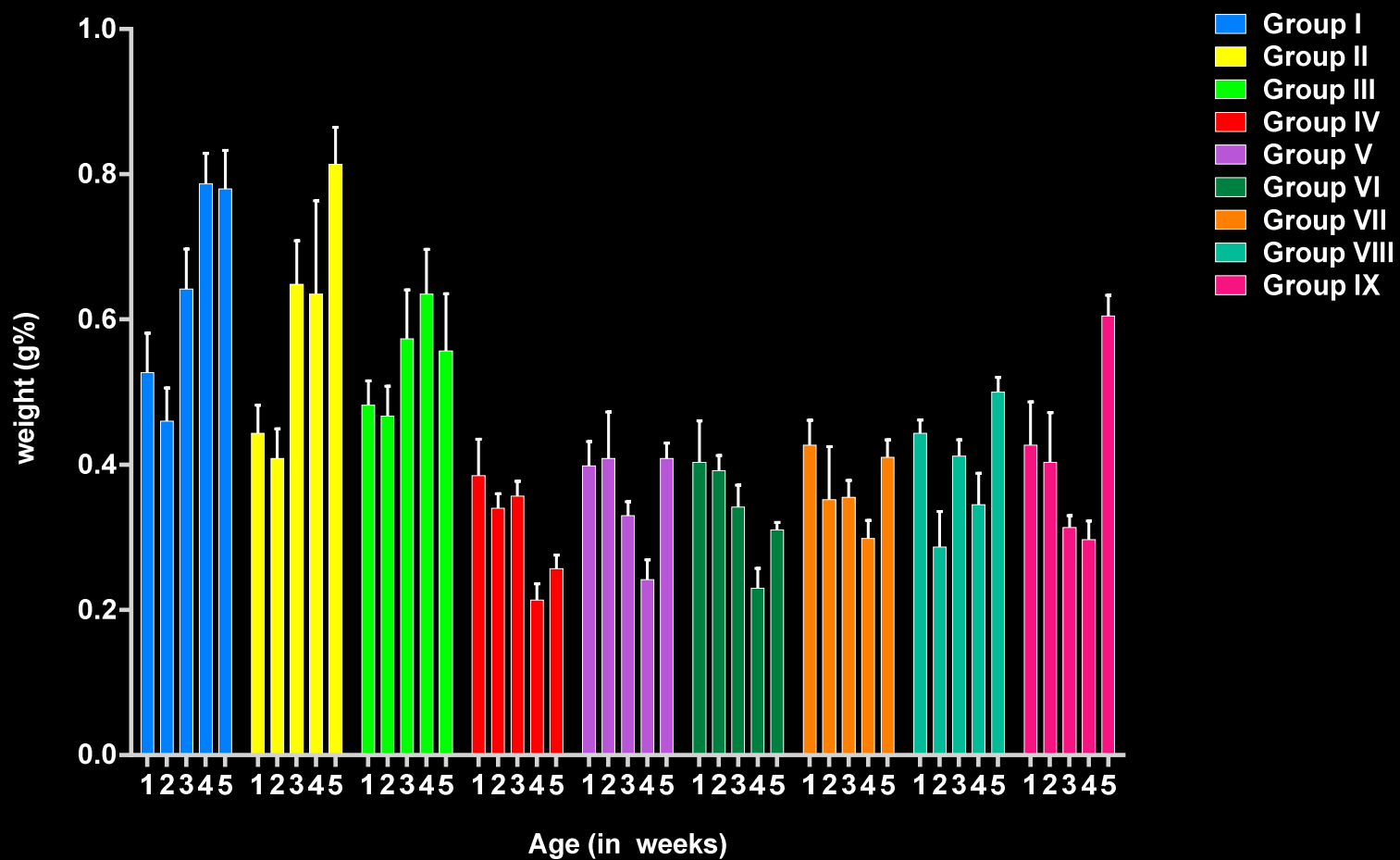


Table 7: Mean ($\pm$ SE) weekly relative weight of thymus (g %) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.

Groups	Aflatoxin (ppm)	DAE (mg/kg feed)	Age in weeks				
			1	2	3	4	5
I	0	0	0.53 $\pm$ 0.06 ^a	0.47 $\pm$ 0.05 ^b	0.65 $\pm$ 0.06 ^b	0.79 $\pm$ 0.05 ^b	0.78 $\pm$ 0.06 ^d
II	0	400	0.45 $\pm$ 0.04 ^a	0.41 $\pm$ 0.05 ^{ab}	0.66 $\pm$ 0.07 ^b	0.64 $\pm$ 0.13 ^b	0.82 $\pm$ 0.06 ^d
III	0	800	0.49 $\pm$ 0.04 ^a	0.47 $\pm$ 0.05 ^b	0.58 $\pm$ 0.07 ^b	0.64 $\pm$ 0.07 ^b	0.80 $\pm$ 0.08 ^c
IV	1	0	0.39 $\pm$ 0.05 ^a	0.35 $\pm$ 0.03 ^{ab}	0.36 $\pm$ 0.03 ^a	0.22 $\pm$ 0.03 ^a	0.26 $\pm$ 0.02 ^a
V	0.5	0	0.4 $\pm$ 0.04 ^a	0.41 $\pm$ 0.07 ^{ab}	0.34 $\pm$ 0.02 ^a	0.25 $\pm$ 0.03 ^a	0.41 $\pm$ 0.03 ^b
VI	1	400	0.41 $\pm$ 0.06 ^a	0.4 $\pm$ 0.03 ^{ab}	0.35 $\pm$ 0.03 ^a	0.24 $\pm$ 0.03 ^a	0.31 $\pm$ 0.02 ^a
VII	1	800	0.43 $\pm$ 0.04 ^a	0.35 $\pm$ 0.08 ^{ab}	0.36 $\pm$ 0.03 ^a	0.3 $\pm$ 0.03 ^a	0.41 $\pm$ 0.03 ^b
VIII	0.5	400	0.45 $\pm$ 0.02 ^a	0.29 $\pm$ 0.05 ^a	0.42 $\pm$ 0.03 ^a	0.35 $\pm$ 0.05 ^a	0.51 $\pm$ 0.03 ^{bc}
IX	0.5	800	0.43 $\pm$ 0.07 ^a	0.41 $\pm$ 0.07 ^{ab}	0.32 $\pm$ 0.02 ^a	0.3 $\pm$ 0.03 ^a	0.61 $\pm$ 0.03 ^c

Mean values bearing at least one common superscript within columns did not differ significantly ($P \geq 0.05$)

Fig. 7: Mean ($\pm$ SE) weekly relative weight of thymus (g %) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.



The relative mean thymus weight of Group I, II and III were almost similar at the end of study period, however there was a significant decrease in relative mean weight of thymus in Group IV and V (only toxin fed groups) as compared to control groups. There was significant ($P<0.05$) increase in relative mean weight of thymus in Group VI, VII, VIII and IX (combined treatment with aflatoxin and toxin binder) in comparison to Group IV and V. The improvement in the relative weight of thymus was significantly ($P<0.05$) higher in the Groups VII and IX (800 mg of DAE/kg feed + aflatoxin) in comparison to Groups VI and VIII fed with 400 mg of DAE/kg feed along with toxin.

4.2.3.6 SPLEEN

Weekly mean relative weights of spleen of different groups are summarized in Table 8 and Fig 8. The mean spleen values of Group I to IX were 0.26 ± 0.02 , 0.23 ± 0.02 , 0.23 ± 0.02 , 0.12 ± 0.01 , 0.16 ± 0.01 , 0.21 ± 0.02 , 0.2 ± 0.02 , 0.22 ± 0.02 and 0.23 ± 0.02 g/100g of body weight, respectively at 35th days of experiment.

The mean relative spleen weights of Group I, II and III were not significantly different during entire period of the experiment. A significant ($P\leq0.05$) decrease in relative spleen weight was seen in toxin fed birds (Group IV and V) from 14th day onwards and continued till the end of experiment (35th day) in comparison to the control birds (Groups I, II and III).

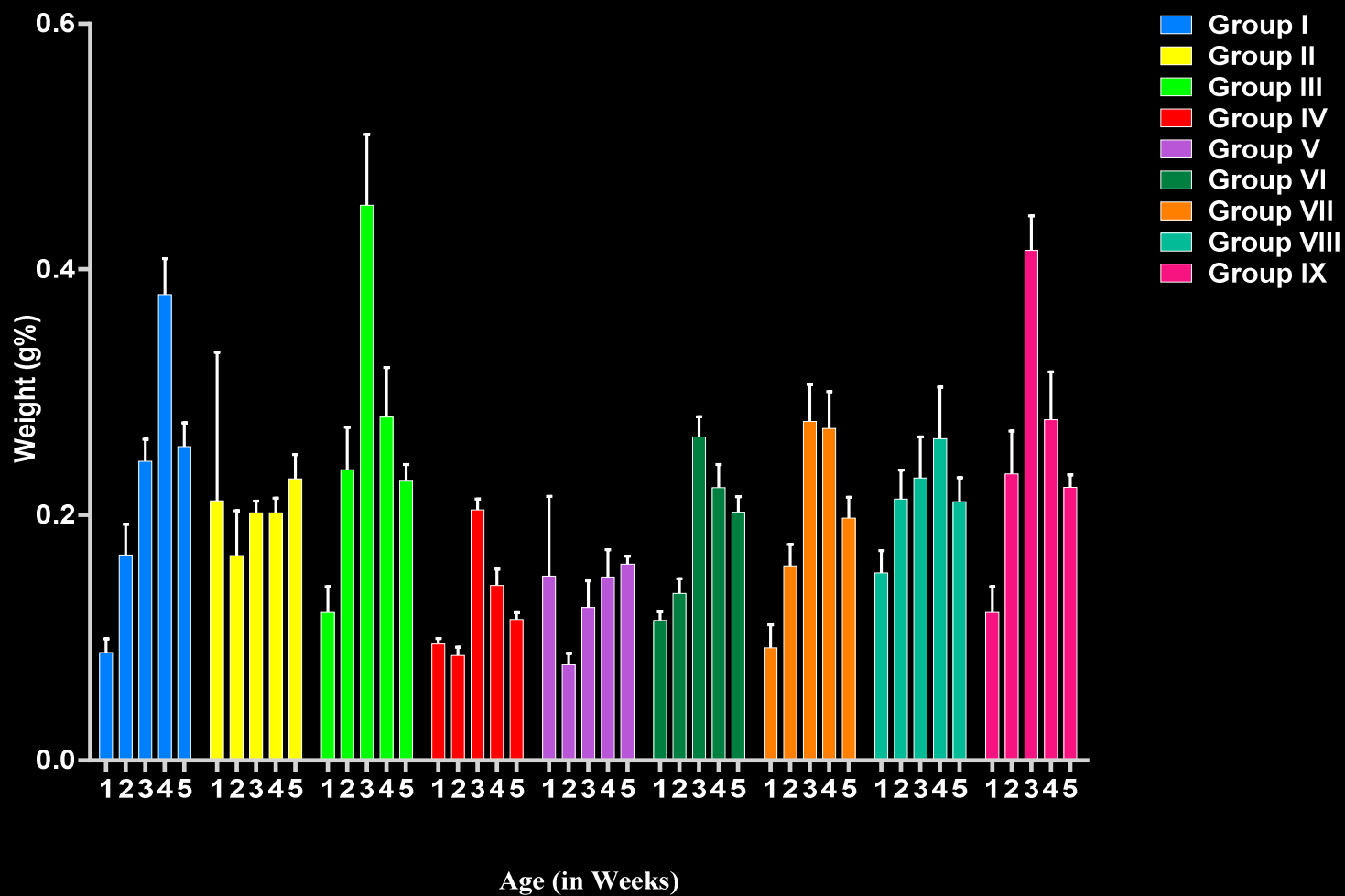
The spleen, in co-treatment groups (VI to IX) fed with DAE and aflatoxin showed significantly higher relative weight in comparison to birds fed with only toxin (Group IV and V). However, the birds supplemented with 800 mg/kg feed (Groups

Table 8: Mean ($\pm$ SE) weekly relative weight of spleen (g %) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.

Groups	Aflatoxin (ppm)	DAE (mg/kg feed)	Age in weeks				
			1	2	3	4	5
I	0	0	0.09 $\pm$ 0.02 ^a	0.17 $\pm$ 0.03 ^{cd}	0.25 $\pm$ 0.02 ^b	0.38 $\pm$ 0.03 ^b	0.26 $\pm$ 0.02 ^d
II	0	400	0.22 $\pm$ 0.13 ^a	0.17 $\pm$ 0.04 ^{cd}	0.21 $\pm$ 0.01 ^{ab}	0.21 $\pm$ 0.02 ^{ab}	0.23 $\pm$ 0.02 ^{cd}
III	0	800	0.13 $\pm$ 0.03 ^a	0.24 $\pm$ 0.04 ^d	0.46 $\pm$ 0.06 ^c	0.28 $\pm$ 0.05 ^b	0.23 $\pm$ 0.02 ^{cd}
IV	1	0	0.1 $\pm$ 0.01 ^a	0.09 $\pm$ 0.01 ^{ab}	0.21 $\pm$ 0.01 ^{ab}	0.15 $\pm$ 0.02 ^a	0.12 $\pm$ 0.01 ^a
V	0.5	0	0.16 $\pm$ 0.07 ^a	0.08 $\pm$ 0.01 ^a	0.12 $\pm$ 0.03 ^a	0.15 $\pm$ 0.03 ^a	0.16 $\pm$ 0.01 ^b
VI	1	400	0.12 $\pm$ 0.01 ^a	0.14 $\pm$ 0.02 ^{abc}	0.27 $\pm$ 0.02 ^b	0.23 $\pm$ 0.02 ^{ab}	0.21 $\pm$ 0.02 ^{bc}
VII	1	800	0.1 $\pm$ 0.02 ^a	0.16 $\pm$ 0.02 ^{bcd}	0.28 $\pm$ 0.04 ^b	0.28 $\pm$ 0.04 ^b	0.2 $\pm$ 0.02 ^{bc}
VIII	0.5	400	0.16 $\pm$ 0.02 ^a	0.22 $\pm$ 0.03 ^{cd}	0.24 $\pm$ 0.04 ^b	0.27 $\pm$ 0.05 ^b	0.22 $\pm$ 0.02 ^{cd}
IX	0.5	800	0.13 $\pm$ 0.03 ^a	0.24 $\pm$ 0.04 ^d	0.42 $\pm$ 0.03 ^c	0.28 $\pm$ 0.04 ^b	0.23 $\pm$ 0.02 ^{cd}

Mean values bearing at least one common superscript within columns did not differ significantly ($P \geq 0.05$)

Fig. 8: Mean ($\pm$ SE) weekly relative weight of spleen (g %) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.



VII and IX) to the aflatoxin mixed feed showed significantly ($P<0.05$) higher relative spleen weight in comparison to Groups VI and VIII fed with 400 mg DAE/kg feed along with toxin.

4.2.3.7. BURSA OF FABRICIUS

The weekly mean relative weights of bursa of Fabricius have been presented in Table 9 and graphically represented in Fig 9. The mean relative weights of bursa of Group I to IX were 0.23 ± 0.02 , 0.24 ± 0.03 , 0.26 ± 0.01 , 0.08 ± 0.01 , 0.09 ± 0.01 , 0.10 ± 0.01 , 0.12 ± 0.01 , 0.11 ± 0.01 and 0.14 ± 0.01 g/100g of body weight, respectively on 35th day of age.

The mean relative weights of bursa of Fabricius of Group I to IX were almost uniform on 7th day of experiment. The mean relative weights of control birds (Group I, II and III) were almost similar till 35th day of observation. However, toxin fed birds (Group IV and V) showed significant ($P<0.05$) decrease in mean relative weight of bursa in comparison to control groups on 35th day of observation.

Birds supplemented with DAE in the toxin mixed feed (Group VI to IX) showed significant ($P<0.05$) improvement in relative bursal weights in comparison to toxin fed birds (Group IV and V).

4.2.4. SERUM BIOCHEMISTRY

4.2.4.1 SERUM ASPARTATE AMINOTRANSFERASE (AST)

The weekly mean serum aspartate aminotransferase (AST) of different groups have been depicted in Table 10 and Fig. 10. The mean serum AST values of Group I to

Table 9: Mean ($\pm$ SE) weekly relative weight of bursa of Fabricius (g %) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.

Groups	Aflatoxin (ppm)	DAE (mg/kg feed)	Age in weeks				
			1	2	3	4	5
I	0	0	0.22 $\pm$ 0.04 ^a	0.24 $\pm$ 0.01 ^a	0.29 $\pm$ 0.03 ^a	0.27 $\pm$ 0.02 ^c	0.23 $\pm$ 0.02 ^d
II	0	400	0.2 $\pm$ 0.02 ^a	0.22 $\pm$ 0.03 ^a	0.22 $\pm$ 0.02 ^a	0.18 $\pm$ 0.02 ^b	0.24 $\pm$ 0.03 ^d
III	0	800	0.2 $\pm$ 0.02 ^a	0.25 $\pm$ 0.02 ^a	0.25 $\pm$ 0.02 ^a	0.28 $\pm$ 0.04 ^c	0.26 $\pm$ 0.01 ^d
IV	1	0	0.25 $\pm$ 0.04 ^a	0.26 $\pm$ 0.02 ^a	0.25 $\pm$ 0.04 ^a	0.09 $\pm$ 0.01 ^a	0.08 $\pm$ 0.01 ^a
V	0.5	0	0.25 $\pm$ 0.03 ^a	0.28 $\pm$ 0.02 ^a	0.25 $\pm$ 0.02 ^a	0.14 $\pm$ 0.01 ^b	0.09 $\pm$ 0.01 ^{ab}
VI	1	400	0.27 $\pm$ 0.05 ^a	0.26 $\pm$ 0.04 ^a	0.28 $\pm$ 0.03 ^a	0.15 $\pm$ 0.03 ^b	0.10 $\pm$ 0.01 ^{ab}
VII	1	800	0.26 $\pm$ 0.02 ^a	0.24 $\pm$ 0.04 ^a	0.26 $\pm$ 0.02 ^a	0.15 $\pm$ 0.01 ^a	0.12 $\pm$ 0.01 ^{bc}
VIII	0.5	400	0.21 $\pm$ 0.03 ^a	0.29 $\pm$ 0.03 ^a	0.24 $\pm$ 0.02 ^a	0.14 $\pm$ 0.02 ^b	0.11 $\pm$ 0.01 ^{ab}
IX	0.5	800	0.25 $\pm$ 0.04 ^a	0.26 $\pm$ 0.02 ^a	0.25 $\pm$ 0.04	0.10 $\pm$ 0.01 ^a	0.14 $\pm$ 0.01 ^c

Mean values bearing at least one common superscript within columns did not differ significantly ($P \geq 0.05$)

Fig. 9: Mean ($\pm$ SE) weekly relative weight of bursa of Fabricius (g %) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.

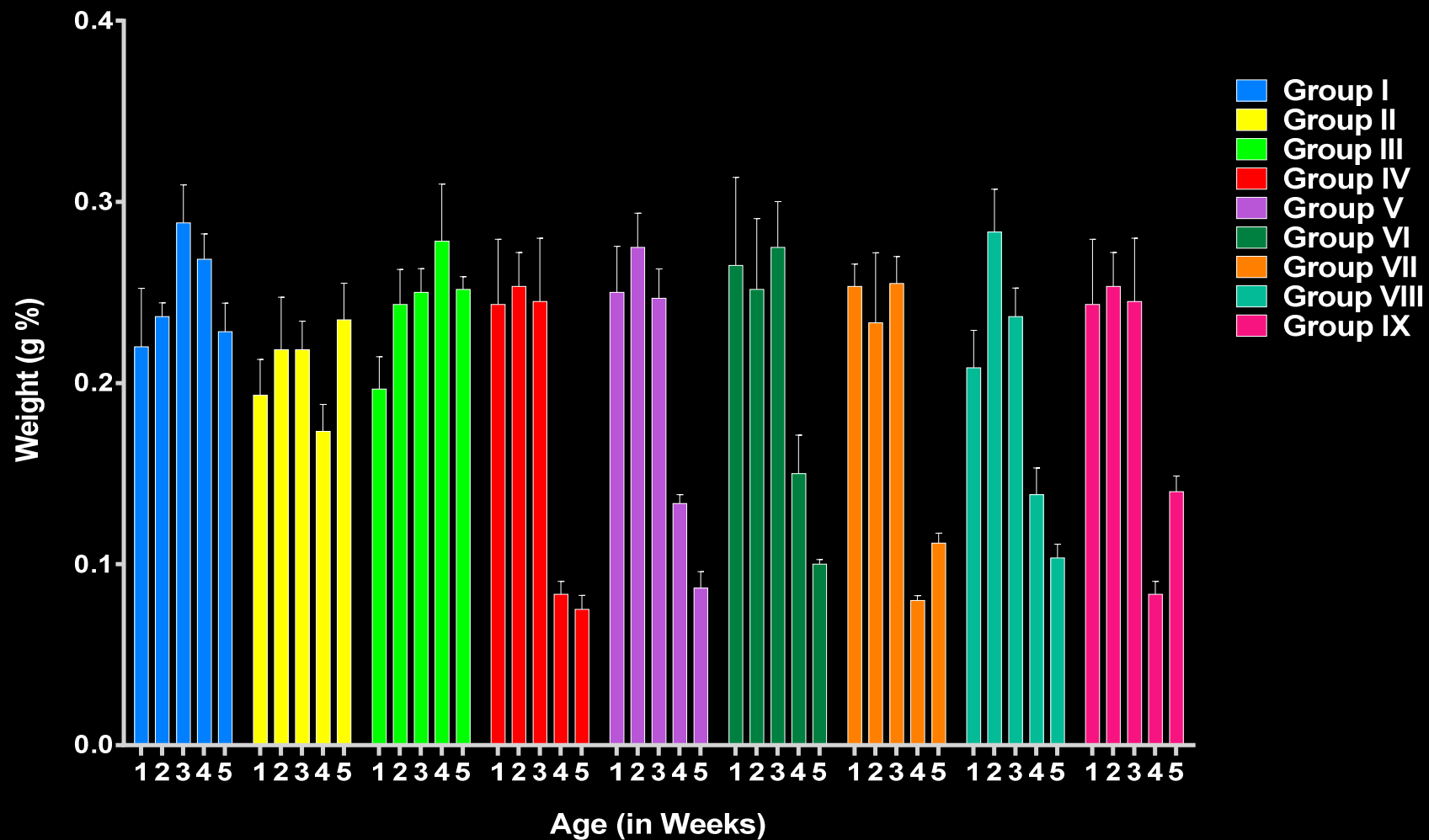
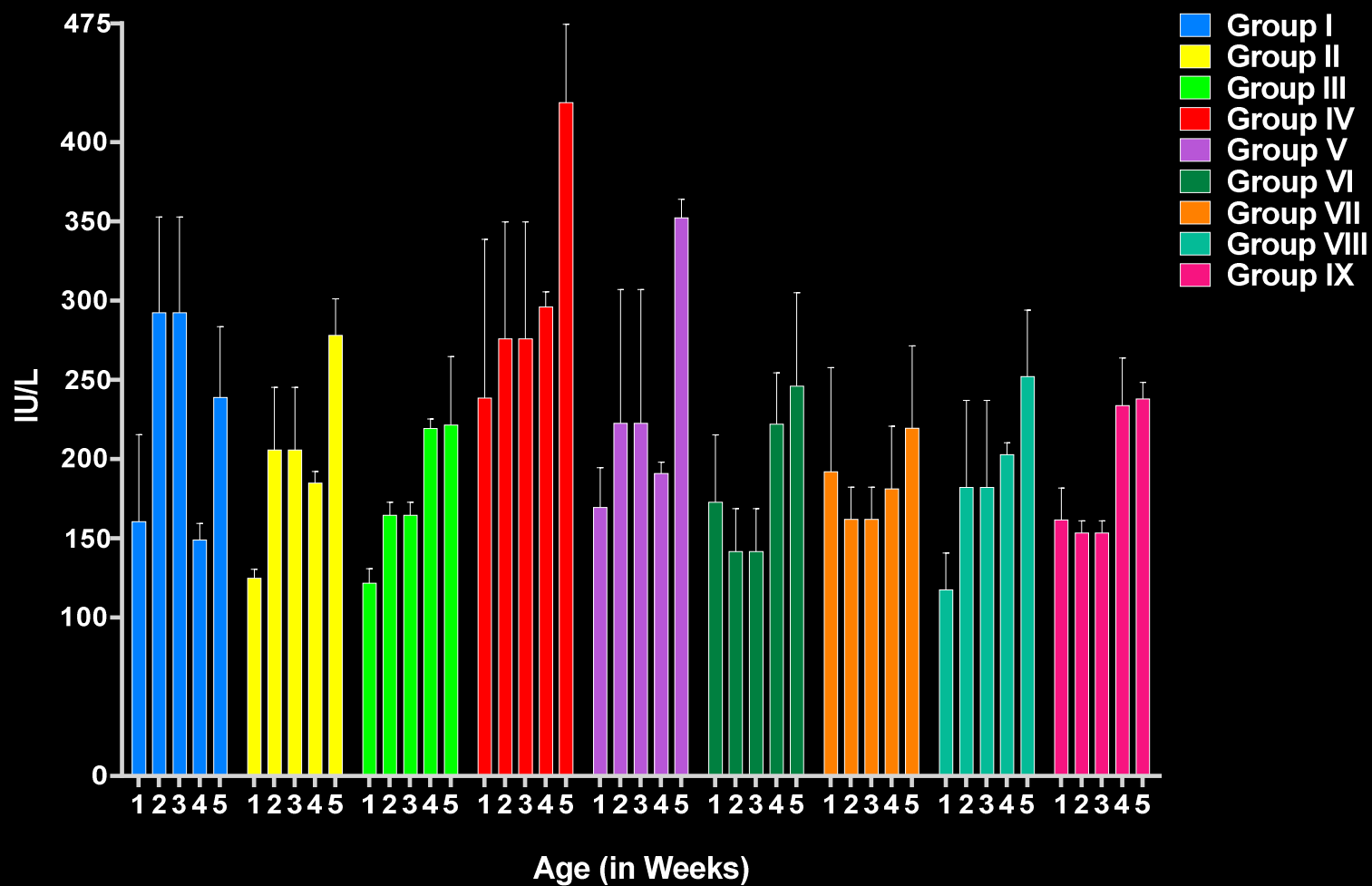


Table 10: Mean ($\pm$ SE) weekly serum AST values (IU/L) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.

Groups	Aflatoxin (ppm)	DAE (mg/kg feed)	Age in weeks				
			1	2	3	4	5
I	0	0	160.4 $\pm$ 55.02 ^a	292.34 $\pm$ 60.46 ^a	292.34 $\pm$ 60.46 ^a	148.97 $\pm$ 10.41 ^a	238.74 $\pm$ 44.77 ^a
II	0	400	124.67 $\pm$ 5.69 ^a	205.8 $\pm$ 39.62 ^a	205.8 $\pm$ 39.62 ^a	184.87 $\pm$ 7.4 ^{ab}	278.04 $\pm$ 22.98 ^a
III	0	800	121.74 $\pm$ 9.13 ^a	164.5 $\pm$ 8.3 ^a	164.5 $\pm$ 8.3 ^a	219.2 $\pm$ 6.21 ^b	221.54 $\pm$ 43.05 ^a
IV	1	0	238.54 $\pm$ 100.09 ^a	275.97 $\pm$ 73.7 ^a	275.97 $\pm$ 73.7 ^a	296.1 $\pm$ 9.56 ^c	424.77 $\pm$ 49.64 ^b
V	0.5	0	169.54 $\pm$ 24.99 ^a	222.5 $\pm$ 84.59 ^a	222.5 $\pm$ 84.59 ^a	190.87 $\pm$ 7.19 ^{ab}	352.27 $\pm$ 11.76 ^b
VI	1	400	172.7 $\pm$ 42.57 ^a	141.67 $\pm$ 26.96 ^a	141.67 $\pm$ 26.96 ^a	222.07 $\pm$ 32.3 ^b	246.1 $\pm$ 58.98 ^a
VIII	1	800	191.97 $\pm$ 65.62 ^a	161.94 $\pm$ 20.3 ^a	161.94 $\pm$ 20.3 ^a	181.24 $\pm$ 39.56 ^{ab}	219.4 $\pm$ 52.08 ^a
VII	0.5	400	117.47 $\pm$ 23.28 ^a	182.1 $\pm$ 54.98 ^a	182.1 $\pm$ 54.98 ^a	202.8 $\pm$ 7.48 ^{ab}	252.1 $\pm$ 41.89 ^a
IX	0.5	800	161.5 $\pm$ 20.17 ^a	153.34 $\pm$ 7.82 ^a	153.34 $\pm$ 7.82 ^a	233.67 $\pm$ 30.12 ^b	237.97 $\pm$ 10.37 ^a

Mean values bearing at least one common superscript within columns did not differ significantly ($P \geq 0.05$)

Fig. 10: Mean ($\pm$ SE) weekly serum AST values (IU/L) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.



IX birds were 238.74 ± 44.77 , 278.04 ± 22.98 , 221.54 ± 43.05 , 424.77 ± 49.64 , 352.27 ± 11.76 , 246.1 ± 58.98 , 219.4 ± 52.08 , 252.1 ± 41.89 and 237.97 ± 10.37 IU/L, respectively at the end of 35th day.

The mean serum AST values in birds fed with toxin (Group IV and V) showed a significant increase in comparison to control group (I, II and III) from first week onwards and continued till the end of the experiment.

However, in co-treatment groups (VI to IX) significant ($P \leq 0.05$) decrease in serum AST level was recorded in comparison to toxin fed birds (Group IV and V) at the end of experiment and the values were comparable with the control birds.

4.2.4.2 SERUM ALANINE AMINOTRANSFERASE (ALT)

The weekly mean serum alanine aminotransferase (ALT) values in birds with different dietary treatments have been summarized in Table 11 and graphically represented in Fig. 11. The mean serum ALT values of Group I to IX birds were 10.27 ± 0.73 , 9.27 ± 0.77 , 10.77 ± 1.3 , 15.97 ± 1.16 , 10.94 ± 0.07 , 11.24 ± 1.14 , 12.47 ± 0.79 , 10.17 ± 1.3 and 12.77 ± 0.93 IU/L, respectively at the end of 35th day of experiment.

The mean serum ALT values of the control Groups I, II and III were almost similar at the end of experiment, however the serum ALT values in toxin fed birds (Group IV and V) increased significantly ($P \leq 0.05$) in comparison to control birds.

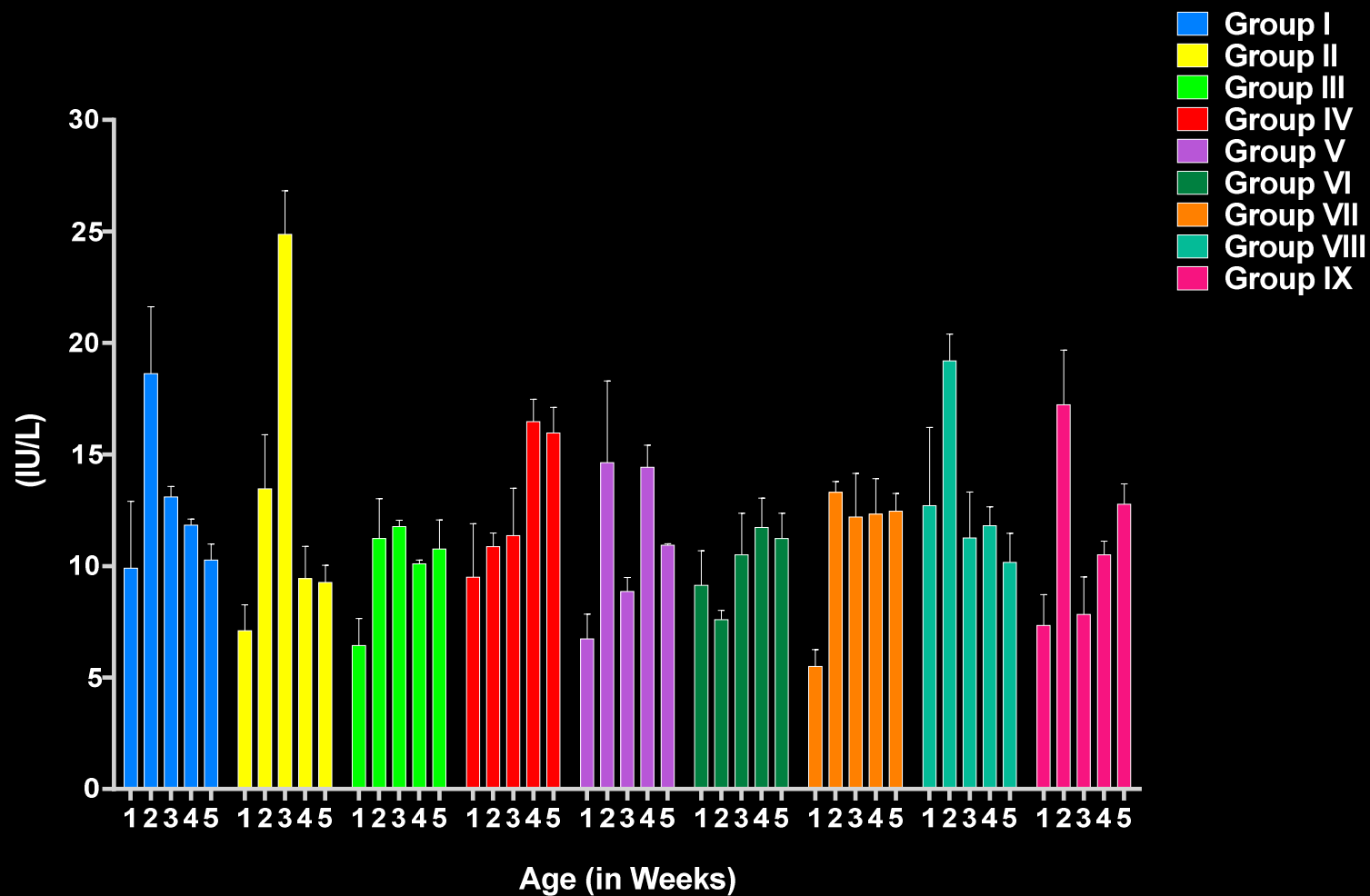
Supplementation with diatomaceous earth to aflatoxin mixed feed could reduce the effect of aflatoxin as the serum ALT values in Group VI to IX decreased significantly ($P \leq 0.05$) in comparison to toxin fed birds (Group IV and V).

Table 11: Mean ($\pm$ SE) weekly serum ALT (IU/L) values of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.

Groups	Aflatoxin (ppm)	DAE (mg/kg feed)	Age in weeks				
			1	2	3	4	5
I	0	0	9.9 $\pm$ 3.01 ^{ab}	18.64 $\pm$ 3 ^c	13.1 $\pm$ 0.47 ^a	11.84 $\pm$ 0.28 ^{ab}	10.27 $\pm$ 0.73 ^{ab}
II	0	400	7.1 $\pm$ 1.16 ^{ab}	13.47 $\pm$ 2.43 ^{abc}	24.87 $\pm$ 1.96 ^b	9.44 $\pm$ 1.46 ^a	9.27 $\pm$ 0.77 ^a
III	0	800	6.44 $\pm$ 1.22 ^{ab}	11.24 $\pm$ 1.79 ^{ab}	11.77 $\pm$ 0.29 ^a	10.1 $\pm$ 0.18 ^a	10.77 $\pm$ 1.3 ^{ab}
IV	1	0	9.5 $\pm$ 2.41 ^{ab}	10.87 $\pm$ 0.62 ^a	11.37 $\pm$ 2.13 ^a	16.47 $\pm$ 1.03 ^c	15.97 $\pm$ 1.16 ^c
V	0.5	0	6.74 $\pm$ 1.12 ^{ab}	14.64 $\pm$ 3.67 ^{bc}	8.87 $\pm$ 0.63 ^a	14.44 $\pm$ 0.98 ^{bc}	10.94 $\pm$ 0.07 ^{ab}
VI	1	400	9.14 $\pm$ 1.55 ^{ab}	7.6 $\pm$ 0.41 ^a	10.5 $\pm$ 1.87 ^a	11.74 $\pm$ 1.32 ^{ab}	11.24 $\pm$ 1.14 ^{ab}
VII	1	800	5.5 $\pm$ 0.76 ^a	13.3 $\pm$ 0.5 ^{abc}	12.2 $\pm$ 1.96 ^a	12.34 $\pm$ 1.58 ^{ab}	12.47 $\pm$ 0.79 ^{ab}
VIII	0.5	400	12.7 $\pm$ 3.53 ^b	19.2 $\pm$ 1.2 ^c	11.27 $\pm$ 2.07 ^a	11.8 $\pm$ 0.86 ^{ab}	10.17 $\pm$ 1.3 ^{ab}
IX	0.5	800	7.34 $\pm$ 1.39 ^{ab}	17.24 $\pm$ 2.46 ^{bc}	7.84 $\pm$ 1.68 ^a	10.5 $\pm$ 0.62 ^a	12.77 $\pm$ 0.93 ^b

Mean values bearing at least one common superscript within columns did not differ significantly ($P \geq 0.05$)

Fig. 11: Mean ($\pm$ SE) weekly serum ALT (IU/L) values of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.



4.2.4.3 SERUM ALKALINE PHOSPHATASE (ALP)

Mean ($\pm$ SE) weekly serum alkaline phosphatase (ALP) values of broiler birds for different dietary treatment groups at weekly intervals are summarized in Table 12 and graphically represented in Fig. 12.

The mean serum ALT values of Group I to IX birds were 946.14 ± 131.5 , 1270.57 ± 60.54 , 1014.17 ± 60.12 , 1921.3 ± 13.37 , 1549.04 ± 7.82 , 1618.34 ± 25.18 , 1354.6 ± 60.82 , 1210.24 ± 57.1 and 1096.97 ± 36.09 IU/L, respectively at the end of 35th day.

The mean serum ALP values of toxin fed birds (Group IV and V) showed a significant ($P \leq 0.05$) increase in comparison to control birds (Group I, II and III) at the end of fifth week. In the combined treatment groups fed with DAE and aflatoxin (Group VI to IX) significant ($P \leq 0.05$) decrease in mean ALP values were observed in comparison with that of toxin fed birds (Group IV and V).

4.2.4.4 TOTAL SERUM PROTEIN

The weekly mean serum total protein levels in different groups have been recorded in Table 13 and Fig.13. The mean Total serum proteins values of Group I to IX birds were 3.84 ± 0.04 , 3.8 ± 0.06 , 3.54 ± 0.13 , 2.2 ± 0.16 , 2.9 ± 0.18 , 2.94 ± 0.15 , 3.12 ± 0.04 , 3.17 ± 0.04 and 3.3 ± 0.06 g/dl, respectively at the end of 35th day of experiment.

The mean serum total protein of Group I to III did not alter significantly throughout the period of experiment. The mean serum total protein of toxin fed groups (IV and V) altered significantly ($P < 0.05$) in comparison with control groups (I, II and III) from 7th day onwards and continued till the end of experiment. However,

Table 12: Mean ($\pm$ SE) weekly serum ALP values (IU/L) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.

Groups	Aflatoxin (ppm)	DAE (mg/kg feed)	Age in weeks				
			1	2	3	4	5
I	0	0	603.4 $\pm$ 85.35 ^a	861 $\pm$ 30.92 ^a	965.47 $\pm$ 39.07 ^a	1038.7 $\pm$ 75.63 ^a	946.14 $\pm$ 131.5 ^a
II	0	400	626.4 $\pm$ 36.29 ^a	862.9 $\pm$ 60.68 ^a	889.14 $\pm$ 62.88 ^a	1070.4 $\pm$ 65.14 ^a	1270.57 $\pm$ 60.54 ^{bc}
III	0	800	609.37 $\pm$ 26.22 ^a	902.54 $\pm$ 43.26 ^{ab}	956.97 $\pm$ 30.19 ^a	1029.87 $\pm$ 38.31 ^a	1014.17 $\pm$ 60.12 ^a
IV	1	0	759 $\pm$ 57.3a	1028.04 $\pm$ 24.71 ^c	1053.27 $\pm$ 30.31 ^a	1630.37 $\pm$ 62.31 ^d	1921.3 $\pm$ 13.37 ^e
V	0.5	0	688.6 $\pm$ 62.24 ^a	986.47 $\pm$ 4.09 ^{bc}	992.27 $\pm$ 65.32 ^a	1371.04 $\pm$ 62.69 ^{bc}	1549.04 $\pm$ 7.82 ^d
VI	1	400	656.64 $\pm$ 27.33 ^a	960.14 $\pm$ 41.93 ^{abc}	1033.07 $\pm$ 44.08 ^a	1487.07 $\pm$ 74.74 ^{cd}	1618.34 $\pm$ 25.18 ^d
VII	1	800	628.9 $\pm$ 45.44 ^a	1001.9 $\pm$ 1.74 ^{bc}	1072.9 $\pm$ 81.27 ^a	1266.4 $\pm$ 30.01 ^b	1354.6 $\pm$ 60.82 ^c
VIII	0.5	400	639.1 $\pm$ 26.85 ^a	979.2 $\pm$ 11.21 ^{bc}	985.87 $\pm$ 33.38 ^a	1089.17 $\pm$ 47.49 ^a	1210.24 $\pm$ 57.1 ^{bc}
IX	0.5	800	653 $\pm$ 32.61 ^a	999.87 $\pm$ 1.54 ^{bc}	1049.54 $\pm$ 48.21 ^a	1045 $\pm$ 41.49 ^a	1096.97 $\pm$ 36.09 ^{ab}

Mean values bearing at least one common superscript within columns did not differ significantly ($P \geq 0.05$)

Fig. 12: Mean ($\pm$ SE) weekly serum ALP values (IU/L) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.

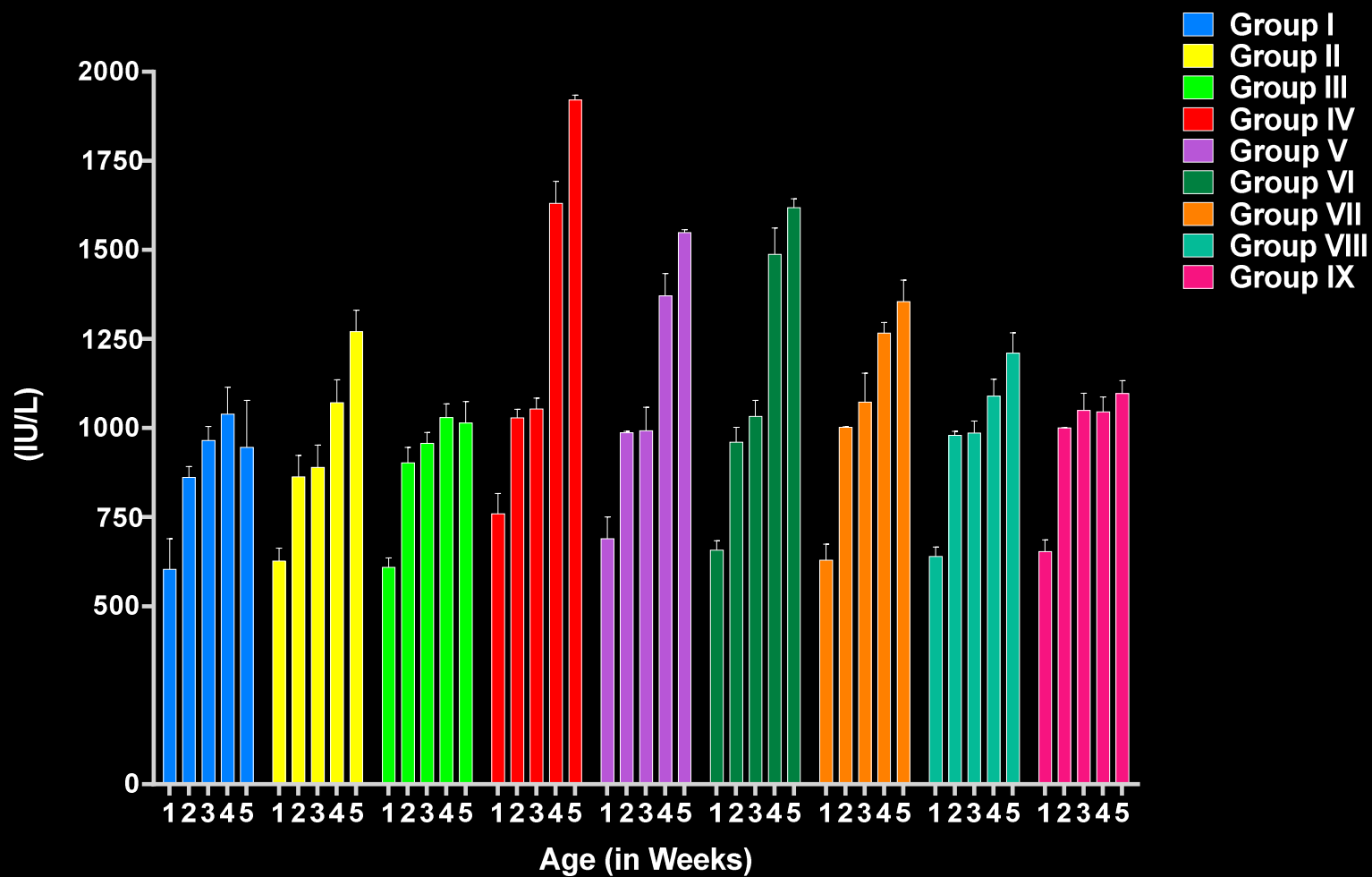
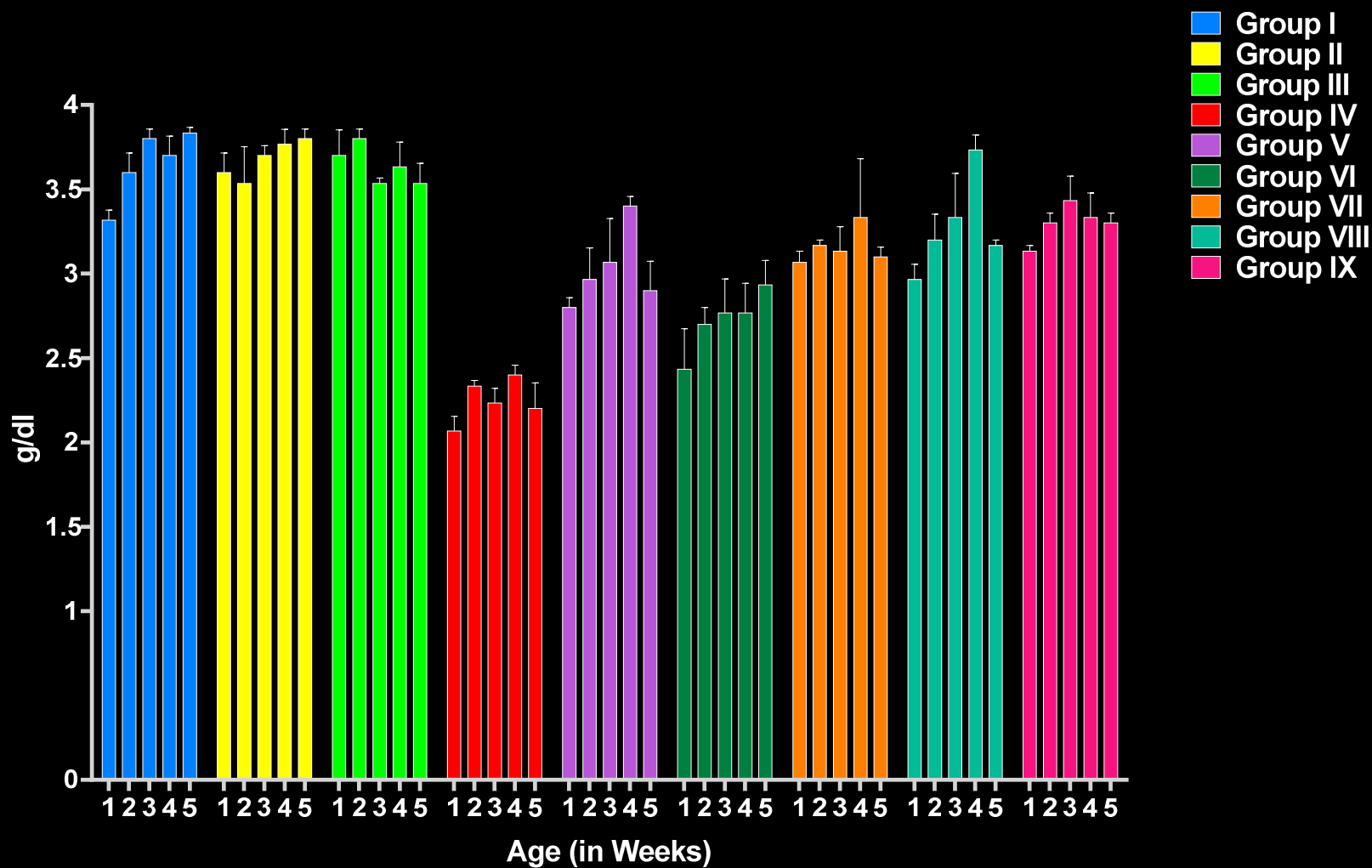


Table 13: Mean ($\pm$ SE) weekly serum total protein values (g/dl) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.

Groups	Aflatoxin (ppm)	DAE (mg/kg feed)	Age in weeks				
			1	2	3	4	5
I	0	0	3.32 $\pm$ 0.07 ^{de}	3.6 $\pm$ 0.12 ^{ef}	3.8 $\pm$ 0.06 ^d	3.7 $\pm$ 0.12 ^b	3.84 $\pm$ 0.04 ^e
II	0	400	3.6 $\pm$ 0.12 ^{ef}	3.54 $\pm$ 0.22 ^{def}	3.7 $\pm$ 0.06 ^d	3.77 $\pm$ 0.09 ^b	3.8 $\pm$ 0.06 ^e
III	0	800	3.7 $\pm$ 0.16 ^f	3.8 $\pm$ 0.06 ^f	3.54 $\pm$ 0.04 ^{cd}	3.64 $\pm$ 0.15 ^b	3.54 $\pm$ 0.13 ^{de}
IV	1	0	2.07 $\pm$ 0.09 ^a	2.34 $\pm$ 0.04 ^a	2.24 $\pm$ 0.09 ^a	2.4 $\pm$ 0.06 ^a	2.2 $\pm$ 0.16 ^a
V	0.5	0	2.8 $\pm$ 0.06 ^c	2.97 $\pm$ 0.19 ^{bc}	3.07 $\pm$ 0.27 ^{bc}	3.4 $\pm$ 0.06 ^b	2.9 $\pm$ 0.18 ^b
VI	1	400	2.44 $\pm$ 0.25 ^b	2.7 $\pm$ 0.1 ^{ab}	2.77 $\pm$ 0.21 ^b	2.77 $\pm$ 0.18 ^a	2.94 $\pm$ 0.15 ^b
VII	1	800	3.07 $\pm$ 0.07 ^{cd}	3.17 $\pm$ 0.04 ^{cd}	3.14 $\pm$ 0.15 ^{bc}	3.34 $\pm$ 0.35 ^b	3.12 $\pm$ 0.04 ^b
VIII	0.5	400	2.97 $\pm$ 0.09 ^{cd}	3.2 $\pm$ 0.16 ^{cd}	3.34 $\pm$ 0.27 ^{cd}	3.74 $\pm$ 0.09 ^b	3.17 $\pm$ 0.04 ^{bc}
IX	0.5	800	3.14 $\pm$ 0.04 ^{cd}	3.3 $\pm$ 0.06 ^{cde}	3.44 $\pm$ 0.15 ^{cd}	3.34 $\pm$ 0.15 ^b	3.3 $\pm$ 0.06 ^{bcd}

Mean values bearing at least one common superscript within columns did not differ significantly ($P \geq 0.05$)

Fig. 13: Mean ($\pm$ SE) weekly serum total proteins values (g/dl) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.



with addition of toxin binder (DAE) to AF contaminated feed, birds of Group VI to IX showed significant ($P<0.05$) increase in total protein as compared to Group IV and V (toxin fed group) on 35th day of observation. The improvement in the serum total protein was higher in the Group VII and IX (800 mg DAE /kg feed + AF) in comparison to Group VI and VIII fed with 400 mg DAE/kg feed along with toxin.

4.2.4.5 SERUM ALBUMIN

The weekly mean serum albumin values in the birds of Group I to IX have been presented in Table 14 and the same have been depicted in Fig.14. The mean serum albumin value of Group I to IX at the end of fifth week were 1.5 ± 0.16 , 1.6 ± 0.06 , 1.47 ± 0.13 , 1.04 ± 0.09 , 1.54 ± 0.13 , 1.54 ± 0.22 , 1.64 ± 0.22 , 1.7 ± 0.16 and 1.87 ± 0.04 g/dl, respectively.

The serum albumin values did not show significant change in birds of Group I, II and III during the entire period of experiment. The mean serum value of Group IV and V (toxin fed group) decreased significantly ($P<0.05$) in comparison to control groups throughout the period of study. However, birds of Groups VI to VII (DAE + AF) showed significant ($P<0.05$) increase in comparison to Group IV throughout the period of experiment. The mean serum values of Group VIII and IX (DAE + AF) also showed significant ($P<0.05$) increase from 14th day onwards in comparison to Group V.

4.2.4.6 SERUM CHOLESTEROL

The weekly mean serum cholesterol values of birds fed with different dietary treatments have been summarized in Table 15 and graphically represented in Fig. 15.

Table 14: Mean ($\pm$ SE) weekly serum albumin values (g/dl) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.

Groups	Aflatoxin (ppm)	DAE (mg/kg feed)	Age in weeks				
			1	2	3	4	5
I	0	0	1.64 $\pm$ 0.04 ^{bc}	1.6 $\pm$ 0.12 ^{ab}	1.44 $\pm$ 0.13 ^c	1.47 $\pm$ 0.09 ^{ab}	1.5 $\pm$ 0.16 ^b
II	0	400	1.5 $\pm$ 0.18 ^{abc}	1.6 $\pm$ 0.06 ^{ab}	1.47 $\pm$ 0.18 ^{cd}	1.6 $\pm$ 0.12 ^{bc}	1.6 $\pm$ 0.06 ^b
III	0	800	1.64 $\pm$ 0.04 ^{bc}	1.64 $\pm$ 0.13 ^{abc}	1.37 $\pm$ 0.09 ^c	1.47 $\pm$ 0.09 ^{ab}	1.47 $\pm$ 0.13 ^b
IV	1	0	1.17 $\pm$ 0.07 ^a	1.5 $\pm$ 0.06 ^a	1 $\pm$ 0.06 ^a	1.14 $\pm$ 0.19 ^a	1.04 $\pm$ 0.09 ^c
V	0.5	0	1.3 $\pm$ 0.06 ^{ab}	1.5 $\pm$ 0.06 ^a	1.27 $\pm$ 0.09 ^{bc}	1.4 $\pm$ 0.16 ^{ab}	1.54 $\pm$ 0.13 ^a
VI	1	400	1.4 $\pm$ 0.12 ^{abc}	1.84 $\pm$ 0.04 ^{cd}	1.3 $\pm$ 0.06 ^{bc}	1.4 $\pm$ 0.16 ^{ab}	1.54 $\pm$ 0.22 ^b
VII	1	800	1.6 $\pm$ 0.12 ^{bc}	1.87 $\pm$ 0.04 ^d	1.54 $\pm$ 0.07 ^{cd}	1.67 $\pm$ 0.13 ^{bc}	1.64 $\pm$ 0.22 ^b
VIII	0.5	400	1.6 $\pm$ 0.18 ^{bc}	1.8 $\pm$ 0.06 ^{bcd}	1.6 $\pm$ 0.12 ^{cd}	1.8 $\pm$ 0.06 ^{bc}	1.7 $\pm$ 0.16 ^b
IX	0.5	800	1.7 $\pm$ 0.16 ^c	1.87 $\pm$ 0.04 ^d	1.77 $\pm$ 0.09 ^d	1.87 $\pm$ 0.04 ^c	1.87 $\pm$ 0.04 ^b

Mean values bearing at least one common superscript within columns did not differ significantly ($P \geq 0.05$)

Fig. 14: Mean ($\pm$ SE) weekly serum albumin values (g/dl) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.

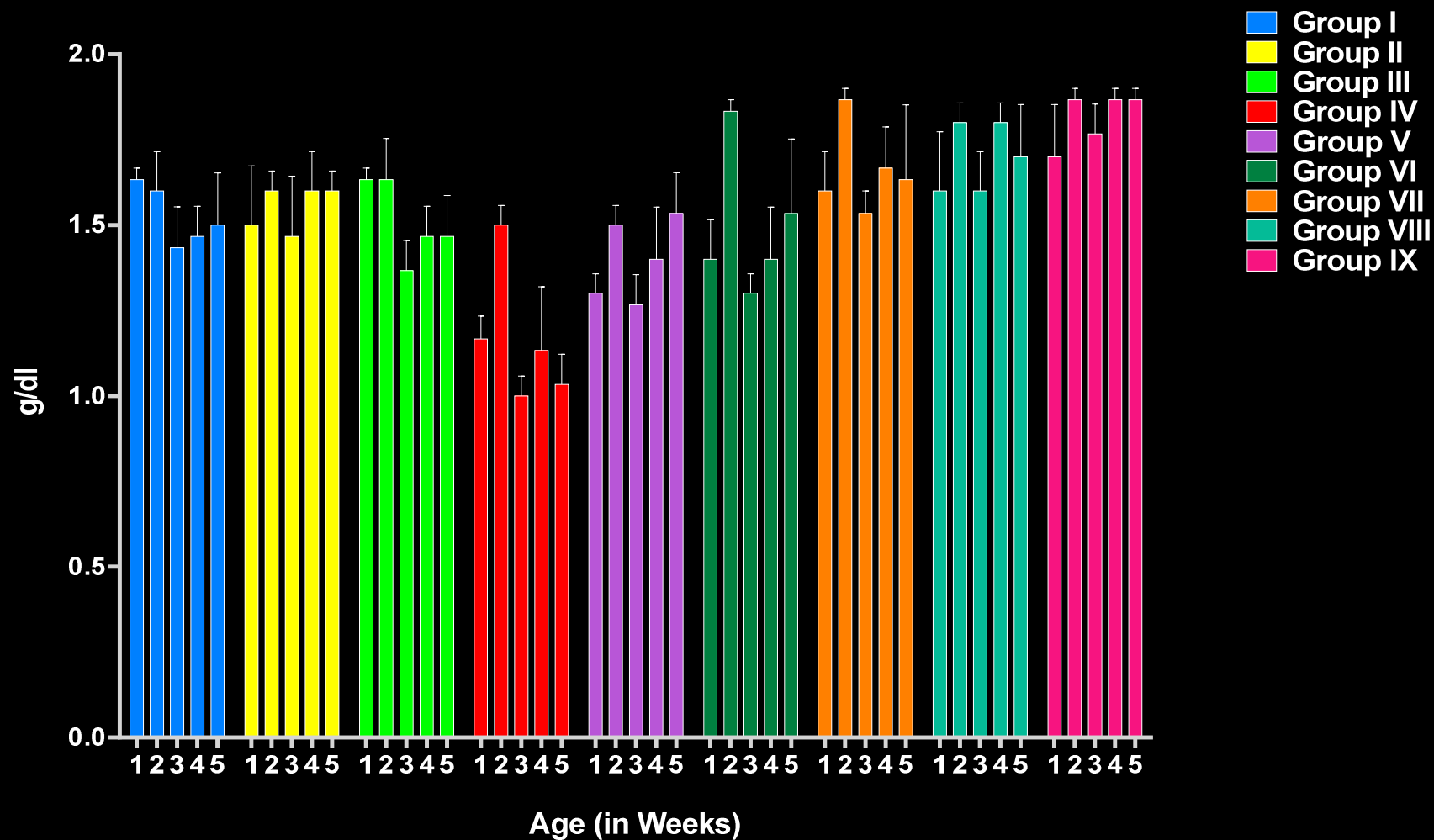
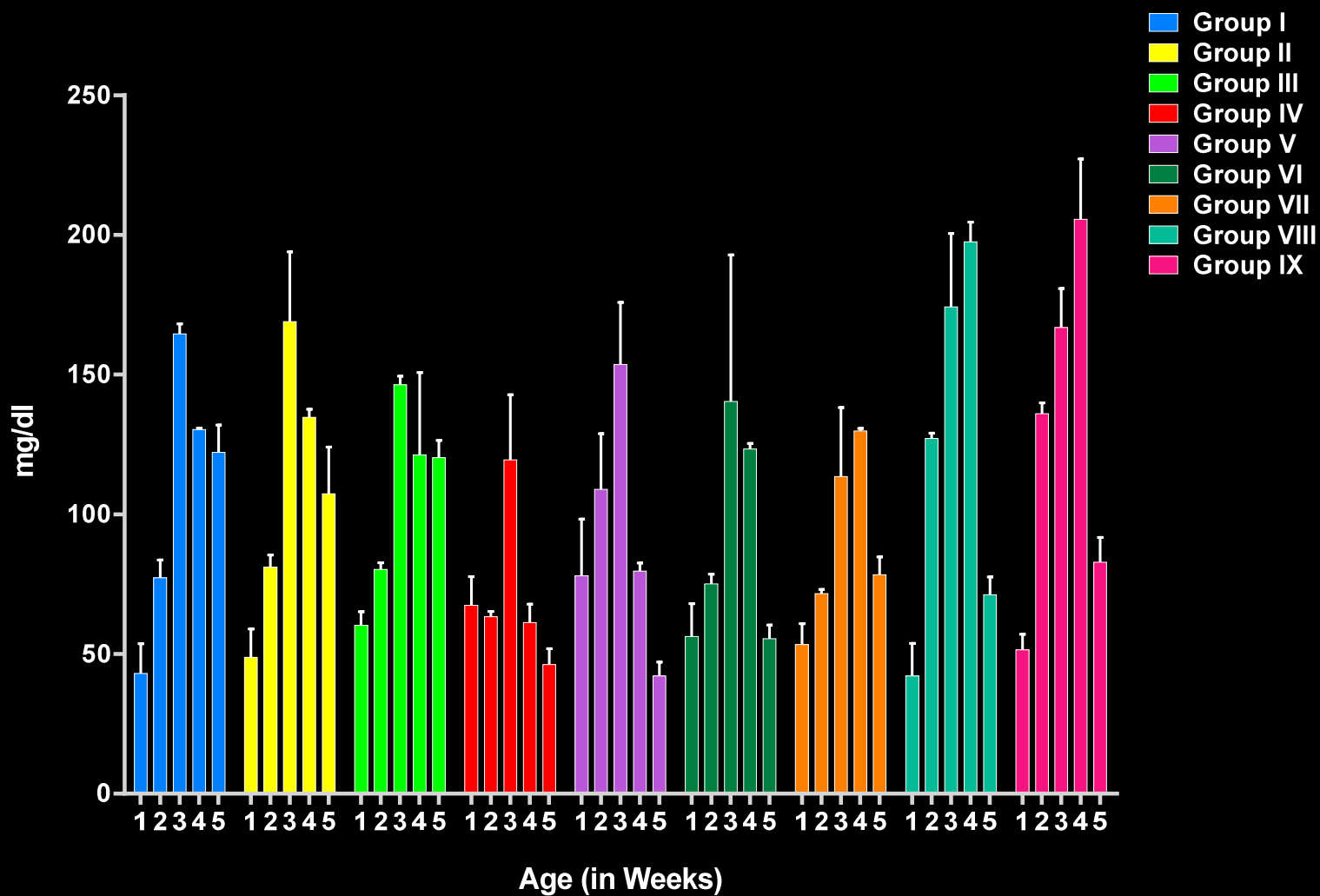


Table 15: Mean ($\pm$ SE) weekly serum cholesterol values (mg/dl) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.

Groups	Aflatoxin (ppm)	DAE (mg/kg feed)	Age in weeks				
			1	2	3	4	5
I	0	0	43.04 $\pm$ 10.69 ^a	77.27 $\pm$ 6.38 ^a	164.57 $\pm$ 3.62 ^a	130.34 $\pm$ 0.53 ^b	122.17 $\pm$ 9.77 ^c
II	0	400	48.9 $\pm$ 10.11 ^a	81.04 $\pm$ 4.42 ^a	168.94 $\pm$ 25.05 ^a	134.74 $\pm$ 2.83 ^b	107.4 $\pm$ 16.69 ^{de}
III	0	800	60.27 $\pm$ 4.91 ^b	80.34 $\pm$ 2.4 ^a	146.37 $\pm$ 3.13 ^b	121.3 $\pm$ 29.45 ^b	120.27 $\pm$ 6.22 ^e
IV	1	0	67.4 $\pm$ 10.34 ^b	63.37 $\pm$ 1.88 ^a	119.5 $\pm$ 23.28 ^c	61.34 $\pm$ 6.49 ^a	46.17 $\pm$ 5.75 ^{ab}
V	0.5	0	78 $\pm$ 20.37 ^c	108.94 $\pm$ 19.9 ^b	153.6 $\pm$ 22.19 ^{ab}	79.67 $\pm$ 2.97 ^a	42.17 $\pm$ 4.99 ^a
VI	1	400	56.3 $\pm$ 11.78 ^b	75.08 $\pm$ 3.56 ^a	140.4 $\pm$ 52.47 ^b	123.37 $\pm$ 2.03 ^b	55.5 $\pm$ 4.87 ^{abc}
VII	1	800	53.47 $\pm$ 7.38 ^a	71.57 $\pm$ 1.55 ^a	113.54 $\pm$ 24.66 ^c	129.9 $\pm$ 0.82 ^b	78.37 $\pm$ 6.38 ^c
VIII	0.5	400	42.1 $\pm$ 11.71 ^a	127.14 $\pm$ 1.99 ^{bc}	174.2 $\pm$ 26.27 ^d	197.5 $\pm$ 7.12 ^c	71.2 $\pm$ 6.41 ^{bc}
IX	0.5	800	51.57 $\pm$ 5.54 ^a	136 $\pm$ 3.97 ^c	166.94 $\pm$ 13.88 ^a	205.6 $\pm$ 21.57 ^c	82.84 $\pm$ 8.9 ^{cd}

Mean values bearing at least one common superscript within columns did not differ significantly ($P \geq 0.05$)

Fig. 15: Mean ($\pm$ SE) weekly serum cholesterol values (mg/dl) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.



The mean serum cholesterol values of Group I to IX birds were 122.17 ± 9.77 , 107.4 ± 16.69 , 120.27 ± 6.22 , 46.17 ± 5.75 , 42.17 ± 4.99 , 55.5 ± 4.87 , 78.37 ± 6.38 , 71.2 ± 6.41 and 82.84 ± 8.9 mg/dl, respectively at the end of 35th day.

The mean serum cholesterol values of birds of Group I, II and III did not alter significantly at the end of experiment. In the toxin fed groups (Group IV and V), the mean serum cholesterol levels decreased significantly ($P \leq 0.05$) as compared with control (Group I, II and III) birds. However, in co-treatment groups (VI to IX) significant ($P \leq 0.05$) increase in cholesterol levels was recorded in comparison to toxin fed birds (Group IV and V) at the end of experiment.

4.2.4.7. SERUM TRIGLYCERIDES

The data pertaining to the mean serum triglycerides in birds of Group I to IX have been furnished in Table 16 and Fig. 16.

The mean serum triglycerides for Group I to IX were 88.28 ± 2.7 , 89.49 ± 2.6 , 90.12 ± 2.8 , 63.04 ± 3.6 , 65.84 ± 2.9 , 69.44 ± 1.9 , 74.72 ± 1.7 , 74.7 ± 1.6 and 81.14 ± 1.9 mg/dl, respectively at 35th days of age.

The triglycerides levels significantly ($P < 0.05$) decreased in toxin fed groups (Group IV and V) in comparison to the control group (I, II and III) at the end of experiment.

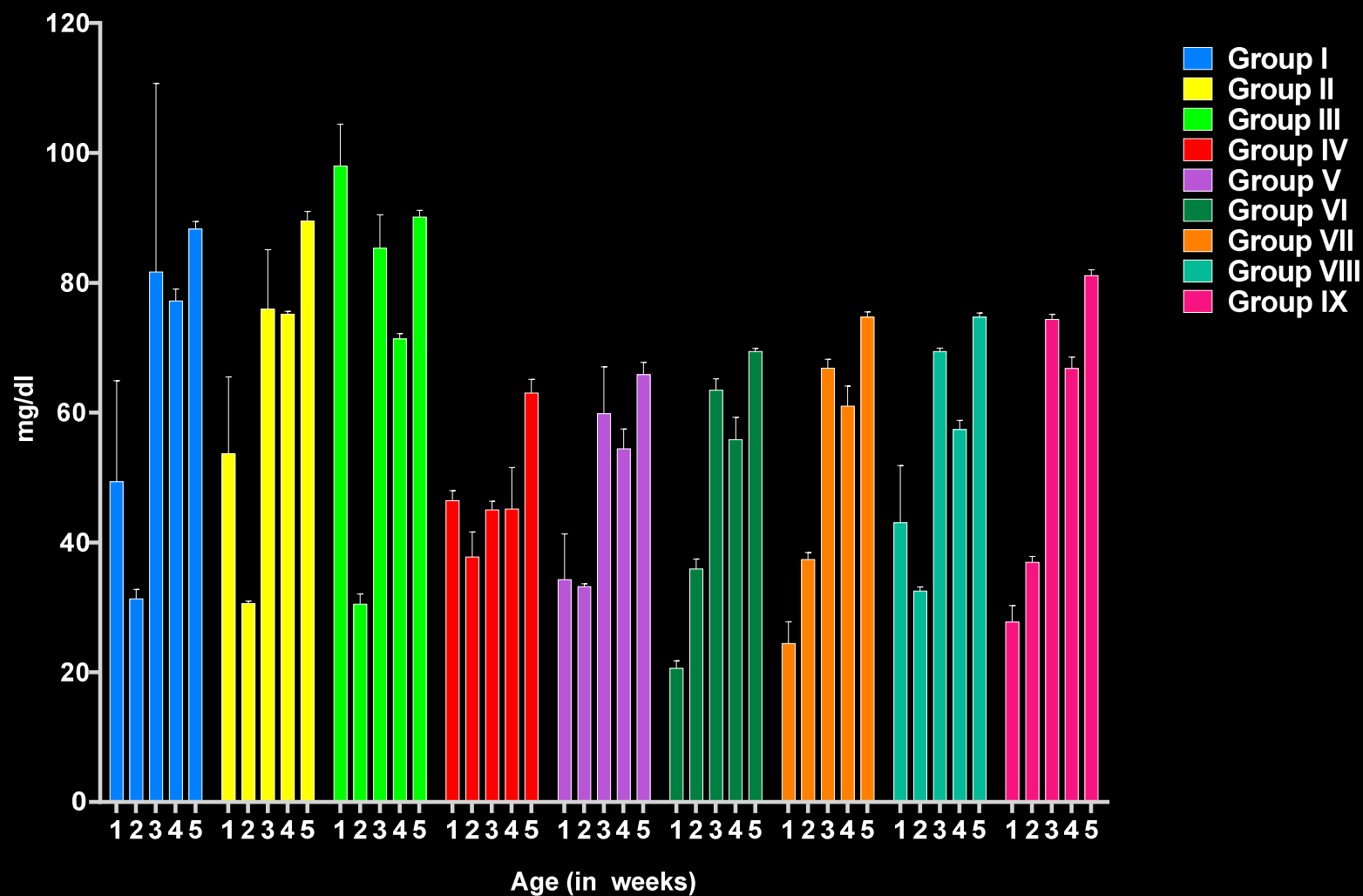
There was significant ($P < 0.05$) increase in mean serum triglycerides in birds supplemented with DAE to AF mixed feed (Group VI, VII, VIII and IX) in comparison to the toxin fed birds (Group IV and V).

Table 16: Mean ($\pm$ SE) weekly serum triglycerides values (mg/dl) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.

Groups	Aflatoxin (ppm)	DAE (mg/kg feed)	Age in weeks				
			1	2	3	4	5
I	0	0	49.37 $\pm$ 16.3 ^a	31.33 $\pm$ 2.9 ^a	81.67 $\pm$ 30 ^a	77.19 $\pm$ 2.5 ^a	88.28 $\pm$ 2.7 ^a
II	0	400	53.67 $\pm$ 12.8 ^b	30.59 $\pm$ 1.7 ^a	75.94 $\pm$ 10 ^b	75.14 $\pm$ 1.8 ^a	89.49 $\pm$ 2.6 ^a
III	0	800	98 $\pm$ 7.1 ^c	30.49 $\pm$ 2.9 ^a	85.34 $\pm$ 6.7 ^a	71.37 $\pm$ 1.6 ^b	90.12 $\pm$ 2.8 ^a
IV	1	0	46.44 $\pm$ 2.8 ^a	37.75 $\pm$ 4.7 ^b	45.01 $\pm$ 2.9 ^c	45.14 $\pm$ 7.8 ^c	63.04 $\pm$ 3.6 ^b
V	0.5	0	34.27 $\pm$ 8.9 ^d	33.2 $\pm$ 1.9 ^c	59.84 $\pm$ 8.1 ^d	54.4 $\pm$ 4.9 ^d	65.84 $\pm$ 2.9 ^{bc}
VI	1	400	20.6 $\pm$ 2.9 ^d	35.9 $\pm$ 2.8 ^{ac}	63.42 $\pm$ 2.3 ^e	55.84 $\pm$ 4.4 ^d	69.44 $\pm$ 1.9 ^{bc}
VII	1	800	24.44 $\pm$ 4.6 ^d	37.34 $\pm$ 2.7 ^b	66.84 $\pm$ 2.6 ^c	60.99 $\pm$ 4.6 ^e	74.72 $\pm$ 1.7 ^d
VIII	0.5	400	43.04 $\pm$ 9.1 ^a	32.5 $\pm$ 1.4 ^{ac}	69.42 $\pm$ 1.9 ^{ae}	57.38 $\pm$ 2.9 ^e	74.7 $\pm$ 1.6 ^d
IX	0.5	800	27.77 $\pm$ 3.7 ^d	36.95 $\pm$ 1.7 ^b	74.37 $\pm$ 1.8 ^a	66.81 $\pm$ 2.4 ^b	81.14 $\pm$ 1.9 ^e

Mean values bearing at least one common superscript within columns did not differ significantly ($P \geq 0.05$)

Fig. 16: Mean ($\pm$ SE) weekly serum triglycerides values (mg/dl) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.



4.2.4.8 SERUM CREATININE

The weekly mean serum creatinine values of birds fed with different dietary treatments have been summarized in Table 17 and graphically represented in Fig.17.

The mean serum creatinine levels for Group I to IX were 1.1 ± 0.1 , 0.9 ± 0.06 , 0.94 ± 0.09 , 1.57 ± 0.18 , 1.14 ± 0.13 , 1.17 ± 0.32 , 0.94 ± 0.09 , 0.94 ± 0.04 and 0.84 ± 0.04 mg/dl, respectively at 35th days of experiment.

The mean serum creatinine values of birds of Group I, II and III did not alter significantly on all the days of experiment. There was significant ($P<0.05$) increase in the mean serum creatinine in toxin fed groups (IV and V) from 28th day onwards of experimental period in comparison to control groups (I to III).

Supplementation with toxin binder to AF mixed feed in Group VI to VII showed significant decrease compared to toxin fed birds (Group IV) from 21st day onwards and continued till the end of experiment. However, in Groups VIII and IX significant ($P<0.05$) decrease in serum creatinine value was seen as compared to group V from 28th day onwards and continued upto 35th day.

4.2.4.9 BLOOD UREA NITROGEN (BUN)

The weekly mean serum BUN levels in different groups have been presented in Table 18 and graphically depicted in Fig. 18.

The mean serum urea values of Group I to IX birds were 14.42 ± 0.33 , 15.2 ± 0.56 , 14.7 ± 1.1 , 24.27 ± 1.32 , 21.25 ± 2.02 , 16.45 ± 0.86 , 14.18 ± 0.9 , 14.03 ± 1.12 and 13.41 ± 0.43 mg/dl, respectively at the end of 35th day.

Table 17: Mean ($\pm$ SE) weekly serum creatinine values (mg/dl) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.

Groups	Aflatoxin (ppm)	DAE (mg/kg feed)	Age in weeks				
			1	2	3	4	5
I	0	0	0.77 $\pm$ 0.04 ^{ab}	0.84 $\pm$ 0.07 ^{bcd}	0.74 $\pm$ 0.07 ^{bc}	0.8 $\pm$ 0.06 ^a	1.1 $\pm$ 0.1 ^a
II	0	400	0.67 $\pm$ 0.04 ^a	0.77 $\pm$ 0.13 ^{abcd}	0.77 $\pm$ 0.07 ^c	0.77 $\pm$ 0.04 ^a	0.9 $\pm$ 0.06 ^a
III	0	800	0.74 $\pm$ 0.04 ^{ab}	0.67 $\pm$ 0.09 ^{ab}	0.74 $\pm$ 0.04 ^{bc}	0.74 $\pm$ 0.09 ^a	0.94 $\pm$ 0.09 ^a
IV	1	0	0.77 $\pm$ 0.04 ^a	0.77 $\pm$ 0.04 ^{abcd}	0.64 $\pm$ 0.04 ^{abc}	1.47 $\pm$ 0.39 ^a	1.57 $\pm$ 0.18 ^b
V	0.5	0	0.74 $\pm$ 0.04 ^{ab}	1.07 $\pm$ 0.13 ^d	0.64 $\pm$ 0.04 ^{abc}	1.34 $\pm$ 0.4 ^a	1.14 $\pm$ 0.13 ^b
VI	1	400	0.74 $\pm$ 0.04 ^{ab}	0.5 $\pm$ 0.06 ^a	0.57 $\pm$ 0.04 ^a	0.94 $\pm$ 0.15 ^a	1.17 $\pm$ 0.32 ^b
VII	1	800	0.77 $\pm$ 0.04 ^{ab}	0.7 $\pm$ 0 ^{abc}	0.6 $\pm$ 0.06 ^{ab}	0.77 $\pm$ 0.07 ^a	0.94 $\pm$ 0.09 ^a
VIII	0.5	400	0.87 $\pm$ 0.04 ^b	1 $\pm$ 0.2 ^{cd}	0.64 $\pm$ 0.04 ^{abc}	1 $\pm$ 0.27 ^a	0.94 $\pm$ 0.04 ^a
IX	0.5	800	0.87 $\pm$ 0.09 ^b	0.67 $\pm$ 0.04 ^{ab}	0.64 $\pm$ 0.04 ^{abc}	1.14 $\pm$ 0.34 ^a	0.84 $\pm$ 0.04 ^a

Mean values bearing at least one common superscript within columns did not differ significantly ($P \geq 0.05$)

Fig. 17: Mean ($\pm$ SE) weekly serum creatinine values (mg/dl) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.

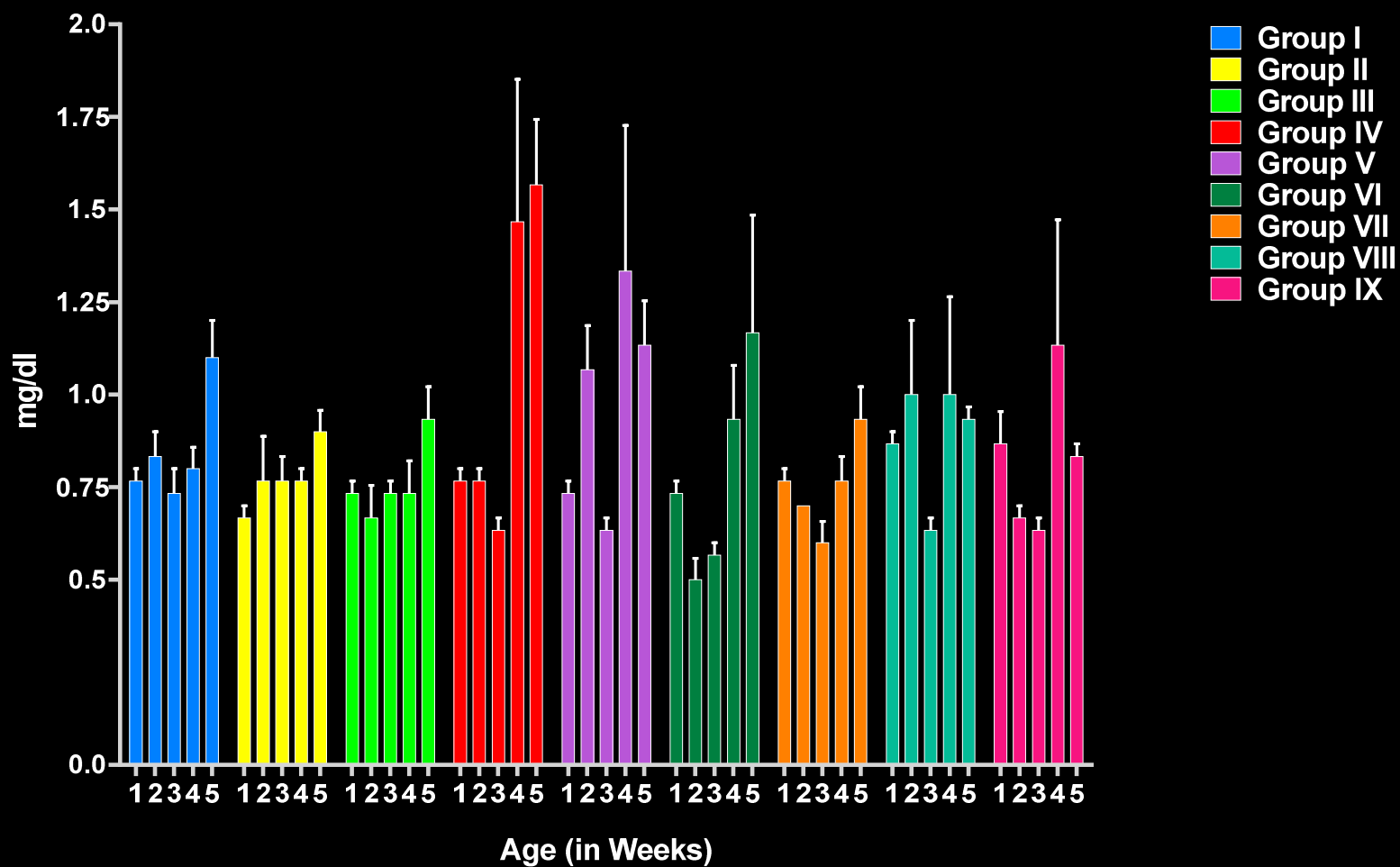
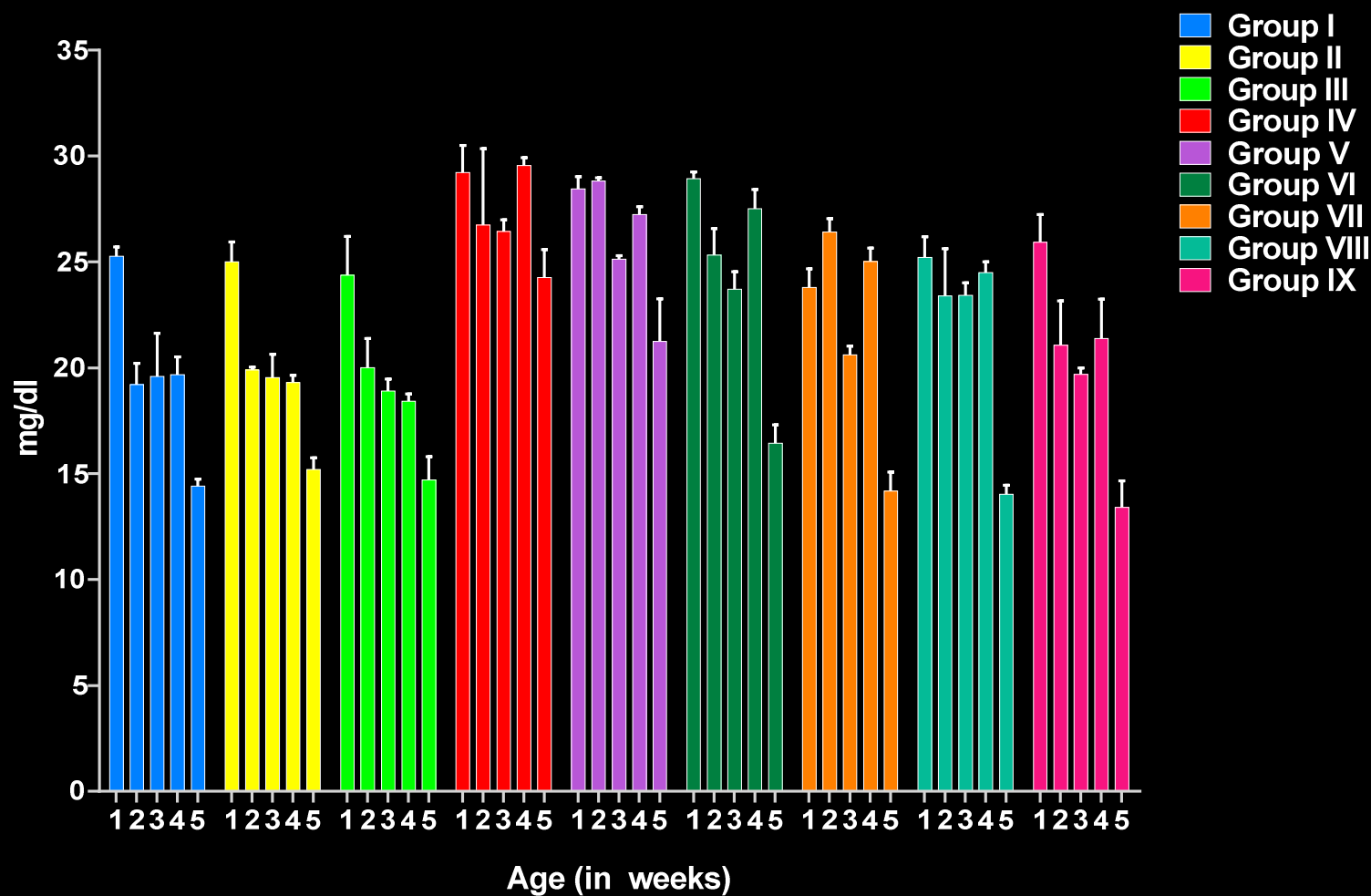


Table 18: Mean ($\pm$ SE) weekly serum BUN values (mg/dl) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.

Groups	Aflatoxin (ppm)	DAE (mg/kg feed)	Age in weeks				
			1	2	3	4	5
I	0	0	25.27 $\pm$ 0.44 ^a	19.22 $\pm$ 1.01 ^a	19.59 $\pm$ 2.05 ^a	19.67 $\pm$ 0.85 ^a	14.42 $\pm$ 0.33 ^a
II	0	400	25 $\pm$ 0.96 ^a	19.9 $\pm$ 0.14 ^a	19.54 $\pm$ 1.11 ^a	19.3 $\pm$ 0.36 ^a	15.2 $\pm$ 0.56 ^a
III	0	800	24.37 $\pm$ 1.85 ^{ad}	20.01 $\pm$ 1.39 ^a	18.91 $\pm$ 0.58 ^a	18.42 $\pm$ 0.35 ^a	14.7 $\pm$ 1.1 ^a
IV	1	0	29.22 $\pm$ 1.28 ^b	26.75 $\pm$ 3.62 ^b	26.45 $\pm$ 0.55 ^b	29.55 $\pm$ 0.39 ^b	24.27 $\pm$ 1.32 ^b
V	0.5	0	28.45 $\pm$ 0.58 ^b	28.83 $\pm$ 0.16 ^b	25.13 $\pm$ 0.17 ^b	27.24 $\pm$ 0.37 ^c	21.25 $\pm$ 2.02 ^c
VI	1	400	28.94 $\pm$ 0.32 ^b	25.32 $\pm$ 1.26 ^c	23.72 $\pm$ 0.82 ^c	27.52 $\pm$ 0.92 ^c	16.45 $\pm$ 0.86 ^d
VII	1	800	23.79 $\pm$ 0.89 ^c	26.42 $\pm$ 0.64 ^c	20.61 $\pm$ 0.43 ^d	25.02 $\pm$ 0.64 ^d	14.18 $\pm$ 0.9 ^e
VIII	0.5	400	25.22 $\pm$ 0.97 ^d	23.4 $\pm$ 2.24 ^d	23.43 $\pm$ 0.59 ^e	24.5 $\pm$ 0.52 ^d	14.03 $\pm$ 1.12 ^e
IX	0.5	800	25.93 $\pm$ 1.31 ^{ad}	21.07 $\pm$ 2.1 ^e	19.69 $\pm$ 0.31 ^a	21.39 $\pm$ 1.87 ^e	13.41 $\pm$ 0.43 ^e

Mean values bearing at least one common superscript within columns did not differ significantly ($P \geq 0.05$)

Fig. 18: Mean ($\pm$ SE) weekly serum BUN values (mg/dl) of broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination.



The mean serum urea levels of birds in control group (I, II and III) did not alter significantly at 35th day of observation. A significant ($P \leq 0.05$) increase in the mean serum urea levels in Group IV and V toxin fed birds was observed as compared with control birds from 7th day onwards and continued till the end of the experiment. However, the birds of co-treatment groups fed with AF and DAE (Group VI to IX) showed significant ($P < 0.05$) decrease in mean serum urea levels as compared to toxin fed birds (Group IV and V) at the end of experiment.

4.2.5. IMMUNOLOGICAL PARAMETERS

4.2.5.1 HUMORAL IMMUNITY

Mean Haemagglutination Inhibition (HI) titres ($\log^2$) to Newcastle disease virus (NDV) in broiler chicken on 35th day of experiment were 6.74 ± 0.05 , 6.39 ± 0.05 , 6.74 ± 0.05 , 3.99 ± 0.07 , 4.4 ± 0.04 , 5.34 ± 0.07 , 5.82 ± 0.05 , 5.67 ± 0.06 and 6.19 ± 0.08 in the treatment Groups I to IX, respectively (Table 19 and Fig 19).

Mean antibody titre (ELISA) to Infectious bursal disease (IBDV) in broiler chicken on 35th day of study were 4554.84 ± 13.3 , 4597.67 ± 3.89 , 4587.5 ± 8.46 , 2922 ± 34.13 , 3324.17 ± 44.53 , 3303.84 ± 32.65 , 3853 ± 23.86 , 3603.17 ± 93.18 and 4095.5 ± 51.04 for different treatment Groups I to IX, respectively (Table 20 and Fig. 20).

The mean antibody titre values against ND and IBD vaccination of the control Groups I, II and III were similar at the end of experiment. However, a non-significant numerical increase in antibody titre values against IBD vaccine was observed in the birds supplemented with DAE alone in the toxin free diet of Group II and III.

Table 19: Mean ($\pm$ SE) values NDV-HI antibody titer ($\log_2$) in broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination at the end of fourth week

Groups	Aflatoxin (ppm)	DAE (mg/kg feed)	NDV-HI Titer ($\log_2$)
I	0	0	6.74 $\pm$ 0.05 ^g
II	0	400	6.39 $\pm$ 0.05 ^f
III	0	800	6.74 $\pm$ 0.05 ^g
IV	1	0	3.99 $\pm$ 0.07 ^a
V	0.5	0	4.4 $\pm$ 0.04 ^b
VI	1	400	5.34 $\pm$ 0.07 ^c
VII	1	800	5.67 $\pm$ 0.06 ^d
VIII	0.5	400	5.82 $\pm$ 0.05 ^d
IX	0.5	800	6.19 $\pm$ 0.08 ^e

Mean values bearing at least one common superscript within columns did not differ significantly ($P \geq 0.05$)

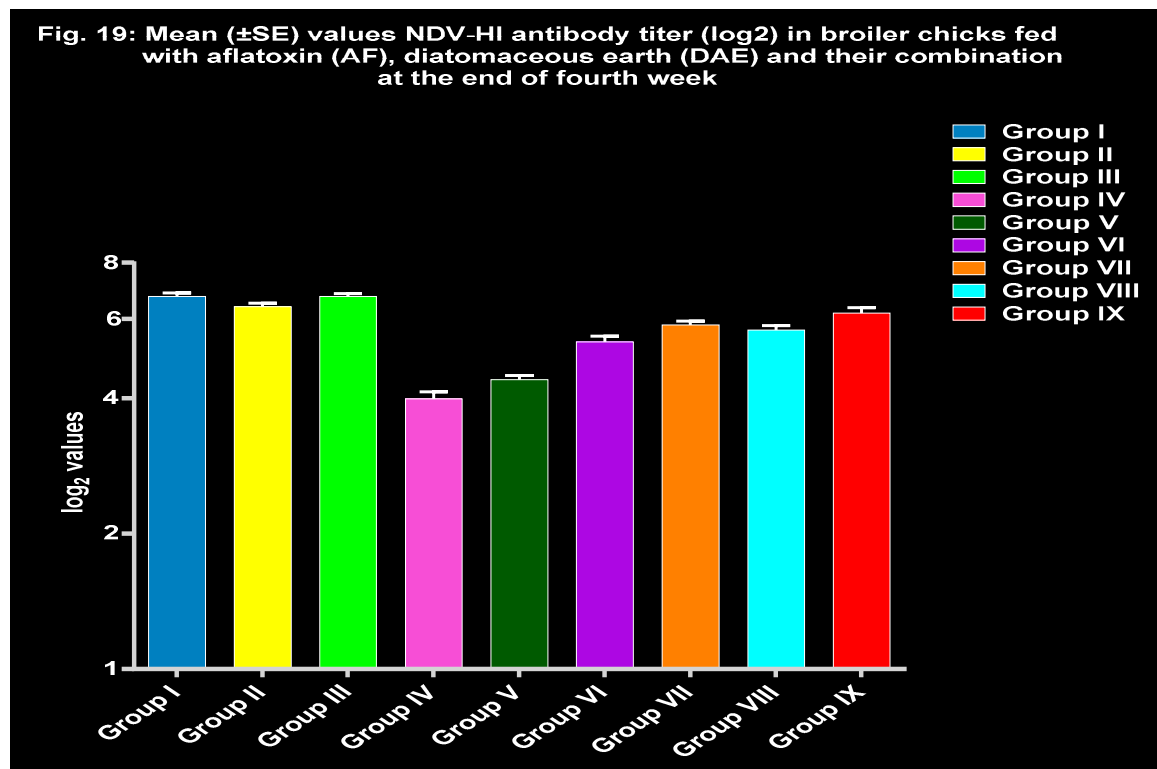
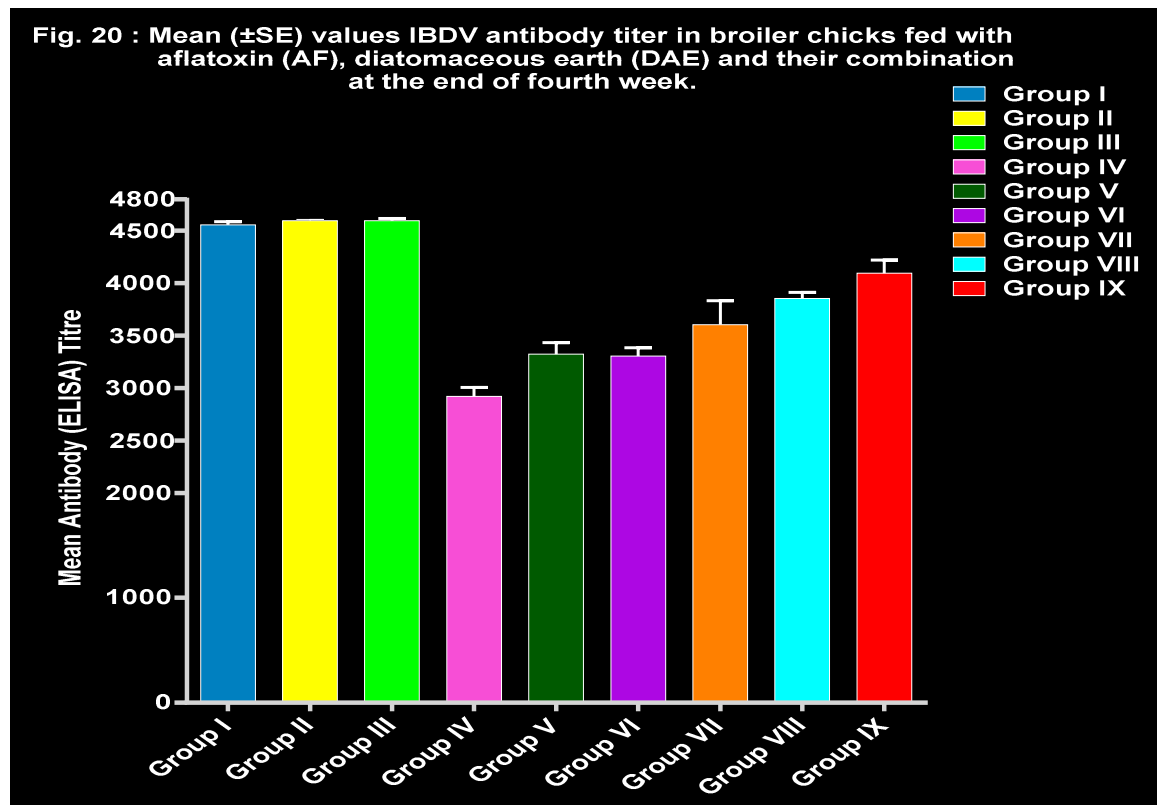


Table 20: Mean ($\pm$ SE) values IBDV antibody titer in broiler chicks fed with aflatoxin (AF), diatomaceous earth (DAE) and their combination at the end of fourth week

Groups	Aflatoxin (ppm)	DAE (mg/kg feed)	IBDV ELISA titre
I	0	0	4554.84 $\pm$ 13.3 ^f
II	0	400	4597.67 $\pm$ 3.89 ^f
III	0	800	4587.5 $\pm$ 8.46 ^f
IV	1	0	2922 $\pm$ 34.13 ^a
V	0.5	0	3324.17 $\pm$ 44.53 ^b
VI	1	400	3303.84 $\pm$ 32.65 ^b
VII	1	800	3853 $\pm$ 23.86 ^d
VIII	0.5	400	3603.17 $\pm$ 93.18 ^c
IX	0.5	800	4095.5 $\pm$ 51.04 ^e

Mean values bearing at least one common superscript within columns did not differ significantly ($P \geq 0.05$)



A significant ($P \leq 0.05$) decrease in antibody titre values against ND and IBD vaccination was observed in birds fed with 0.5 ppm and 1 ppm of AF in their diet in comparison to birds maintained on control diet.

Supplementation of DAE @ 400 and 800 mg/kg feed in the diet which contained 0.5 and 1 ppm of AF significantly ($P \leq 0.05$) improved the antibody titres against ND and IBD vaccines in comparison to the birds fed 0.5 and 1 ppm of AF alone. In the combined treatment (AF + DAE) groups, the birds supplemented with 800 mg of DAE/kg (Groups VII and IX) to the aflatoxin mixed feed showed significantly higher antibody titres against ND and IBD vaccines in comparison to Group VI and VIII fed with 400 mg/kg of DAE along with toxin.

4.2.5.2 CELL MEDIATED IMMUNITY

4.2.2.2 CD₄⁺ AND CD₈⁺ CELLS IN PERIPHERAL BLOOD

Lymphocytes were separated from the diluted peripheral blood after adding lymphocyte separating medium (Histopaque) and centrifuged at 1500 rpm for 30 min at room temperature.

4.6.2.2.1 OPTIMIZATION OF ANTI CHICKEN CD4 AND CD8 ANTIBODIES

The optimum concentration of mouse antichickens CD4: FITC and CD8a: RPE monoclonal antibody to be used to bind lymphocytes was determined by end point titration. Different concentrations 2.5, 5.0, 7.5 and 10 μ l containing 1 μ g/ μ l of mouse anti-chicken CD4: FITC and CD8a: RPE monoclonal antibodies were used. The assay indicated that 7.5 μ l of CD4: FITC and CD8a: RPE monoclonal antibodies were optimal

for effective separation of CD4 and CD8 cells in flow cytometry. Therefore, 7.5 µl of each of the conjugates was used in the present study.

4.6.2.2.2 CD4⁺ CELLS

The mean ($\pm$ SE) per cent CD4⁺ cells in birds in Groups I to IX at the end of 35th day were 14.04 $\pm$ 0.13, 13.87 $\pm$ 0.09, 14.15 $\pm$ 0.12, 8.24 $\pm$ 0.13, 9.85 $\pm$ 0.15, 10.04 $\pm$ 0.16, 10.89 $\pm$ 0.14, 11.49 $\pm$ 0.13 and 12.37 $\pm$ 0.08, respectively (Table 21 and Fig. 21, 23, 24, 25).

Results obtained from flow cytometric cell segregation indicated that CD4⁺ cells were significantly ($P \leq 0.05$) decreased in birds fed 0.5 (Group V) and 1 ppm AF (Group IV) in comparison to per cent CD4⁺ cells in control birds (Group I). Among the AF fed birds, Group IV (1ppm AF) showed significantly lower percentage of CD4⁺ cells in comparison to Group V (0.5 ppm AF).

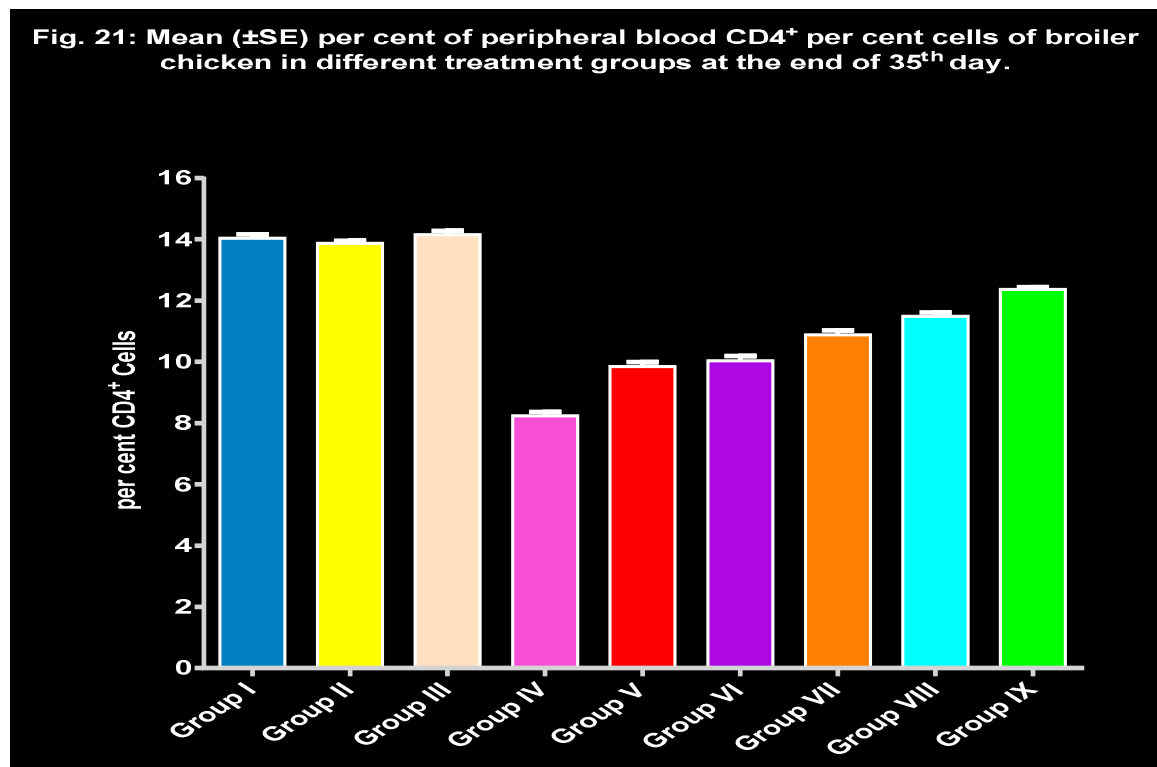
Birds which received DAE supplementation in the diet with zero level of aflatoxin (Group II and III) recorded almost similar per cent of CD4⁺ cells in peripheral blood in comparison to the CD4⁺ values in control birds (Group I).

In the co-treatment groups fed with DAE @ 400 and 800 mg/kg feed in the diet mixed with 0.5 and 1 ppm of AF (Groups VI to IX) significantly ($P \leq 0.05$) increased per cent of CD4⁺ cells in peripheral blood was observed in comparison to toxin fed birds (Group IV and V) at the end of experiment. In the co-treatment groups (AF + DAE) groups, the birds supplemented with 800 mg/kg feed (Groups VII and IX) to the aflatoxin mixed feed showed significantly higher per cent of CD4⁺ cells in peripheral blood in comparison to Group VI and VIII fed with 400 mg DAE/kg feed along with toxin.

Table 21: Mean ($\pm$ SE) values of peripheral blood CD4⁺ per cent cells in broiler chicken in different treatment groups at the end of fifth week of experiment

Groups	Aflatoxin (ppm)	DAE (mg/kg feed)	CD4 ⁺ (%)
I	0	0	14.04 $\pm$ 0.13 ^f
II	0	400	13.87 $\pm$ 0.09 ^f
III	0	800	14.15 $\pm$ 0.12 ^f
IV	1	0	8.24 $\pm$ 0.13 ^a
V	0.5	0	9.85 $\pm$ 0.15 ^b
VI	1	400	10.04 $\pm$ 0.16 ^b
VII	1	800	10.89 $\pm$ 0.14 ^c
VIII	0.5	400	11.49 $\pm$ 0.13 ^d
IX	0.5	800	12.37 $\pm$ 0.08 ^e

Means bearing at least one common superscript within column do not differ significantly ($P \leq 0.05$)



4.6.2.2.3 CD8⁺ Cells

Mean ($\pm$ SE) per cent CD8⁺ cells in peripheral blood of broiler chicken in Groups I to IX were 6.6 ± 0.06 , 6.67 ± 0.07 , 6.55 ± 0.11 , 3.32 ± 0.05 , 3.8 ± 0.08 , 4.27 ± 0.1 , 4.82 ± 0.04 , 4.67 ± 0.05 and 5.24 ± 0.1 , respectively (Table 22 and Fig. 22, 23, 24, 25).

Birds which received DAE supplementation in the diet with zero level of aflatoxin (Group II and III) recorded almost similar per cent of CD8⁺ cells in peripheral blood in comparison to the CD8⁺ values in control birds (Group I). However, per cent CD8⁺ cells in Group II and III differed numerically, which was non-significantly different in comparison to control birds (Group I).

The per cent CD8⁺ cells were significantly ($P \leq 0.05$) decreased in birds fed 0.5 (Group V) and 1 ppm AF (Group IV) in comparison to control birds (Group I, II and III). Among the AF fed birds, Group IV (1ppm AF) showed significantly ($P \leq 0.05$) lower CD8⁺ cells as compared to Group V (0.5 ppm AF).

Incorporation of DAE in the aflatoxin mixed feed (Group VI to IX) caused significant ($P \leq 0.05$) increase in per cent CD8⁺ cells in comparison to birds in toxin fed group (Group IV and V). Among the birds fed with AF and supplemented with DAE, Group IX (0.5 ppm AF and 800 mg DAE) recorded the highest per cent of CD8⁺ cells in peripheral blood, which was significantly ($P \leq 0.05$) higher than the values of Group VI to VIII.

4.2.6. CYTOKINE ASSAY

The chicken IL-2, IL-6 and TNF α cytokine concentrations in the serum samples were determined using commercially available ELISA kits procured from Cusabio,

Table 22: Mean ($\pm$ SE) values of peripheral blood CD8⁺ per cent cells in broiler chicken in different treatment groups at the end of fifth week of experiment

Groups	Aflatoxin (ppm)	DAE (mg/kg feed)	CD8 ⁺ (%)
I	0	0	6.6 $\pm$ 0.06 ^f
II	0	400	6.67 $\pm$ 0.07 ^f
III	0	800	6.55 $\pm$ 0.11 ^f
IV	1	0	3.32 $\pm$ 0.05 ^a
V	0.5	0	3.8 $\pm$ 0.08 ^b
VI	1	400	4.27 $\pm$ 0.1 ^c
VII	1	800	4.82 $\pm$ 0.04 ^d
VIII	0.5	400	4.67 $\pm$ 0.05 ^d
IX	0.5	800	5.24 $\pm$ 0.1 ^e

Means bearing at least one common superscript within column do not differ significantly ($P \leq 0.05$)

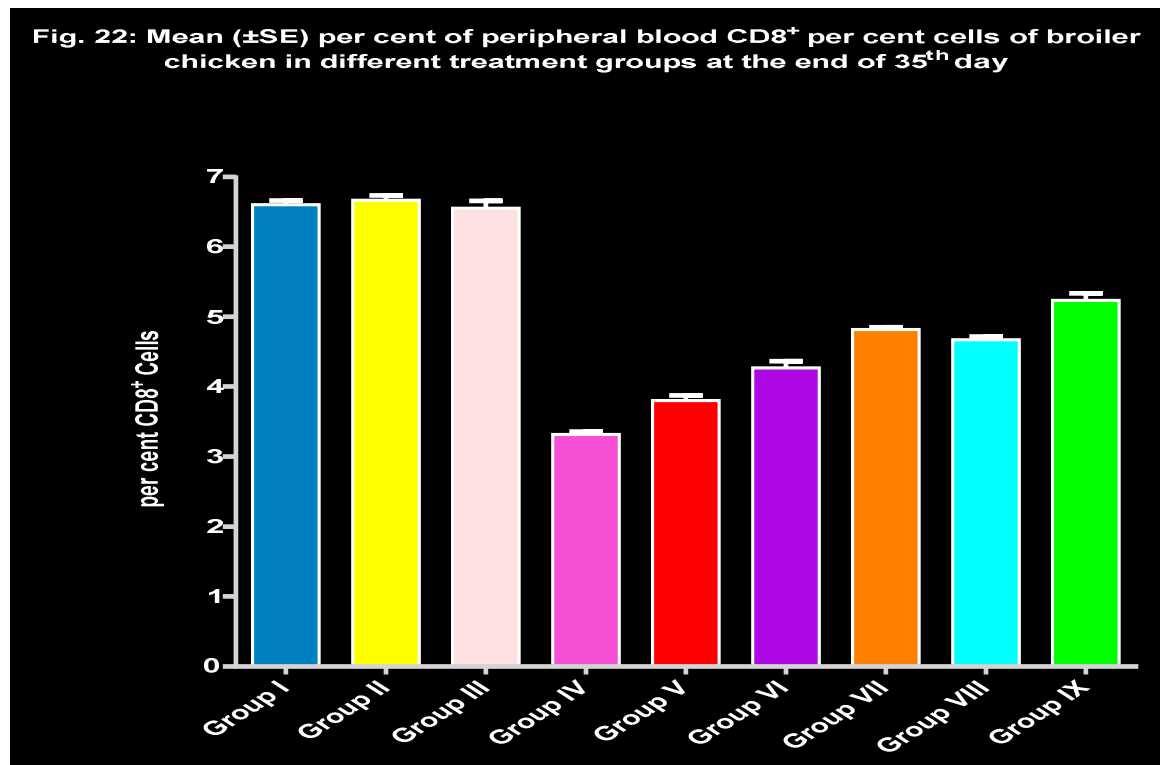
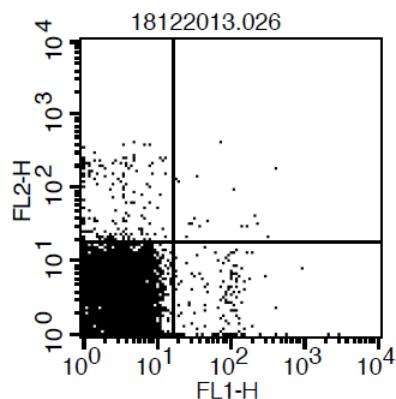


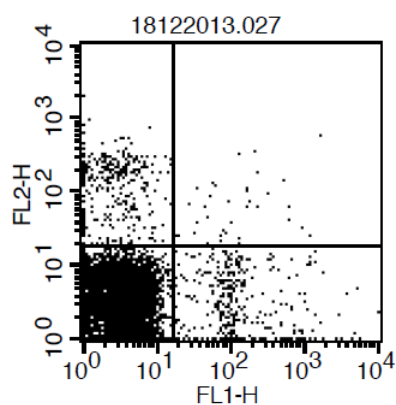
Fig. 23. Profile of CD4⁺ and CD8⁺ cells in peripheral blood of broiler chicken in Groups I, II and III at the end of fourth week of experiment



Quadrant Statistics

File: 18122013.026 Sample ID: B
 Acquisition Date: 18-Dec-13 Gated Events: 7534
 Total Events: 12759 X Parameter: FL1-H (Log)
 Y Parameter: FL2-H (Log)

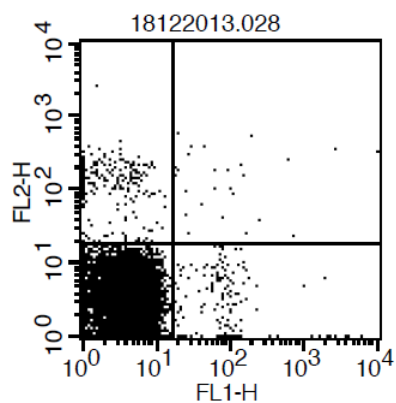
Quad	Events	% Gated	% Total	X Geo Mean	Y Mean
UL	157	2.08	1.23	2.75	110.01
UR	18	0.24	0.14	67.49	82.52
LL	7209	95.69	56.50	2.87	4.56
LR	150	1.99	1.18	75.32	4.04



Quadrant Statistics

File: 18122013.027 Sample ID: C
 Acquisition Date: 18-Dec-13 Gated Events: 6659
 Total Events: 15818 X Parameter: FL1-H (Log)
 Y Parameter: FL2-H (Log)

Quad	Events	% Gated	% Total	X Geo Mean	Y Mean
UL	339	5.09	2.14	2.50	173.32
UR	36	0.54	0.23	113.70	92.40
LL	5877	88.26	37.15	3.07	3.95
LR	407	6.11	2.57	123.60	4.11



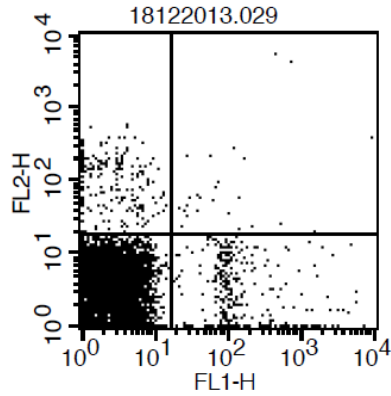
Quadrant Statistics

File: 18122013.028 Sample ID: D
 Acquisition Date: 18-Dec-13 Gated Events: 7417
 Total Events: 12782 X Parameter: FL1-H (Log)
 Y Parameter: FL2-H (Log)

Quad	Events	% Gated	% Total	X Geo Mean	Y Mean
UL	216	2.91	1.69	2.35	155.50
UR	26	0.35	0.20	92.22	195.09
LL	7001	94.39	54.77	3.57	3.99
LR	174	2.35	1.36	78.56	4.09

Fig. 24. Profile of CD4⁺ and CD8⁺ cells in peripheral blood of broiler chicken in Groups IV, V and VI at the end of fourth week of experiment.

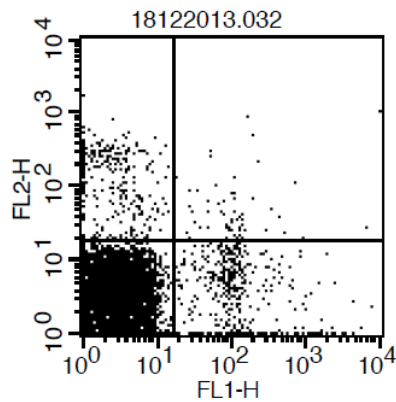
IV, V, VI GROUP



Quadrant Statistics

File: 18122013.029 Sample ID: E
 Acquisition Date: 18-Dec-13 Gated Events: 7213
 Total Events: 13365 X Parameter: FL1-H (Log)
 Y Parameter: FL2-H (Log)

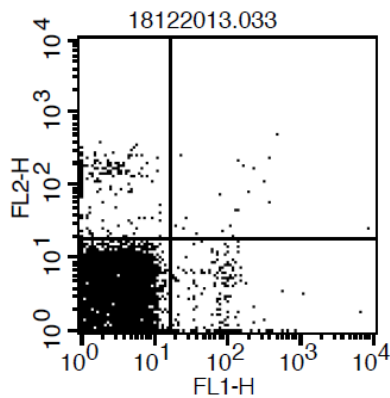
Quad	Events	% Gated	% Total	X Geo Mean	Y Mean
UL	198	2.75	1.48	2.62	124.62
UR	26	0.36	0.19	108.54	462.63
LL	6643	92.10	49.70	2.24	3.90
LR	346	4.80	2.59	145.32	4.15



Quadrant Statistics

File: 18122013.032 Sample ID: H
 Acquisition Date: 18-Dec-13 Gated Events: 7396
 Total Events: 17381 X Parameter: FL1-H (Log)
 Y Parameter: FL2-H (Log)

Quad	Events	% Gated	% Total	X Geo Mean	Y Mean
UL	277	3.75	1.59	2.48	179.72
UR	58	0.78	0.33	111.66	97.05
LL	6636	89.72	38.18	2.60	3.84
LR	425	5.75	2.45	116.17	5.27

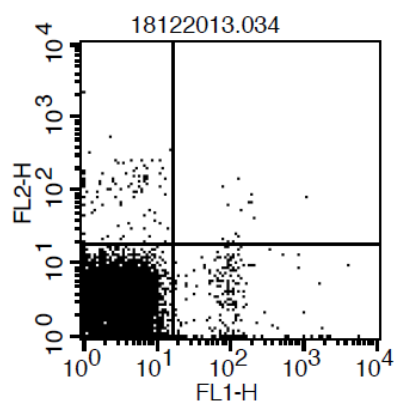


Quadrant Statistics

File: 18122013.033 Sample ID: I
 Acquisition Date: 18-Dec-13 Gated Events: 6324
 Total Events: 18911 X Parameter: FL1-H (Log)
 Y Parameter: FL2-H (Log)

Quad	Events	% Gated	% Total	X Geo Mean	Y Mean
UL	161	2.55	0.85	2.26	134.46
UR	22	0.35	0.12	109.81	103.24
LL	5935	93.85	31.38	2.92	4.18
LR	206	3.26	1.09	74.65	4.14

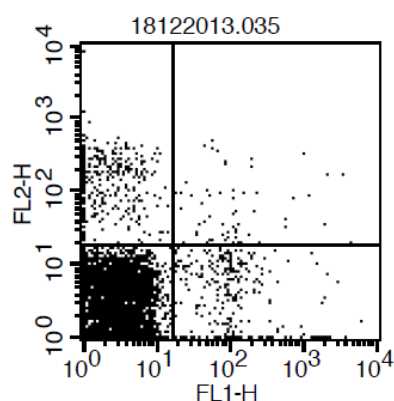
Fig. 25. Profile of CD4⁺ and CD8⁺ cells in peripheral blood of broiler chicken in Groups VI, VIII and IX at the end of fourth week of experiment.



Quadrant Statistics

File: 18122013.034 Sample ID: J
 Acquisition Date: 18-Dec-13 Gated Events: 7453
 Total Events: 14520 X Parameter: FL1-H (Log)
 Y Parameter: FL2-H (Log)

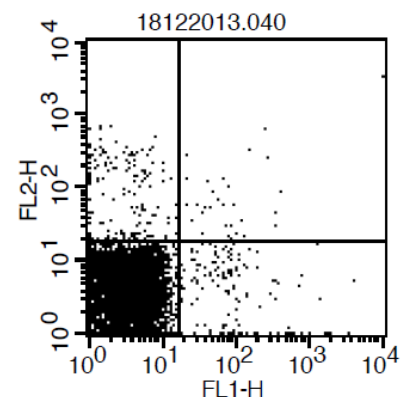
Quad	Events	% Gated	% Total	X Geo Mean	Y Mean
UL	91	1.22	0.63	3.47	145.53
UR	19	0.25	0.13	111.45	60.91
LL	7110	95.40	48.97	3.03	3.32
LR	233	3.13	1.60	93.39	4.56



Quadrant Statistics

File: 18122013.035 Sample ID: K
 Acquisition Date: 18-Dec-13 Gated Events: 5573
 Total Events: 19770 X Parameter: FL1-H (Log)
 Y Parameter: FL2-H (Log)

Quad	Events	% Gated	% Total	X Geo Mean	Y Mean
UL	319	5.72	1.61	2.14	209.38
UR	62	1.11	0.31	115.55	92.73
LL	4893	87.80	24.75	2.71	3.98
LR	299	5.37	1.51	97.85	5.78



Quadrant Statistics

File: 18122013.040 Sample ID: P
 Acquisition Date: 18-Dec-13 Gated Events: 7402
 Total Events: 16091 X Parameter: FL1-H (Log)
 Y Parameter: FL2-H (Log)

Quad	Events	% Gated	% Total	X Geo Mean	Y Mean
UL	167	2.26	1.04	2.75	137.31
UR	32	0.43	0.20	86.14	196.52
LL	7062	95.41	43.89	3.24	4.37
LR	141	1.90	0.88	80.39	5.60

United Kingdom (IL-2: Catalog No. CSB-E06755Ch, IL-6: Catalog No. CSB-E08549Ch and TNF α : CSB-E112312Ch).

4.2.6.1. INTERLEUKIN 6 (IL-6)

The mean $\pm$ SE serum levels of IL-6 of Group I to IX birds were 56.1 $\pm$ 1.57, 59.93 $\pm$ 1.92, 65.3 $\pm$ 4.59, 217.98 $\pm$ 9.11, 172.13 $\pm$ 5.84, 173.03 $\pm$ 3.61, 130.88 $\pm$ 5.11, 136.1 $\pm$ 6.65 and 105.83 $\pm$ 3.59 pg/ml, respectively at the end of 35th day of experiment (vide Table 23 and Fig. 26).

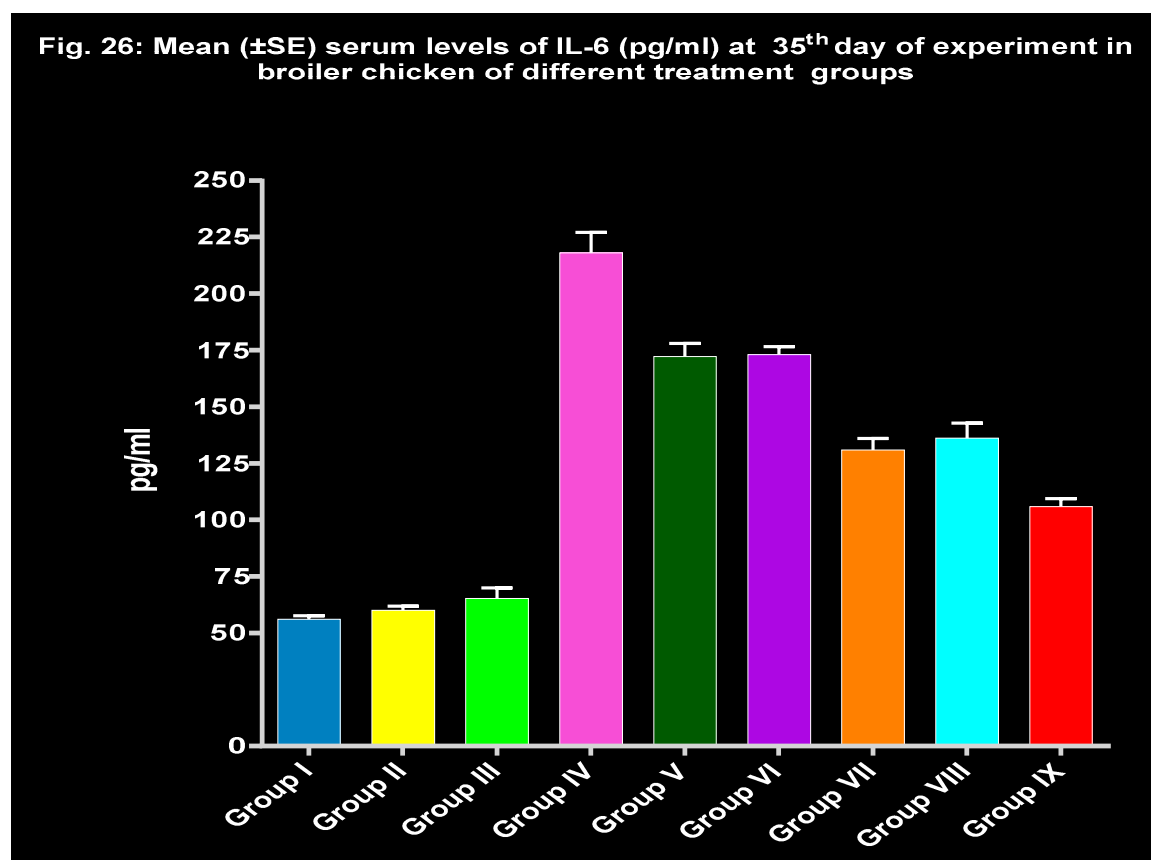
Birds which received DAE supplementation in the diet with zero level of AF (Group II and III) recorded significantly ($P \leq 0.05$) higher level of IL-6 in comparison to the control birds (Group I). However, in DAE treated birds marginal increase in IL-6 of 10 pg/ml was recorded in comparison to the control birds.

In the toxin fed groups (IV and V), fourfold increase in IL-6 level was recorded and the values were significantly ($P \leq 0.05$) altered in comparison to the untreated control (Group I) and DAE fed birds (Group II and III). However, with addition of toxin binder (DAE) to the aflatoxin mixed feed, birds of Group VI and VII showed significantly ($P \leq 0.05$) lower levels of IL-6 as compared to Group IV fed with 1 ppm of AF. In addition, a significant ($P \leq 0.05$) reduction in IL-6 level was also observed in DAE supplemented birds of Group VIII and IX as compared to Group V fed with 0.5 ppm of aflatoxin. In the co-treatment groups, birds supplemented with 800 mg/kg feed of DAE to the aflatoxin mixed feed (Group VII and IX) recorded significant ($P \leq 0.05$) reduction in IL-6 levels in comparison to toxin fed groups (IV and V) as well as Group VI and VIII (Aflatoxin + DAE @ 400 mg/kg feed).

Table 23: Mean ($\pm$ SE) serum levels of Interleukin-6 (pg/ml) at 35th day of experiment in broiler chicken in different treatment groups.

Groups	Aflatoxin (ppm)	DAE(mg/kg feed)	IL-6 (pg/ml)
I	0	0	56.1 $\pm$ 1.57 ^a
II	0	400	59.93 $\pm$ 1.92 ^b
III	0	800	65.3 $\pm$ 4.59 ^c
IV	1	0	217.98 $\pm$ 9.11 ^e
V	0.5	0	172.13 $\pm$ 5.84 ^d
VI	1	400	173.03 $\pm$ 3.61 ^d
VII	1	800	130.88 $\pm$ 5.11 ^c
VIII	0.5	400	136.1 $\pm$ 6.65 ^c
IX	0.5	800	105.83 $\pm$ 3.59 ^b

Mean values bearing at least one common superscript within columns did not differ significantly ($P \geq 0.05$)



4.2.6.2. INTERLEUKIN-2 (IL-2)

The mean serum levels of IL-2 of different treatment groups (I to IX) were 1.5 ± 0.03 , 1.53 ± 0.02 , 1.9 ± 0.04 , 0.63 ± 0.02 , 0.72 ± 0.01 , 0.86 ± 0.02 , 1.1 ± 0.03 , 1.12 ± 0.04 and 0.95 ± 0.04 pg/ml, respectively at the end of 35th day (Table 24 and Fig. 27).

The mean serum levels of IL-2 in Group I, II and III did not alter significantly. A significant ($P \leq 0.05$) decrease was recorded in birds fed with aflatoxin (Group IV and V). The mean serum levels of IL-2 in birds supplemented with toxin binder (DAE) along with afltoxin (Groups VI, VII, VIII and IX) showed significant ($P \leq 0.05$) increase in levels of interleukin-6 in comparison to toxin fed birds (Group IV and V).

Within the co-treatment groups, birds supplemented with 800 mg of DAE/kg feed to the aflatoxin mixed feed (Group VII and IX) showed significant ($P \leq 0.05$) increase in IL-6 levels in comparison to toxin fed groups (IV and V) as well as Group VI and VIII (aflatoxin + DAE @ 400 mg kg⁻¹)

4.2.6.3. TUMOUR NECROSIS FACTOR (TNF- α)

The mean serum levels of TNF- α of different treatment groups(I to IX) were 44.13 ± 0.67 , 45.58 ± 0.52 , 45.68 ± 0.67 , 101.83 ± 1.67 , 92.8 ± 1.46 , 84.1 ± 1.15 , 62.33 ± 0.87 , 75.25 ± 1.25 and 55.88 ± 0.89 pg/ml, respectively (Table 25 and Fig. 28).

The mean serum levels of TNF- α in Group I, II and III did not show significant difference. A significant ($P \leq 0.05$) increase in levels of TNF- α was recorded in birds fed with aflatoxin (Group IV and V) in comparison to control groups. Significantly ($P \leq 0.05$) a higher level of TNF α was recorded following feeding of 1ppm AF in comparison to 0.5 ppm of AF.

Table 24: Mean ($\pm$ SE) serum levels of Interleukin-2 (pg/ml) at 35th day of experiment in broiler chicken in different treatment groups.

Groups	Aflatoxin (ppm)	DAE(mg/kg feed)	IL-2 (pg/ml)
I	0	0	1.5 $\pm$ 0.03 ^a
II	0	400	1.53 $\pm$ 0.02 ^a
III	0	800	1.9 $\pm$ 0.04 ^b
IV	1	0	0.63 $\pm$ 0.02 ^c
V	0.5	0	0.72 $\pm$ 0.01 ^d
VI	1	400	0.86 $\pm$ 0.02 ^e
VII	1	800	1.1 $\pm$ 0.03 ^f
VIII	0.5	400	1.12 $\pm$ 0.04 ^f
IX	0.5	800	0.95 $\pm$ 0.04 ^g

Mean values bearing at least one common superscript within columns did not differ significantly ($P \geq 0.05$)

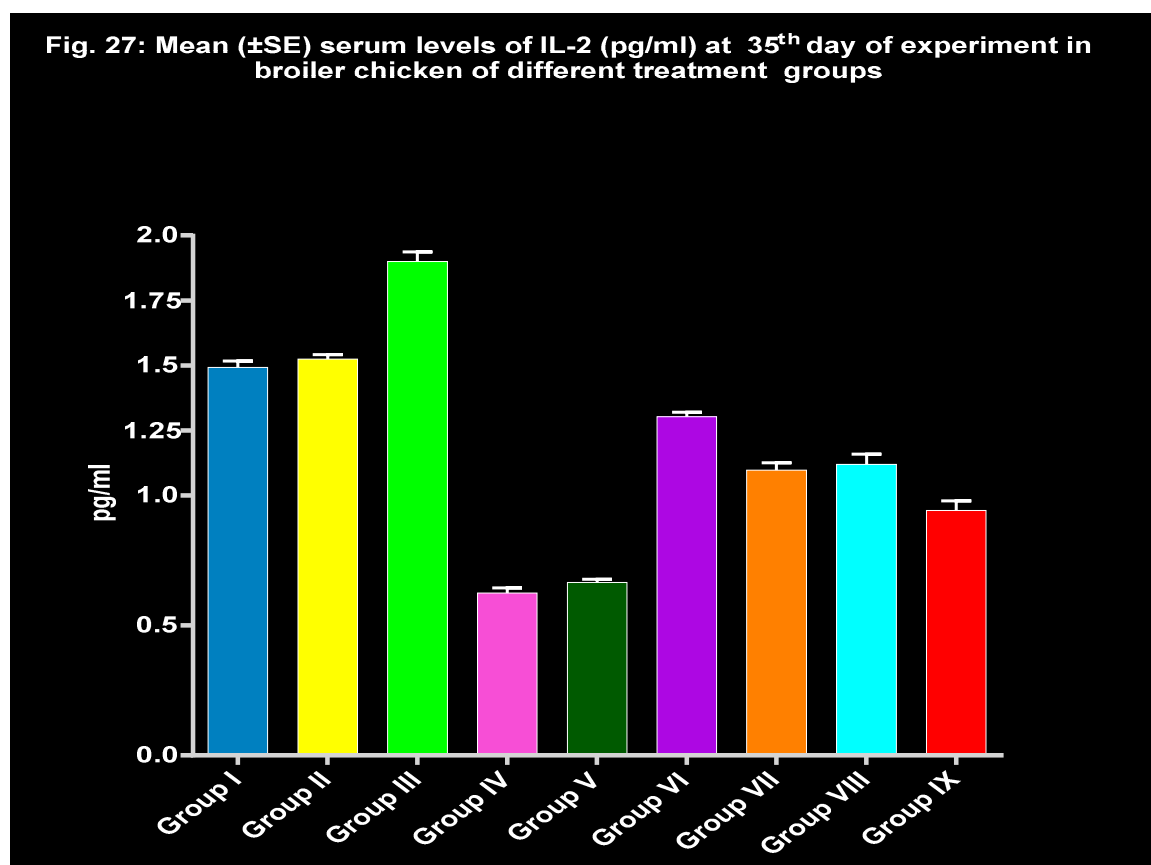
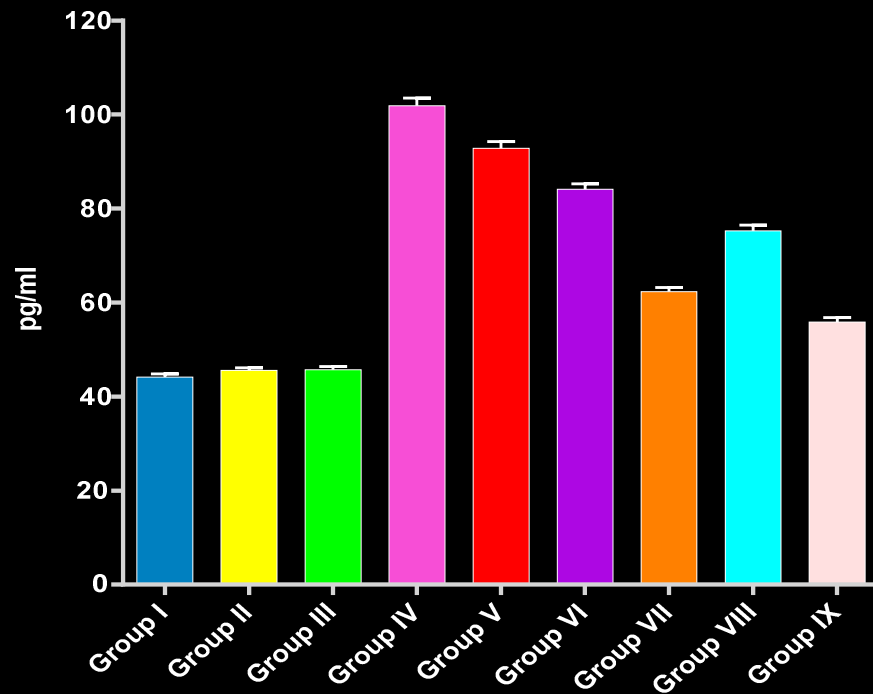


Table 25: Mean ($\pm$ SE) serum levels of Tumour Necrosis Factor- α (pg/ml) at 35th day of experiment in broiler chicken in different treatment groups.

Groups	Aflatoxin (ppm)	DAE(mg/kg feed)	TNF α (pg/ml)
I	0	0	44.13 $\pm$ 0.67 ^a
II	0	400	45.58 $\pm$ 0.52 ^a
III	0	800	45.68 $\pm$ 0.67 ^a
IV	1	0	101.83 $\pm$ 1.67 ^g
V	0.5	0	92.8 $\pm$ 1.46 ^f
VI	1	400	84.1 $\pm$ 1.15 ^e
VII	1	800	62.33 $\pm$ 0.87 ^c
VIII	0.5	400	75.25 $\pm$ 1.25 ^d
IX	0.5	800	55.88 $\pm$ 0.89 ^b

Mean values bearing at least one common superscript within columns did not differ significantly ($P \geq 0.05$)

Fig. 28: Mean ($\pm$ SE) serum levels of TNF α (pg/ml) at 35th day of experiment in broiler chicken of different treatment groups



Co-treatment with DAE and aflatoxin (Group VI, VII, VIII and IX) resulted in significant ($P \leq 0.05$) decrease in levels of TNF- α as compared to toxin fed birds (Group IV and V). In the combined treatment groups, birds of Group VIII and IX showed lowest levels of TNF- α in comparison to VI and VII.

4.2.7. GENE EXPRESSION OF IL-8 CYTOKINE:

The mRNA expression of IL-8 cytokines was measured by real-time RT-PCR in peripheral blood lymphocytes samples collected at the end of the experiment (35th day).

The integrity of RNA was confirmed in denaturing agarose gel (Fig. 29); the 28 S and 18 S rRNA bands were visible in all the samples and no contamination of DNA or RNA degradation was observed. Afterwards, the RNA was reverse-transcribed and the real-time PCR was set-up together with a standard curve (Fig. 30). The melt curve analysis indicated higher amplification of IL8 m-RNA in comparison to the GAPDH acting as internal control (Fig. 31, 32, 33).

The normalized values of IL-8 with respect to the house keeping gene GAPDH is represented in the Table 26 and Fig. 34. Birds fed with the diet containing both AF demonstrated a significant ($P \leq 0.05$) up regulation in mRNA expression for IL-8 cytokines in comparison to the control birds. The cytokines expression was significantly ($P \leq 0.05$) higher in birds fed with 1ppm of AF (Group IV) in comparison to 0.5 ppm aflatoxin (Group V).

Supplementation with DAE to the aflatoxin mixed feed (Groups VI to IX) demonstrated significant ($P \leq 0.05$) decrease in mRNA encoding for IL-8 in comparison to AF fed birds (Group IV and V). Within the co-treatment groups, IL-8 mRNA

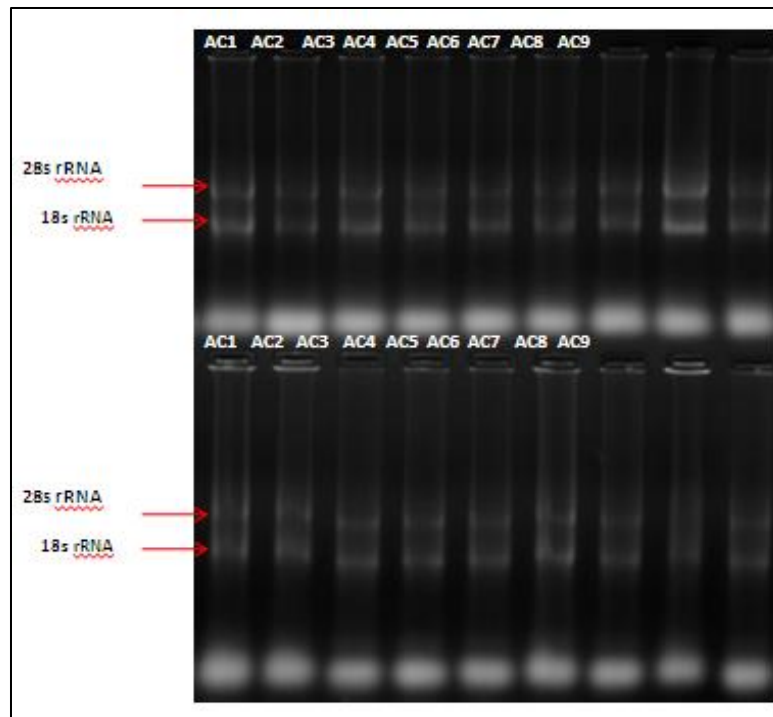
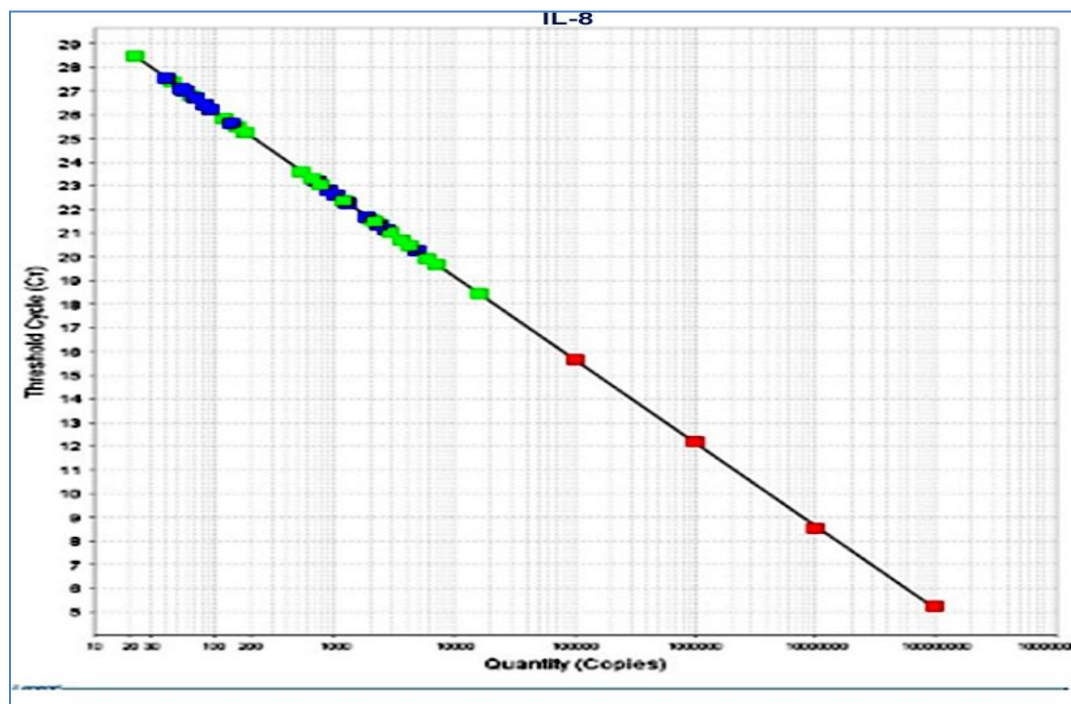


Fig. 29. Denaturing Agarose gel (Plate 16) showing the 28 S and 18 S rRNA bands visible in all the samples and no degradation of DNA or RNA



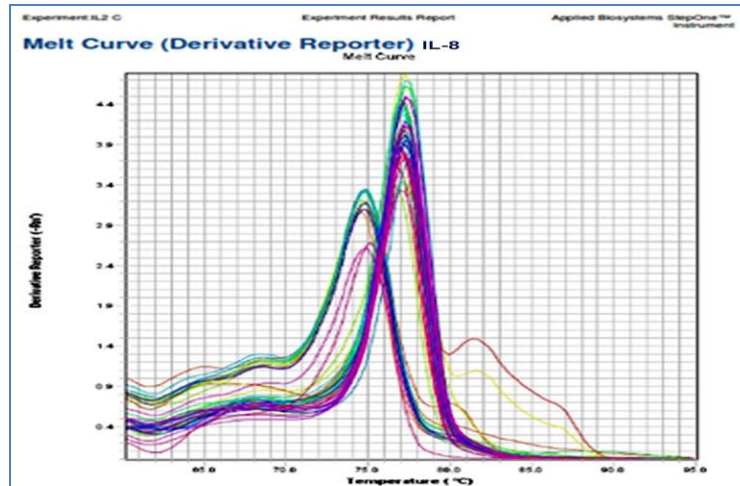


Fig. 31. Photograph showing reproducibility of derivative melting curve of IL-8 in melting curve analysis of SYBR Green real time PCR assay.

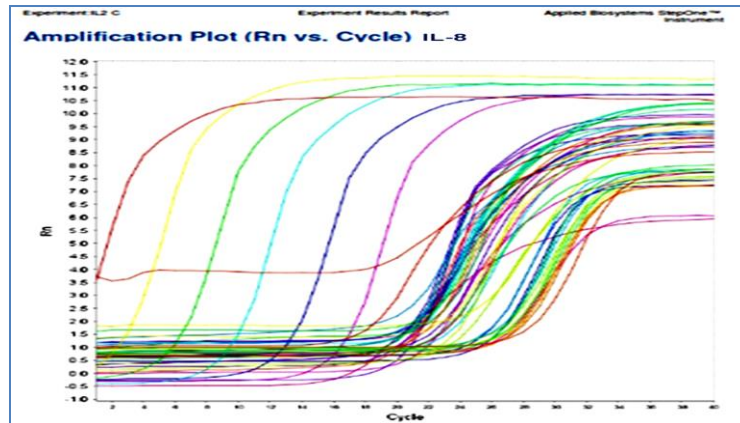


Fig. 32. Photograph showing amplification plot showing Ct values of IL-8 of blood lymphocytes by SYBR Green real time PCR assay

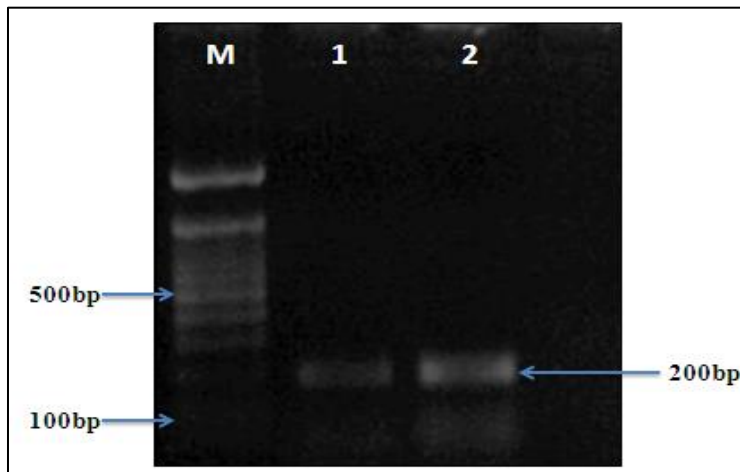
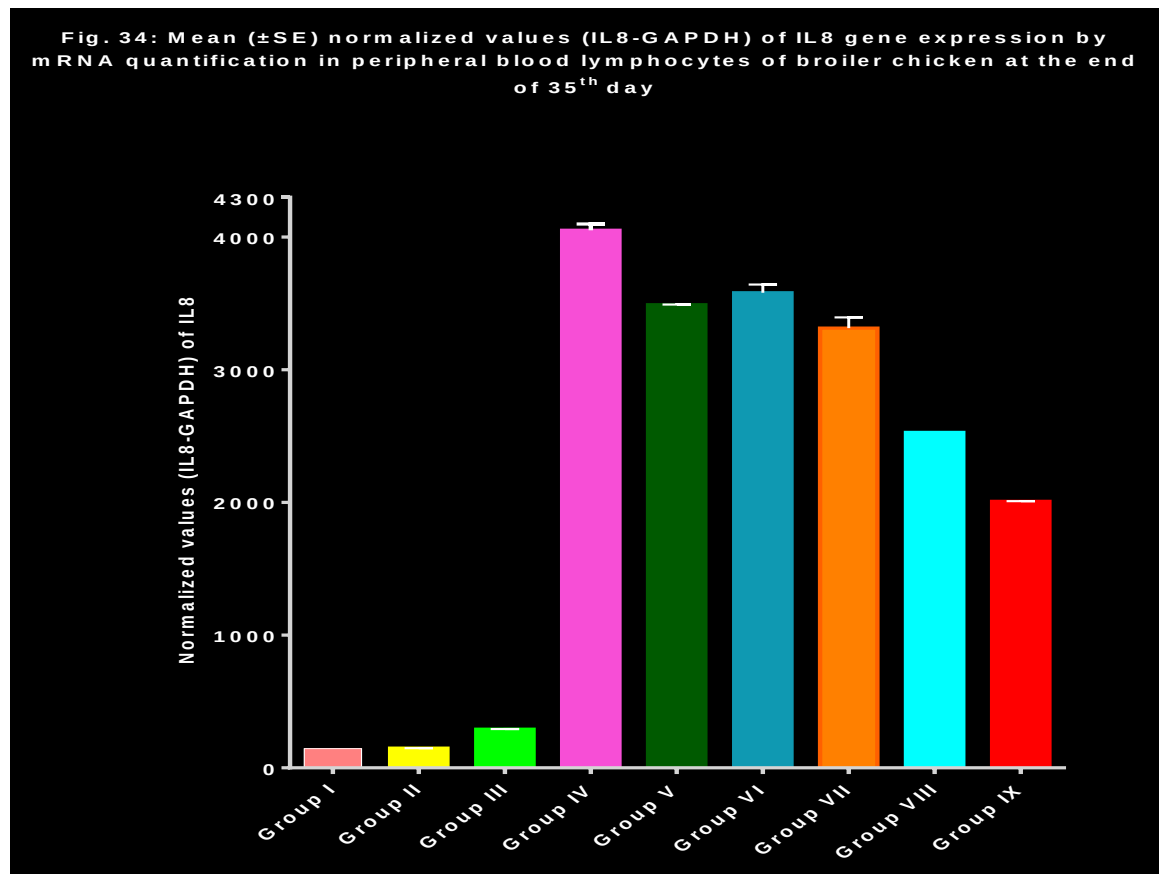


Fig. 33. Photograph of gel showing the amplification of IL8 using real time PCR primer (M-100bp ladder, 1,2- PCR product, 3- negative control)

Table 26: Normalized values (IL8-GAPDH) of IL8 gene expression by mRNA quantification in different treatment groups at the end of 35th day.

Groups	Aflatoxin(ppm)	DAE (mg/kg feed)	IL-8 expression
I	0	0	144.34±0.21 ^a
II	0	400	148.2±0.5 ^a
III	0	800	291.92±1 ^b
IV	1	0	4051.38±47.78 ^c
V	0.5	0	3490.92±0.62 ^d
VI	1	400	3580.5±62.31 ^e
VII	1	800	3314.6±80 ^d
VIII	0.5	400	2528.14±0.57 ^f
IX	0.5	800	2008.63±1.03 ^g

Mean values bearing at least one common superscript within columns did not differ significantly ($P \geq 0.05$)



expression in peripheral lymphocytes was comparatively lower in the birds supplemented with 800 mg DAE/kg feed (Groups VII and IX) than that of 400 mg DAE/kg feed (Groups VI and VIII) to the aflatoxin mixed feed in comparison to the toxin fed birds. However, the mRNA expression for IL-8 in Groups VI to IX (AF+DAE fed birds) was comparatively higher than the control groups (Group I, II and III).

4.2.8. DNA FRAGMENTATION ASSAY

The results of quantification of isolated DNA from various organs as expressed in ng/μl by Nanodrop method using spectrophotometer has been summarized in Table 27 and 28. The fragmentation of DNA in various tissues of broiler chickens in different treatment groups at the end of experiment (35th day) have been analysed and the same is presented as below.

The DNA fragmentation in AF toxin fed groups was observed in all the organs examined such as liver (Fig. 35 and Table 29), kidneys (Fig. 36 and Table 30), thymus (Fig. 37 and Table 31), bursa (Fig. 37 and Table 31) and spleen (Fig. 38 and Table 32).

AF feeding caused severe damage to DNA of liver and lymphoid organs namely thymus, spleen and bursa of Fabricius; followed by kidneys and the least damage was observed to the DNA of heart and pancreas. The damage was more pronounced at 1 ppm (Group IV) in comparison to 0.5 ppm (Group V) of dietary aflatoxin.

The DNA from various tissues of birds fed diet containing 0 level of Aflatoxin (Group I) and DAE alone to toxin free feed (Group II and III) showed no damage and can be compared with the control group.

Table 27: DNA quantification in various visceral organs of different groups by Spectrophotometer (Nanodrop method)

GROUPS	LIVER		KIDNEY		HEART		PANCREAS	
	Nucleic Acid Conc. (ng/μl)	260/280	Nucleic Acid Conc. (ng/μl)	260/280	Nucleic Acid Conc. (ng/μl)	260/280	Nucleic Acid Conc. (ng/μl)	260/280
BLANK	-0.6	0.66	-0.6	0.66	-0.6	0.66	-0.6	0.66
I	202.6	1.65	352	1.77	172.4	1.92	888.4	1.83
II	464.9	1.64	445.5	1.79	168.8	1.89	632.7	1.71
III	126.4	1.74	451.6	1.79	100.8	1.98	1369.4	1.8
IV	518.6	1.78	114.9	1.67	245.9	1.77	2976.2	1.87
V	298.6	1.82	251.2	1.79	179	1.89	585.9	1.68
VI	610.4	1.79	723.9	1.82	790.7	1.97	1086.9	1.82
VII	346	1.93	1212.9	1.89	332	1.85	969.8	1.84
VIII	300.1	1.9	239.2	1.79	348.4	1.95	378.3	1.8
IX	204.3	1.79	328.9	1.71	2589.2	1.92	755.4	1.69

Table 28: DNA quantification in various lymphoid organs of different groups by Spectrophotometer (Nanodrop method)

GROUPS	THYMUS		SPLEEN		BURSA OF FABRICIUS	
	Nucleic Acid Conc. (ng/μl)	260/280	Nucleic Acid Conc. (ng/μl)	260/280	Nucleic Acid Conc. (ng/μl)	260/280
BLANK	-0.6	0.66	-0.6	0.66	-0.6	0.66
I	3751.3	1.89	2168.8	1.91	703.1	1.76
II	590.7	1.78	1052.4	1.92	2536.1	1.87
III	5909.5	1.88	223.3	1.87	6223.6	1.9
IV	7809.7	1.81	1528.6	1.9	2485.1	1.79
V	3794.3	1.87	495.2	1.89	789.8	1.85
VI	1558.9	1.73	892.9	1.85	509.1	1.86
VII	3881.1	1.84	1822.9	1.89	471.3	1.92
VIII	4454.3	1.9	2904.7	1.88	1130.8	1.89
IX	12377.8	1.86	274.2	1.85	749.9	1.89

Fig. 35. DNA fragmentation in liver at the end of fifth week in different treatment groups

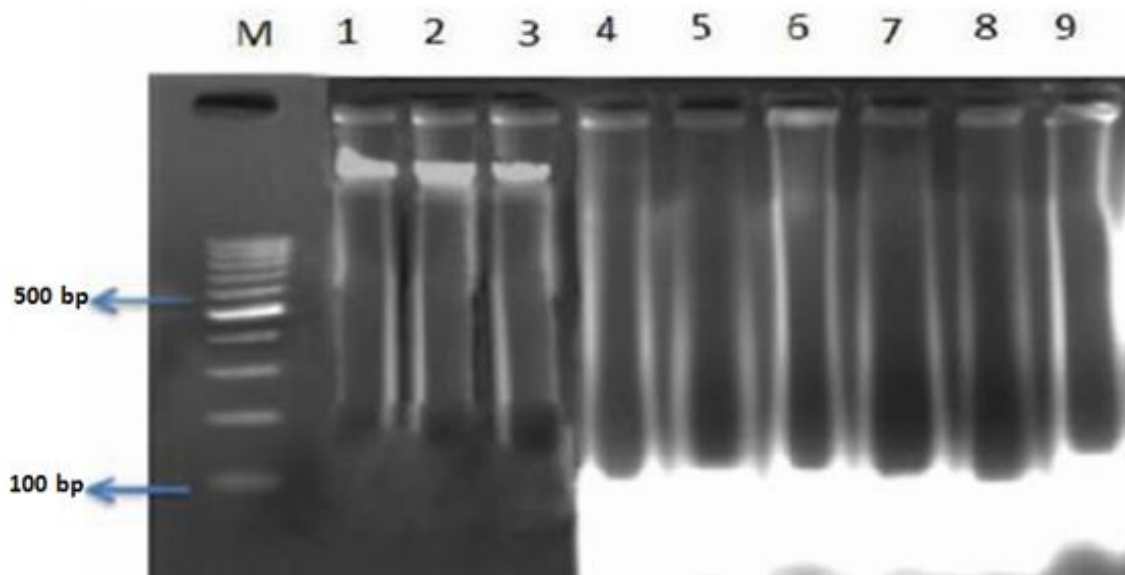


Table 29: Degree of DNA fragmentation in liver at the end of fifth week in different treatment groups

Lanes	Particulars	Level of Fragmentation in base pair (bp)	Degree of DNA Fragmentation
M	100 bp Ladder		
1	Control group	DNA Streak/Smear	No fragmentation
2	Control+400 mg DAE	DNA Streak/Smear	No fragmentation
3	Control+800mg DAE	DNA Streak/Smear	No fragmentation
4	Aflatoxin 1ppm	<100	2+
5	Aflatoxin 0.5 ppm	<100	2+
6	Aflatoxin 1ppm+400mg DAE	<100, >1200	2+
7	Aflatoxin 1 ppm+800mg DAE	<100, >1200	1+
8	Aflatoxin 0.5 ppm+400mg DAE	<100, >1200	2+
9	Aflatoxin 0.5ppm+800mg DAE	>100, >1200	1+

Fig. 36: DNA fragmentation in kidneys at the end of fifth week in different treatment groups

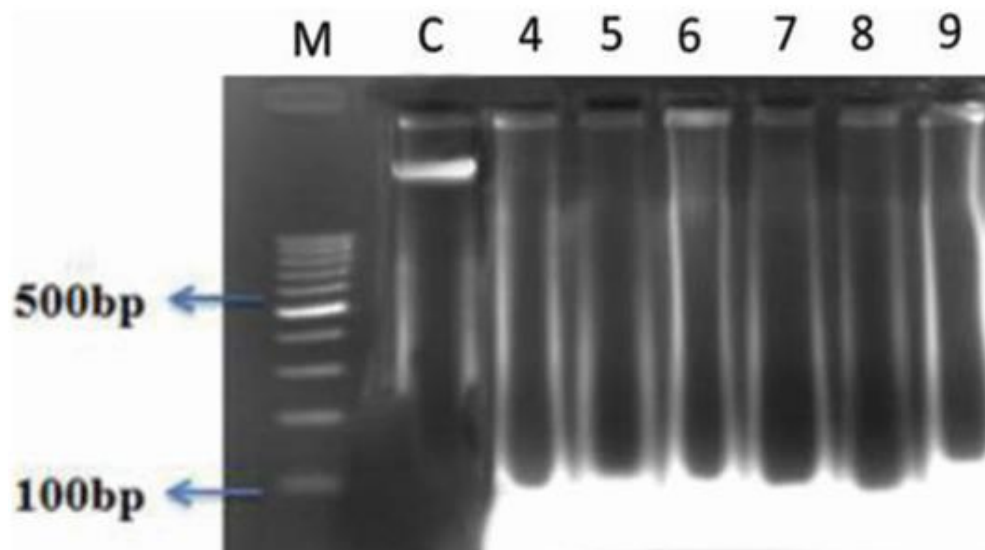


Table 30. Degree of DNA fragmentation in kidneys at the end of fifth week in different treatment groups

Lanes	Particulars	Level of Fragmentation in base pair (bp)	Degree of DNA Fragmentation
M	100 bp Ladder	Kidney	Kidney
1	Control group	DNA Streak/Smear	No fragmentation
2	Control+400 mg DAE	DNA Streak/Smear	No fragmentation
3	Control+800mg DAE	DNA Streak/Smear	No fragmentation
4	Aflatoxin 1ppm	DNA Streak/Smear	2+
5	Aflatoxin 0.5 ppm	<100	1+
6	Aflatoxin 1ppm+400mg DAE	DNA Streak/Smear	No fragmentation
7	Aflatoxin 1 ppm+800mg DAE	<100	1+
8	Aflatoxin 0.5 ppm+400mg DAE	>100	1+
9	Aflatoxin 0.5ppm+800mg DAE	100-200	1+

Fig. 37: DNA fragmentation in bursa of Fabricius and thymus at the end of fifth week in different treatment groups

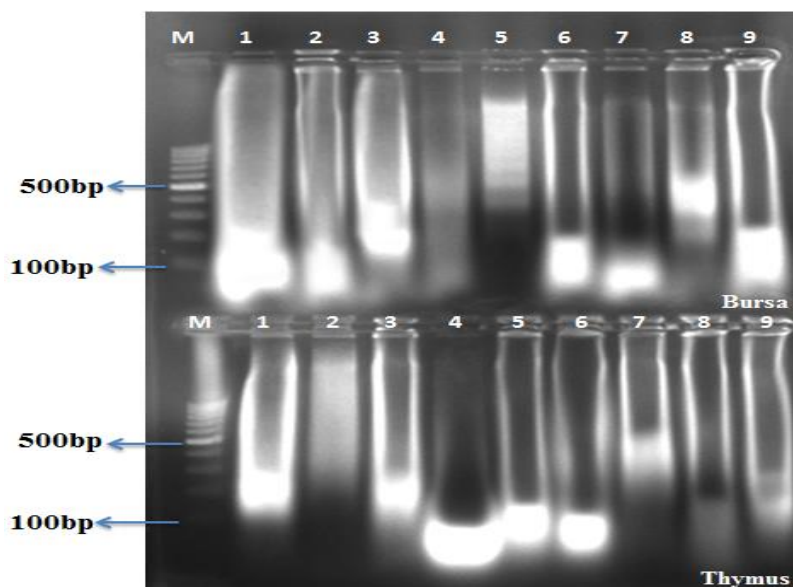


Table 31: Degree of DNA fragmentation in bursa of Fabricius and thymus at the end of fifth week in different treatment groups

Lanes	Particulars	Level of Fragmentation in base pair (bp)		Degree of DNA Fragmentation	
		Bursa	Thymus	Bursa	Thymus
M	100 bp Ladder				
1	Control group	DNA Streak/Smear	DNA Streak/Smear and 150-300	No fragmentation	No fragmentation
2	Control+400 mg DAE	DNA Streak/Smear	DNA Streak/Smear	No fragmentation	No fragmentation
3	Control+800mg DAE	DNA Streak/Smear and 200-400	DNA Streak/Smear and 150-300	1+	No fragmentation
4	Aflatoxin 1ppm	DNA Streak/Smear and >1200	<100	1+	1+
5	Aflatoxin 0.5 ppm	400,500,600,700	>100	4+	1+
6	Aflatoxin 1ppm+400mg DAE	100-200	<100	1+	1+
7	Aflatoxin 1 ppm+800mg DAE	<100	200-500	1+	1+
8	Aflatoxin 0.5 ppm+400mg DAE	200-500	200	1+	1+
9	Aflatoxin 0.5ppm+800mg DAE	100-250	200-300	1+	1+

Fig. 38: DNA fragmentation in spleen at the end of fifth week in different treatment groups

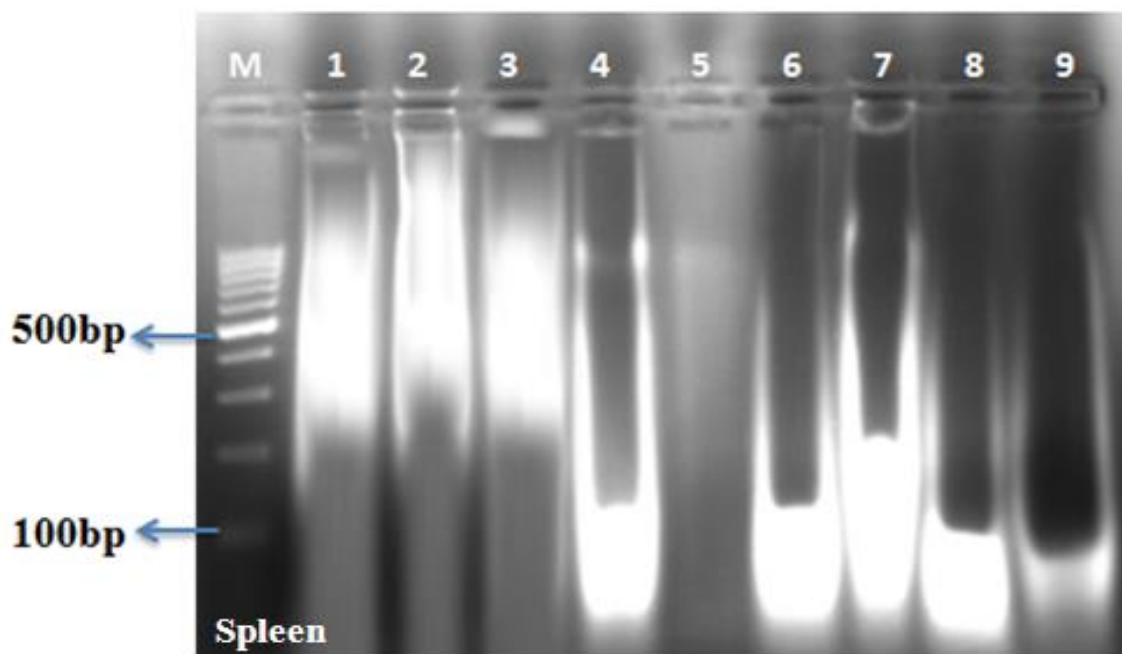


Table 32: Degree of DNA fragmentation in spleen at the end of fifth week in different treatment groups

Lanes	Spleen -Particulars	Level of Fragmentation in base pair (bp)	Degree of DNA Fragmentation
M	100 bp Ladder		
1	Control group	DNA Streak/Smear	No fragmentation
2	Control+400 mg DAE	DNA Streak/Smear	No fragmentation
3	Control+800mg DAE	DNA Streak/Smear	No fragmentation
4	Aflatoxin 1ppm	>100 and 1000	2+
5	Aflatoxin 0.5 ppm	DNA Streak/Smear and 1000	1+
6	Aflatoxin 1ppm+400mg DAE	>100	1+
7	Aflatoxin 1 ppm+800mg DAE	>200	1+
8	Aflatoxin 0.5 ppm+400mg DAE	<100	1+
9	Aflatoxin 0.5ppm+800mg DAE	<100	1+

The damage to DNA in most of the organs was strikingly reduced in bird of co-treatment groups fed 0.5 and 1ppm of aflatoxin and DAE @ 400 and 800 mg/kg feed (Group VI to IX). The birds fed with DAE @ 800 mg/kg feed and aflatoxin (0.5 and 1 ppm) showed reduced damage to DNA in comparison to broilers fed with DAE @ 400 mg/kg feed and aflatoxin @ 0.5 and 1 ppm.

4.2.9. GROSS PATHOLOGY

In the present study, gross pathological changes were recorded in the liver, kidneys, spleen, bursa of Fabricius, thymus, pancreas and intestines on 7th, 14th, 21st, 28th and 35th day of the experimental study. The birds of Group I, II and III revealed normal morphological appearance of all the organs throughout the experimental study. The aflatoxin fed birds of Group IV and V appeared dehydrated and emaciated on all the days of observation. The pathomorphological changes observed in different organs in various treatment groups have been presented as hereunder organ wise.

4.2.9.1 LIVER

The liver in Group I, II and III appeared normal (Plate 13) without of any gross lesions throughout the period of experiment.

The macroscopic changes observed at the end of 5th week in the liver of aflatoxin fed birds included pale to yellowish discolouration, enlargement, soft and friable texture throughout the experimental study (Plate 14, 15). In addition, presence of hematoma measuring around 0.25-0.5 cm in diameter along the borders of liver with distended gall bladder containing thick greenish bile was also observed in the Group IV birds.

The changes observed in the liver of Group VI to IX showed gradual improvement in the condition and intensity of gross pathological changes (Plate 16) and could be compared with the control group at the end of 35th day.

4.2.9. 2 KIDNEYS

The kidneys in control Groups (I, II and III) appeared apparently normal and showed no gross lesions during experimental period.

In Group IV and V birds, the kidneys appeared slightly pale and swollen during the first and second weeks. Kidneys appeared mildly congested by the end of third week onwards and moderately congested at the end of the experiment. The gross changes observed in the kidneys at the end of the experiment included paleness with yellowish discoloration and enlargement (Plate 17, 18, 19). The changes observed in birds of Group VI to IX were less pronounced and showed reduction in size and improvement in condition of kidneys (Plate 20).

4.2.9.3 HEART

The pathomorphological changes observed in heart consisted of mild enlargement, congestion and focal areas of petechial haemorrhages in the epicardium in aflatoxin fed birds (Group IV and V). In the control groups (I, II and III) and in co-treatment groups (VI to IX), the heart remained apparently normal.

4.2.9.4 INTESTINES

The intestines from birds of Groups I, II and III appeared normal throughout the study period of 35 days. In the toxin fed birds (Group IV and V), the lesions observed

were serosal and mucosal congestion. In addition, the contents were catarrhal in nature. Areas of haemorrhages and ulcers were also noticed in the distal portion of small intestines (Plate 21). The condition of intestines improved on supplementation with DAE to the aflatoxin administered Groups VI to IX (Plate 22).

4.2.9.5 PANCREAS

The gross lesions observed in the pancreas of the toxin fed birds (Group IV and V) were mild to moderate degree of congestion and mild focal haemorrhages. The pancreas in control groups (I, II and III) as well as in all the combined treatment groups (VI to IX) fed with aflatoxin and DAE remained apparently normal during the entire period of experimentation.

4.2.9.6 LYMPHOID ORGANS

The morphological changes observed in the lymphoid organs viz., thymus, spleen and bursa of Fabricius included reduction in size which was more pronounced in aflatoxin fed (Group IV and V) birds (Plate 23, 24). In addition to this, petechial haemorrhages were also observed on the surface of the thymus. The spleen showed mild congestion. The sizes of the lymphoid organs in birds of Group VI to IX (aflatoxin with supplementation with DAE) were increased compared to toxin alone fed birds (Plate 25, 26, 27). These lymphoid organs remained apparently normal in Groups I, II and III.

4.2.10. HISTOPATHOLOGY

The histopathological changes observed in different dietary treatments at different time intervals have been detailed out as hereunder. The birds of group I (Control), group II and III (DAE treated) revealed normal architecture of liver, kidneys, heart, pancreas

and lymphoid organs at different intervals of time. In addition, the lymphoid organs also revealed widened lymphoid component, secondary follicle formation in spleen and presence of compact and normal sized follicles in the bursa of Fabricius and widening of cortex in thymus during the latter part of experimental study in birds.

4.2.10.1. LIVER

Birds in Groups I, II and III did not reveal any histological lesions in liver and appeared apparently normal throughout the study period of 35 days. Microscopically, the livers of AF fed bird's revealed vacuolar degeneration, fatty vacuoles coupled with mild congestion of vessels and distension of sinusoidal spaces during first week of the study in birds fed 0.5 and 1 ppm of AF (Group IV and V, respectively).

During the second week, hepatocytes were severely swollen and vacuolated (Plate 28) along with hyperplasia of bile duct epithelium. There was infiltration of inflammatory cells around periductular and perivascular region in Group IV. The birds fed with 0.5 ppm AF revealed mild congestion, cellular swelling, cytoplasmic vacuolation and degeneration of hepatocytes (Plate 29).

During third week, these changes progressed in severity followed by varying degree of necrosis of hepatocytes with infiltration of mononuclear cells, biliary hyperplasia, periductular fibrosis and increased Kupffer cell activity in Group IV and V. In addition to the above, other changes in Group IV included fatty change; cholangitis with sloughing of bile duct epithelium into the lumen, severe biliary hyperplasia and periductular fibrosis were noticed at the end of fourth week. Hepatocytes showed tendency to arrange in a glandular or acinar pattern at the end of fourth week (Plate 30).

Birds in Group IV at fifth week showed presence of fatty cysts formed by coalescence of multiple severely distended hepatocytes with large fatty vacuoles, focal to diffuse areas of necrosis, large number of heterophils and mononuclear cell infiltration in the periportal region as well as around the necrotic areas (Plate 31, 32). The surviving hepatocytes were arranged in glandular or acinar pattern. In addition, there was bile duct epithelial hyperplasia in the periportal area, which also appeared as bridging type between central vein and portal region and mild degree of fibrous connective tissue proliferation in the portal region.

Birds in Group V fed with 0.5 ppm AF in their diet showed moderate biliary hyperplasia with periductular fibrosis along with perivascular infiltration of inflammatory cells (Plate 33), necrosis of hepatocytes and fatty cysts formation (Plate 34) were also evident at the end of experiment.

Birds fed AF (0.5 and 1 ppm) and supplemented with different levels of DAE (Groups VI to IX) showed similar changes of aflatoxin induced damage, but of reduced magnitude and severity. The changes observed in liver during second week of the experiment were mild degree of congestion of blood vessels and sinusoidal spaces, occasional hemorrhages, mild vacuolar degeneration of hepatocytes (Plate 35), mild biliary hyperplasia at perivascular and periportal region, occasional areas of necrosis, infiltration of few heterophils and mononuclear cells. These changes were progressive in nature and were of moderate severity during third week of the experiment. However, marked improvement in the architecture of liver and decrease in the severity of lesions (Plate 36) were observed at the end of fourth week. During the fifth week of experiment the lesions were of mild nature and comprised of almost normal architecture of liver with

mild congestion and haemorrhages, occasional individual cell necrosis, heterophil and mononuclear infiltration and persistence of mild biliary hyperplasia (Plates 37, 38).

4.2.10.2. KIDNEYS

The sections of kidneys in birds fed with toxin and DAE free diet (Group I) and DAE alone in feed (Group II and III) appeared normal. The histological lesions observed during second and third week included congestion, focal hemorrhages and degeneration of tubular epithelium (Plate 39, 40) in toxin fed birds (Group IV and V). At the end of fourth week, moderate degree of congestion and hemorrhages, focal degeneration and desquamation of tubular epithelium along with infiltration of heterophils and lymphocytes were also observed. During the fifth week, there were haemorrhages, multifocal areas of tubular degeneration (Plates 41, 42) and necrosis with collections of mononuclear cells and macrophages. In addition, glomeruli appeared swollen and showed hypercellularity as well as hyaline changes at the end of experiment.

Birds of combined treatment groups fed with aflatoxin and DAE (group VI to IX) revealed mild degree of congestion and occasional haemorrhages, mild vacuolar degeneration of epithelial cells of renal tubules (Plate 43) with focal collections of mononuclear cells in the interstitium during the third and fourth week of experiment. During the fifth week of experiment, the kidneys revealed almost normal architecture with minimal congestion and mild vacuolar changes in tubular epithelium (Plates 44, 45).

4.2.10.3. HEART

Microscopic examination of heart revealed no histological lesions and the sections appeared normal in Groups I, II and III at all the time intervals examined. During the

third and fourth week, birds in Group IV and V showed hemorrhages, congestion and infiltration of mononuclear cells (Plate 46).

At the end of experiment (35th day), the cardiac musculature of the birds in Group IV (1 ppm AF) revealed mild to moderate degree of haemorrhages and degenerative changes (Plate 47) along with occasional infiltration of mononuclear and heterophils. The sections from Group V (0.5 ppm AF) also revealed mild degree of congestion, hemorrhages and degenerative changes in the myocardium.

The birds supplemented with DAE in the aflatoxin mixed feed (Group VI to IX) showed marked improvement and revealed mild congestion and degenerative changes of the cardiac muscles (Plates 48, 49, 50).

4.2.10.4. PANCREAS

Sections of pancreas Groups I, II and III revealed no histologic lesions and appeared apparently normal. Aflatoxin fed birds revealed mild acinar cell degeneration and reduced zymogen granules during the third and fourth week of experiment. At the end of fifth week (35th day), there was severe vascular congestion, acinar cell degeneration (Plate 51) around the pancreatic duct along with infiltration of mononuclear cells and fibroblast proliferation around pancreatic duct in birds fed with 1 ppm of AF. In addition, the pancreatic duct epithelial cells showed hyperplastic changes (Plate 52). Similar lesions of mild nature (Plate 53) were noticed in birds fed with 0.5 ppm of aflatoxin during the fifth week.

Birds in Group VI to IX fed with combination of AF and DAE, showed normal appearance of pancreas with mild degree of vascular congestion (Plate 54).

4.2.10.5. INTESTINES:

Intestinal sections in Group I, II and III appeared normal with no microscopical lesions throughout the period of experiment.

Birds fed 1 ppm of AF revealed progressive severity of catarrhal changes in intestines characterized by increased goblet cell activity from beginning to the end of the experiment. Hyperplasia of crypts epithelium with marked basophilia and mild lymphoid cell infiltration in lamina propria was observed at the end of third and fourth week. Degeneration and necrosis of villus epithelium (Plate 55) with infiltration of large number of mononuclear cells in lamina propria was seen at the end of the experiment (35th day). Cecal tonsils revealed mild to moderate degree of lymphoid depletion in fourth and fifth week of experiment. Group V birds fed with 0.5 ppm AF toxin showed similar changes of lesser severity throughout the experimental study (Plate 56).

In the combination groups (VI to IX) fed with aflatoxin and DAE, no significant changes was observed within the villi and appeared almost normal except for mild congestion (Plates 57, 58).

4.2.10.6. THYMUS

Microscopic examination of thymus from birds of Group I, II and III revealed distinctly large uniform sheet of thickly populated lymphoid cells in compact thymic lobes with distinct cortex and medulla.

The microscopic examination of thymus of aflatoxin fed birds revealed mild to moderate degree of congestion and haemorrhages in the medulla from second week

onwards (Plate 59). A mild degree of lymphocytolysis and depletion in the medullary area and lymphoid necrosis was observed during second and third week in birds of Group IV fed with 1 ppm AF (Plate 60). At the end of fourth and fifth week, severe lymphoid depletion with homogenous cellular debris in the medulla and prominent reticulo-endothelial proliferation were recorded. In addition, thinning of cortical areas, widening of medulla, degeneration of Hassall's corpuscles was visualized as eosinophilic mass and increase in the number of histiocytes containing cellular debris in cortex were also observed at the end of the experiment (Plate 61).

Mild degree of lymphoid necrosis (Plate 62) was observed during third and fourth week in Group V (AF @ 0.5 ppm). At the end of 35th day, moderate degree of lymphoid necrosis with an increase in number of Hassall's corpuscles comprising of degenerated cells.

In Group VI to IX (Aflatoxin + DAE fed birds), mild to moderate degree of congestion of medulla along with mild histiocytic filtration was observed from 14th day till the end of experiment. At the end of experiment, mild lymphoid depletion with hyperplastic changes, repopulation of lymphoid cells and widening of cortex with compact arrangement of lymphoid cells were also observed (Plates 63, 64, 65) at the end of experiment.

4.2.10.7. SPLEEN

Birds of Group I, II and III showed normal histology of spleen with no evidence of any lesions throughout the experimental period. Spleen of aflatoxin fed birds showed mild to moderate degree of congestion with cellular sparcity of the periarteriolar

lymphoid component along with mild histiocytosis from 14th day onwards and continued till the end of the experiment.

Lymphoid cell necrosis around the periarteriolar sheath with eosinophilic debris and depletion in the germinal centres and reticular cell hyperplasia were consistently observed in spleen of the toxin fed birds (Plates 66, 67). The lesions were diffuse in birds fed with 1 ppm (Group IV), while focal distribution was observed at 0.5 ppm of AF from fourth week onwards. In addition, degenerating cells with pyknotic and fragmented nuclei with macrophage accumulation were observed at the end of experiment.

Birds supplemented with DAE in aflatoxin mixed feed (Groups VI to IX) revealed mild degree of congestion of red pulp on 14th day of experiment. There was widening of lymphoid component of some of the periarteriolar sheath along with formation of occasional secondary lymphoid follicles appreciable from 14th day onwards and then continued till the end of the experiment. The widening of lymphoid component and number of secondary follicle increased causing repopulation of lymphoid cells was observed by the end of fourth week. At the end of experiment, the follicles showed varying degree of regeneration and repopulation (Plates 68, 69).

4.2.10.8 BURSA OF FABRICIUS

Sections of bursa of Fabricius in Group I, II and III appeared normal with no microscopical lesions throughout the period of experiment.

A mild lymphoid necrosis, sparse cellularity in occasional follicles and depletion in medulla were noticed in bursa of Group IV (AF @ 1 ppm) birds during the second week. A moderate degree of lymphoid depletion with formation of multiple varying sized

cysts with well-defined wall within follicle (Plate 70) and corrugation of plical epithelium was observed during the third and fourth week. At the end of experiment, a marked lymphoid depletion, increase in number of histiocytes, single cell necrosis, lymphocytolysis with atrophy of bursal follicles (Plate 71), microcyst formation (Plate 72) and interfollicular fibrous tissue proliferation were noticed. The plical epithelium showed hyperplastic changes with corrugations and occasional microcyst formation. Similar changes along with less severe lymphoid tissue depletion (Plate 73) were observed in Group V fed with 0.5 ppm of aflatoxin.

The bursa of Fabricius in aflatoxin fed birds with DAE supplementation revealed cellular sparsity in a few follicles with histiocytic infiltration from 14th day of the experiment till the end of third week. The number and size of the microcyst in the plical epithelium wall also reduced on 28th day of experiment. At the end of experiment, there was marked hyperplasia with repopulation of lymphocytes and formation of large follicles consisting of compactly arranged lymphoid cells and numerous mitotic cells (Plates 74, 75).

Plate 1: Photograph showing day old chicks in cage brooder with proper housing and light arrangement.

Plate 2: *Aspergillus parasiticus* (MTCC-2796) culture (7th day) on Sabourads dextrose agar in Petri (Top) and on potato dextrose agar slants (Bottom). (Note: Dark green colour mycelial mat on the agar surface).

Plate 3: *Aspergillus parasiticus* (MTCC-2796) culture (14th day) on rice in conical flasks. (Note: Dark green colour of rice following growth of fungus on surface of rice).

Plate 4: Photograph showing pinkish amorphous diatomaceous earth (DAE).

Plate 5: Photograph showing good growth broilers from control group at 35th day of age.

Plate 6: Photograph showing ruffled feathers, marked emaciation, poor growth and reduced body weight in 1 ppm AF fed broiler bird at 21 days of age.

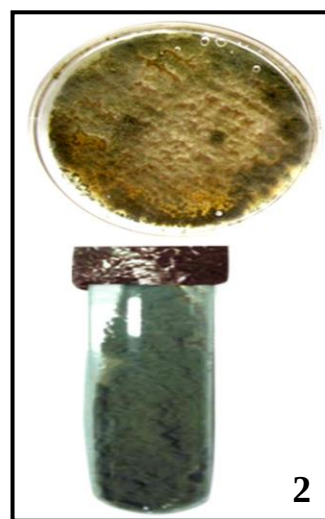


Plate 7: Photograph showing normal growth and good feathers in bird fed with AF 0.5 ppm + DAE 800 mg/kg feed at 35th day of age.

Plate 8: Photograph showing stunted growth in 1ppm aflatoxin treated bird (Left) in comparison with untreated control (Right) at 21st day of the experiment.

Plate 9: Photograph showing reduced growth in 1 ppm aflatoxin fed bird showing reduced growth at 35th day of the experiment.

Plate 10: Photograph showing reduced growth in 0.5 ppm aflatoxin treated bird (Left) in comparison with untreated control (Right) at 35th day of the experiment.

Plate 11: Photograph showing better performance of growth in bird fed with 0.5 ppm AF and 800 mg DAE/kg feed on 35th day of the experiment.

Plate 12: Photograph showing comparative body weight of control (Right) and 0.5ppm AF+ 400 mg DAE (Left).

Plate 13: Gross photograph showing normal liver from control bird (Group I) at 35th day of age.

Plate 14: Gross Photograph showing pale, yellowish discoloration with soft and friable consistency of liver from 1ppm aflatoxin fed bird on 35th day of age.

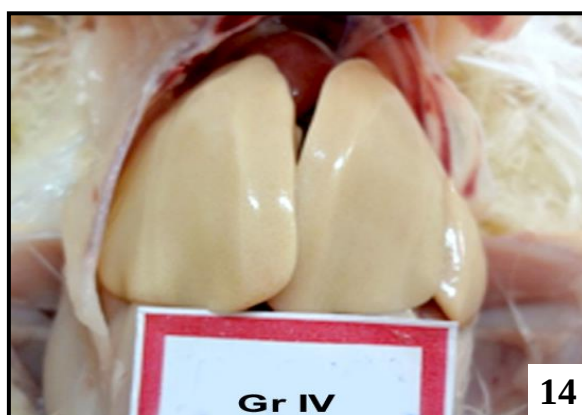


Plate 15: Gross Photograph showing pale, yellowish discoloration with soft and friable consistency of liver (Group IV and V) from bird fed with aflatoxin in comparison to normal liver from control bird (Group I) at 35th day of age.

Plate 16: Gross Photograph showing slight paleness and gradual improvement in colour and texture of liver in combined treatment groups (VI to IX) at 35th day of age.

Plate 17: Gross Photograph showing enlargement and pale yellowish discoloration of kidneys from bird fed with 1ppm of aflatoxin at 35th day of age.

Plate 18: Gross Photograph showing swollen, enlarged and congested kidneys in bird fed with 0.5 ppm of aflatoxin at 35th day of age.

Plate 19: Gross Photograph showing swelling, enlargement and paleness in kidneys in birds fed with aflatoxin 1 ppm (Group IV) and 0.5 ppm (Group V) in comparison to control birds (Group I and II).

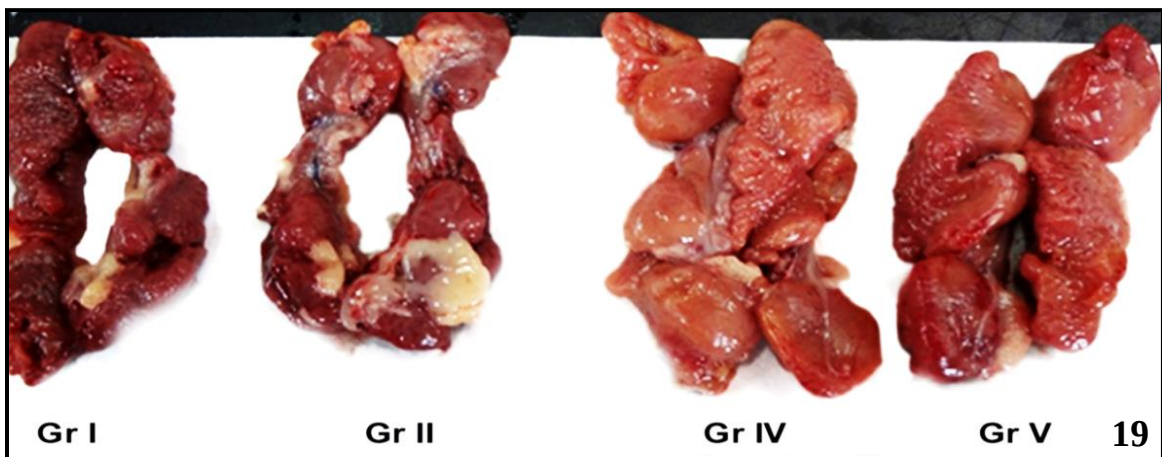


Plate 20: Gross Photograph of kidneys at 35th day of age with enlargement and paleness for comparison between control (Group I) and co-treatment with AF and DAE supplemented groups (Group VI to IX). (Note: Gradual improvement in the condition of kidneys in DAE supplemented groups (VI to IX)).

Plate 21: Gross photograph showing areas of haemorrhages in intestine from bird fed with 0.5 ppm of aflatoxin at 35th day of age.

Plate 22: Gross photograph showing mild catarrhal contents with congestion of intestine in combined treatment group fed with aflatoxin 1 ppm and 400 mg DAE/kg feed at 35th day of age.

Plate 23: Gross photograph showing marked reduction in size of thymus from bird fed with 1ppm AF (Group IV) in comparison to control (Group I) and DAE control (Group II) at 35th day of age.

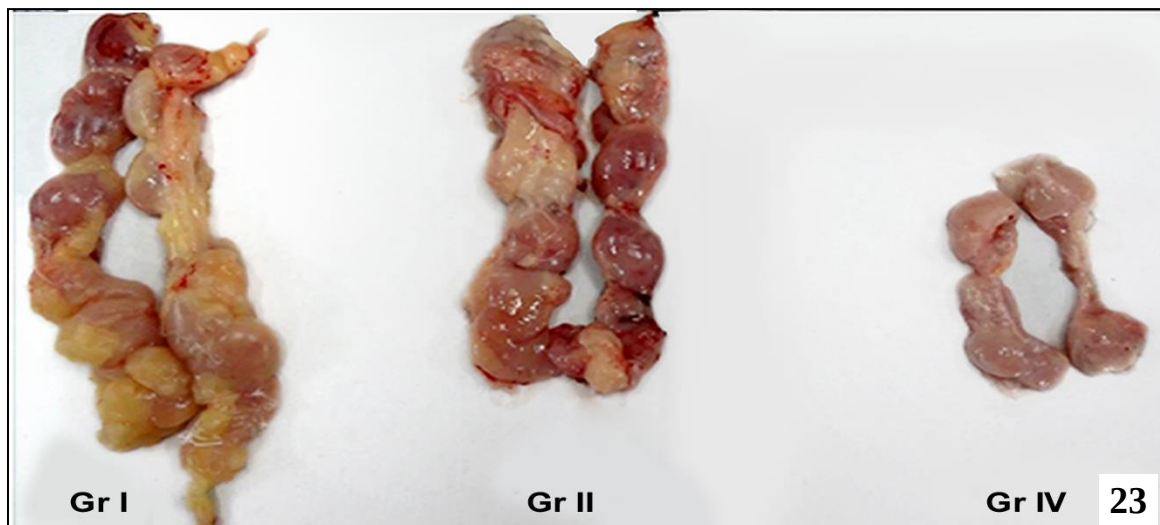
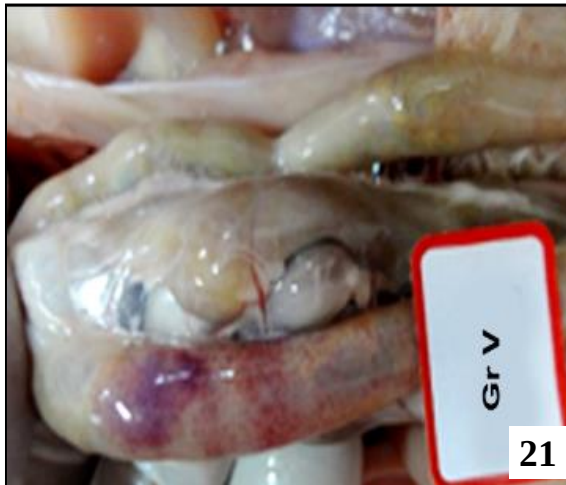
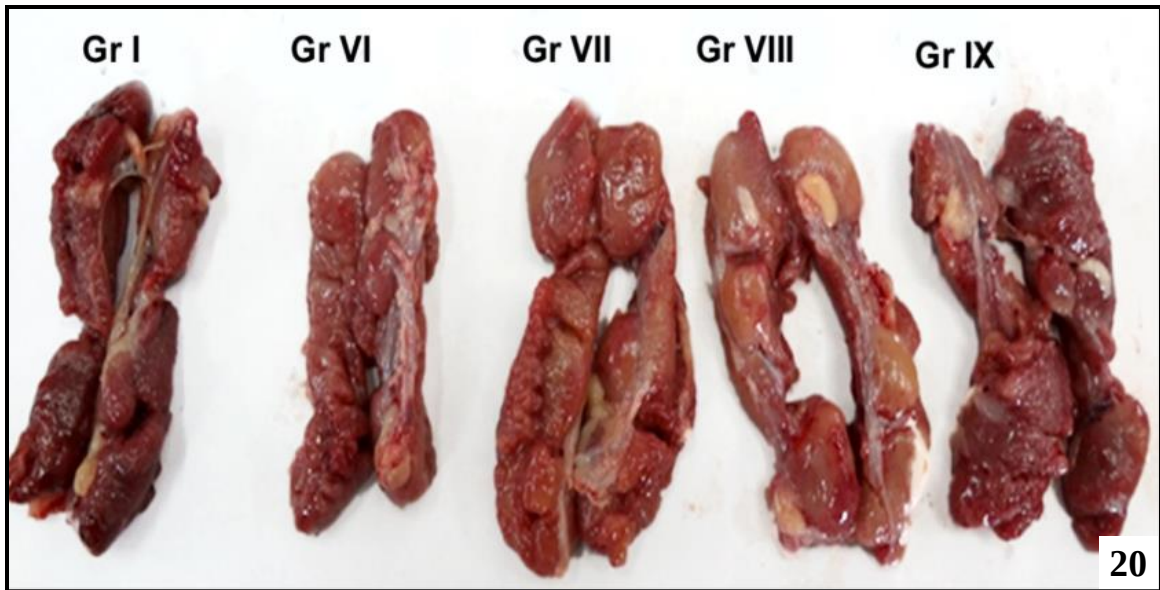


Plate 24: Gross photograph showing marked reduction in size of bursa of Fabricius of bird fed with 1ppm AF (Group IV) and 0.5 ppm (Group V) in comparison to control (Group I) and DAE control (Group II) at 35th day of age.

Plate 25: Gross photograph showing marked improvement in the size and condition of thymus of co-treatment groups (VI to IX) in comparison to reduced size of thymus of bird fed with 1ppm AF at 35th day of age.

Plate 26: Gross photograph at 35th day of age showing marked reduction in size of spleen of bird fed with 1ppm AF in comparison to control (Left). Improvement in the size and condition of spleen (Right) from bird fed with AF 1 ppm + DAE @ 800 mg/kg feed.

Plate 27: Gross photograph showing comparison of bursa of Fabricius between control (Group I) and combined treatment groups fed with AF + DAE (Groups VI to IX) at 35th day of age. (Note: Gradual improvement in the size and condition in combined treatment (AC-6 to AC-9) groups.

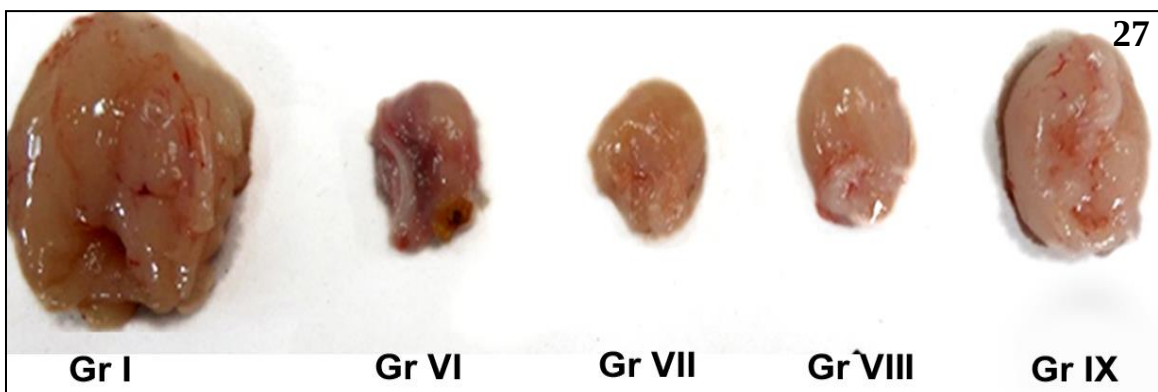
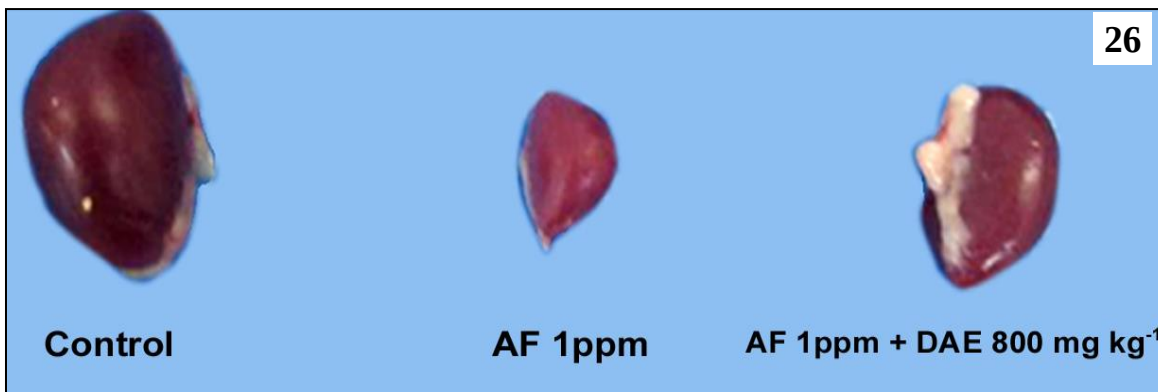
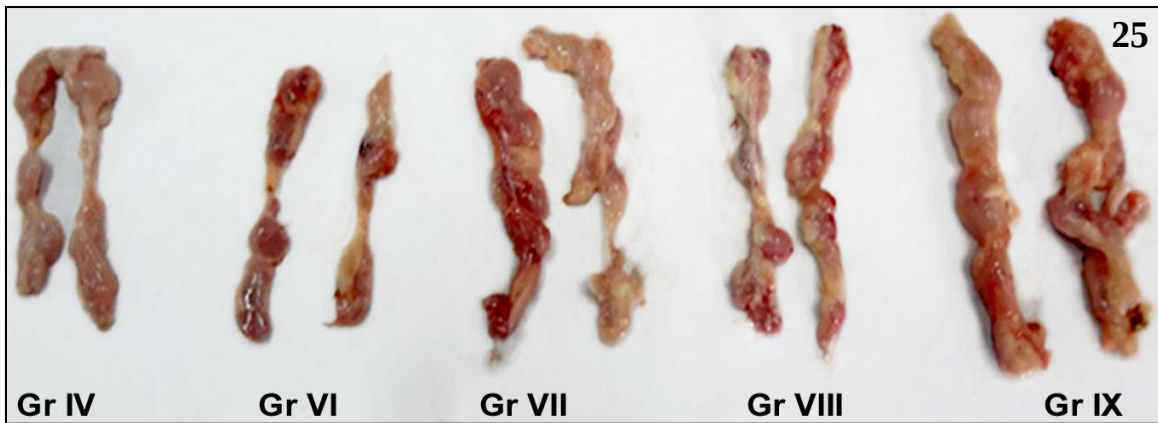


Plate 28: Section of liver from 1 ppm AF fed bird showing marked cellular swelling, cytoplasmic vacuolation, degeneration/necrosis of hepatocytes on day 14th of the experiment. (H & E x 200)

Plate 29: Section of liver from 0.5 ppm AF fed bird showing mild congestion, cellular swelling, cytoplasmic vacuolation and hepatocyte degeneration on 14th day of the experiment. (H & E x 100)

Plate 30: Section of liver from 1ppm AF fed bird showing degeneration/necrosis and glandular appearance of hepatocytes and severe infiltration of mixed inflammatory cells on 28th day of the experiment. (H & E x 200)

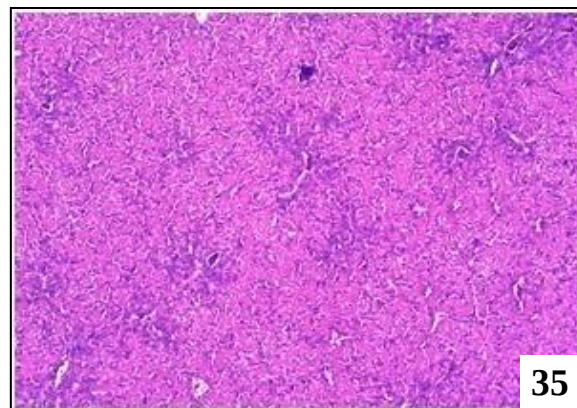
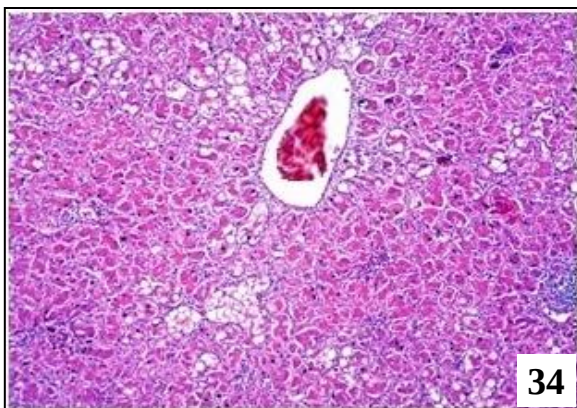
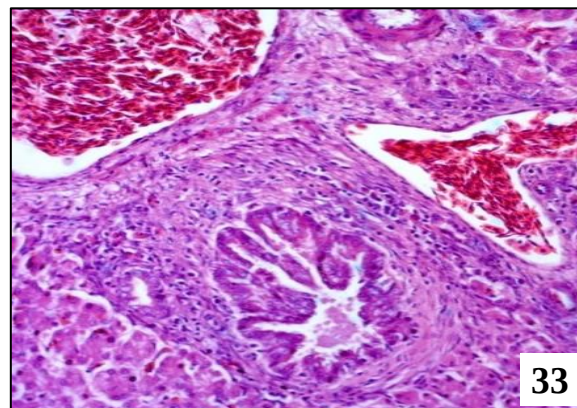
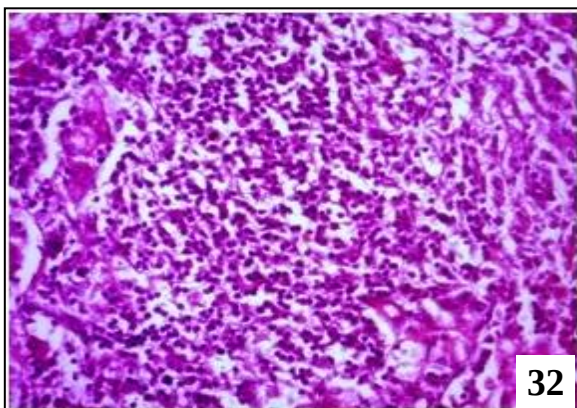
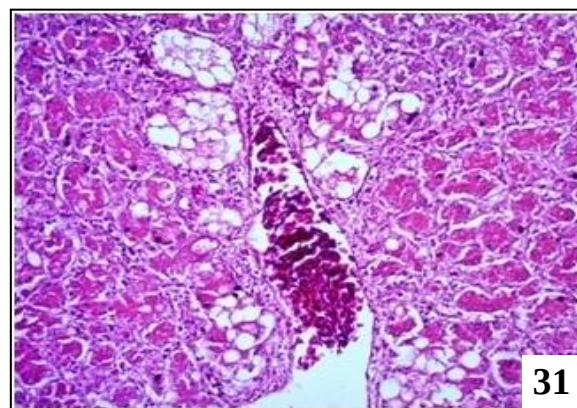
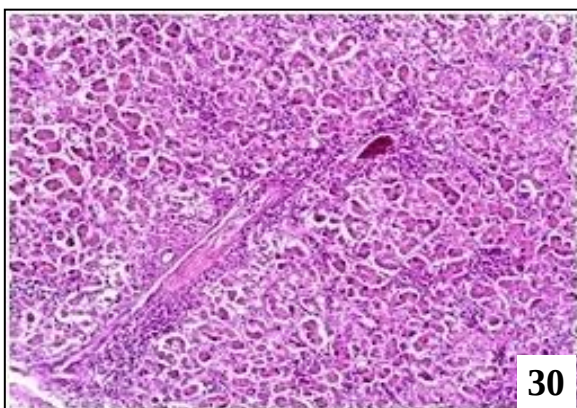
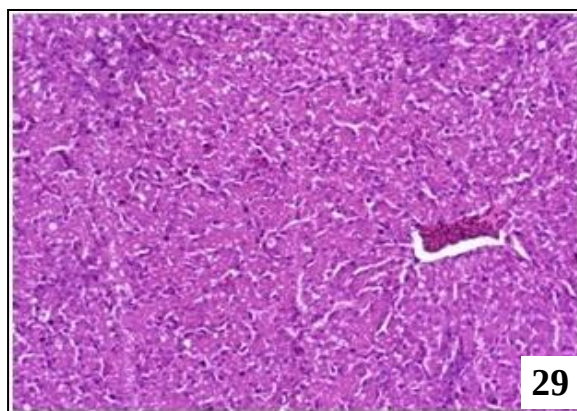
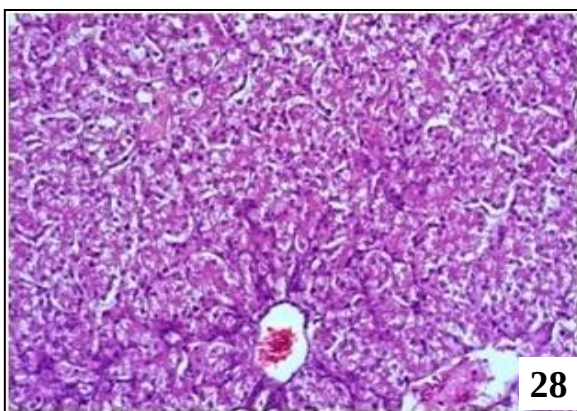
Plate 31: Section of liver from 1ppm AF fed bird showing congestion, severe fatty change, necrosis and glandular arrangement of hepatocytes on 35th day of the experiment. (H & E x 200)

Plate 32: Section of liver from 1ppm AF fed bird showing degeneration/necrosis, glandular arrangement of hepatocytes and severe inflammatory reaction (predominantly mononuclear cells) at 35th day of the experiment. (H & E x 400)

Plate 33: Section of liver from 0.5 ppm AF fed bird showing vascular congestion, bile duct hyperplasia, periductular fibroplasia and lymphocytic infiltration, and degeneration of hepatocyte at 35th day of the experiment. (H & E x 200)

Plate 34: Section of liver from bird fed with 0.5 ppm AF showing congestion, fatty change, degeneration/necrosis, glandular arrangement of hepatocytes and inflammatory reaction on 35th day of the experiment. (H& E x 200)

Plate 35: Section of liver from bird fed with 1ppm AF and 800 mg DAE/kg feed showing near normal hepatocytes and minimal sinusoidal and vascular congestion on 14th day of the experiment. (H & E x 100)



- Plate 36: Section of liver from bird fed with 0.5 ppm AF and 400 mg of DAE/ kg feed showing improvement in architecture of liver on 28th day of the experiment. (Also note mild congestion of sinusoidal space) (H & E x 100)
- Plate 37: Section of liver from bird fed with 1ppm AF and 800 mg DAE/ kg feed showing marked improvement in hepatocytes and minimal sinusoidal congestion on 35th day of the experiment (H & E x 200)
- Plate 38: Section of liver from bird fed with 0.5 ppm AF and 800 mg DAE/kg feed showing improvement in hepatocytes on 35th day of the experiment. (Also note mild congestion of sinusoidal space) (H & E x 100)
- Plate 39: Section of kidney from bird fed with 1ppm AF showing moderate haemorrhages and tubular epithelial degeneration/desquamation on 14th day of the experiment. (H & E x 200)
- Plate 40: Section of kidney from bird fed with 0.5 ppm AF showing mild haemorrhages, glomerular swelling and tubular epithelial degeneration/desquamation on 14th day of the experiment. (H & E x 200)
- Plate 41: Section of kidney from bird fed with 1 ppm AF showing mild haemorrhages, glomerular swelling and tubular epithelial degeneration/desquamation on 35th day of the experiment. (H & E x 200)
- Plate 42: Section of kidney from bird fed with 0.5 ppm AF showing congestion, haemorrhages, glomerular degeneration and desquamation of tubular epithelial cells on 35th days of the experiment. (H & E x 400)
- Plate 43: Section of kidney from bird fed with 0.5 ppm AF supplemented with 800 mg DAE/kg showing almost normal architecture of renal tubules and mild congestion on 28th days of the experiment. (H & E x 100)

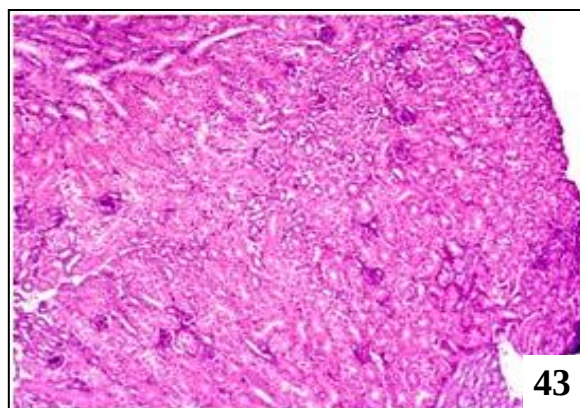
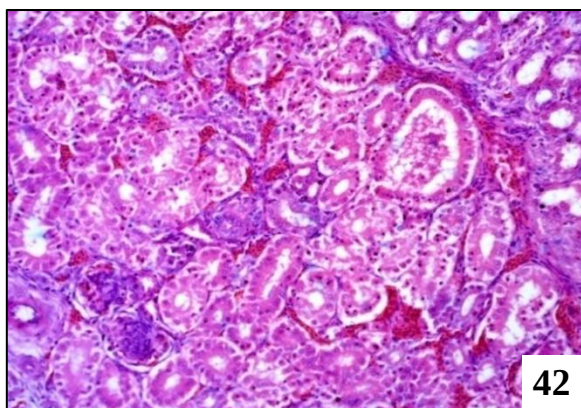
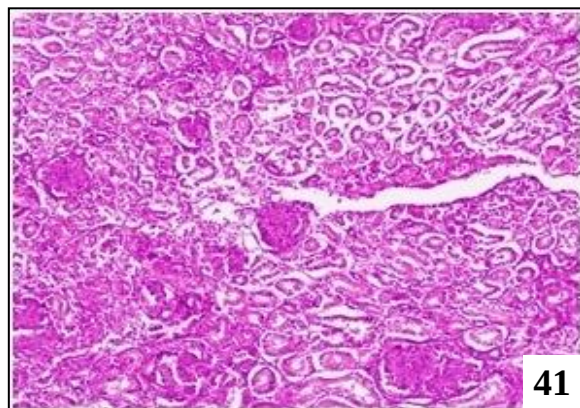
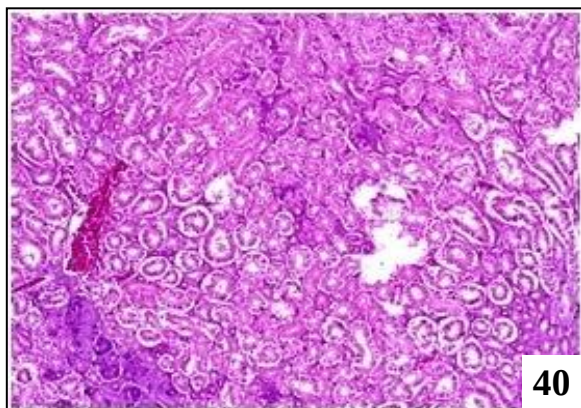
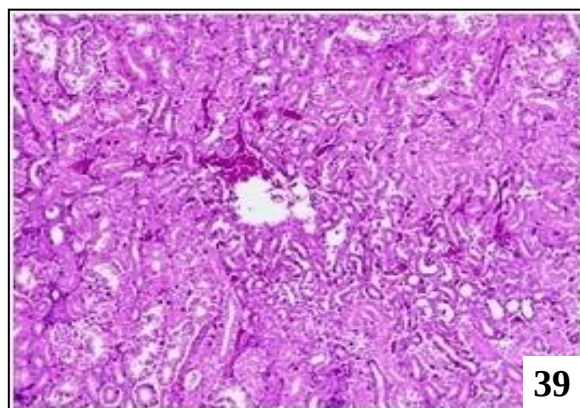
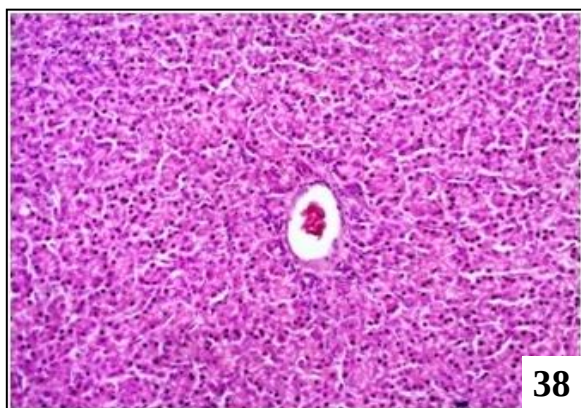
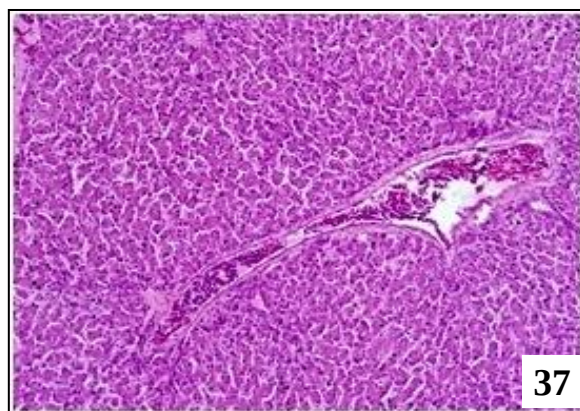
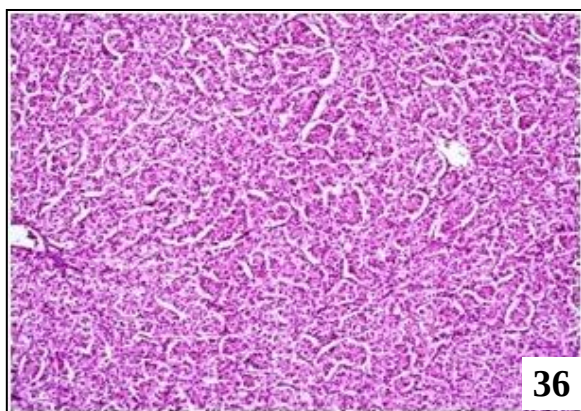


Plate 44: Section of kidney from bird fed with 1 ppm AF and 800 mg of DAE/kg feed showing mild haemorrhages and tubular degeneration on 35th day of the experiment. (H & E x 200)

Plate 45: Section of kidney from bird fed with 0.5 ppm AF and 800 mg DAE/kg feed showing almost normal architecture of glomeruli and tubules on 35th day of the experiment. (H & E x 200)

Plate 46: Section of heart from bird fed with 0.5 ppm AF showing mild haemorrhages and a few inflammatory cells on 28th day of the experiment. (H & E x 200)

Plate 47: Section of heart from bird fed with 1 ppm AF showing moderate haemorrhages on 35th day of the experiment. (H & E x 400)

Plate 48: Section of heart from bird fed with 1 ppm AF and 400 mg DAE/kg feed showing mild congestion and haemorrhages on 35th day of the experiment. (H & E x 100)

Plate 49: Section of heart from bird fed with 0.5 ppm AF and 400 mg DAE/kg feed showing mild congestion, haemorrhages and a few inflammatory cells on 35th day of the experiment. (H & E x 100)

Plate 50: Section of heart from bird fed with 1 ppm AF and 800 mg DAE/kg feed showing mild haemorrhages on 35th day of the experiment. (H & E x 100)

Plate 51: Section of pancreas from bird fed with 1 ppm AF showing severe congestion and moderate degeneration of acinar cells on 35th day of the experiment. (H & E x 200)

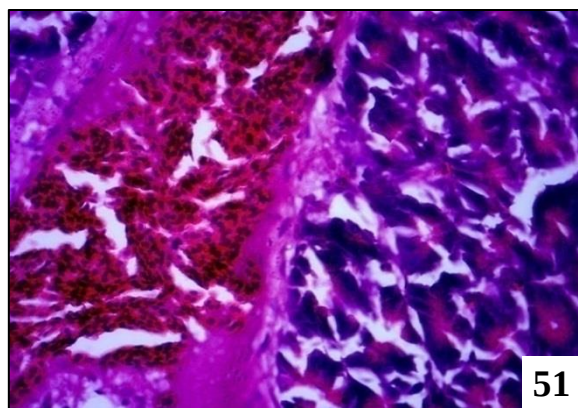
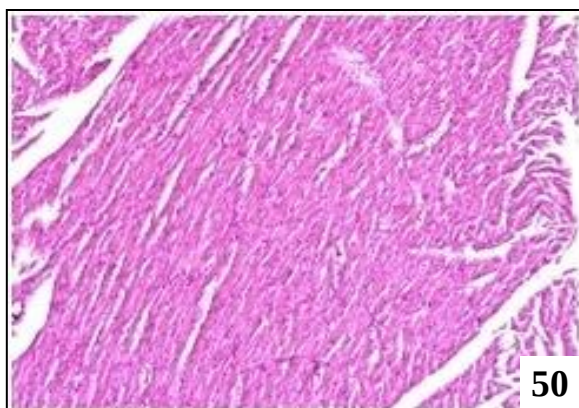
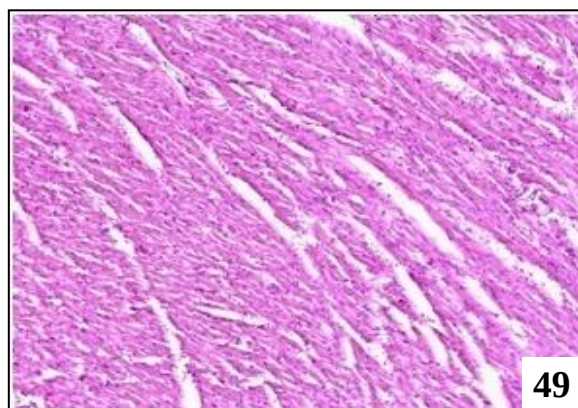
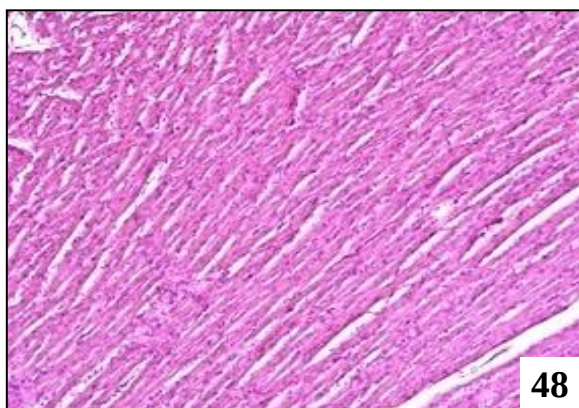
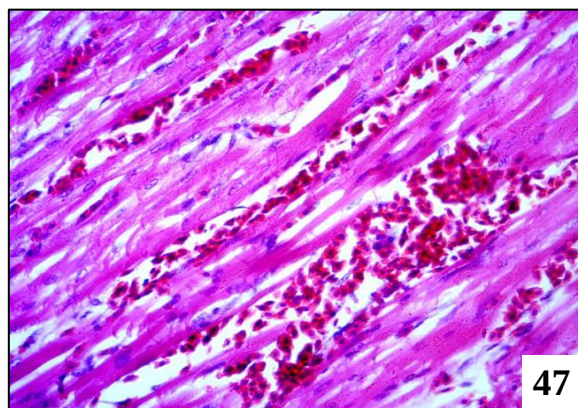
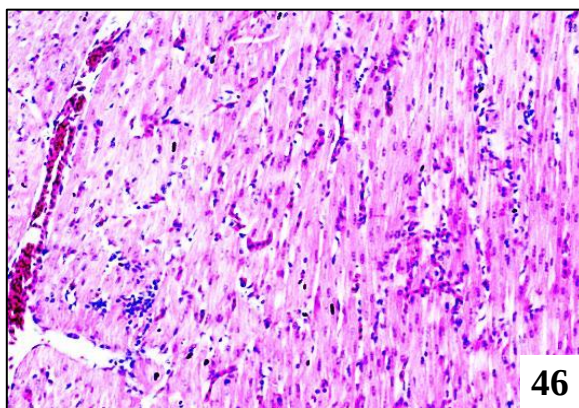
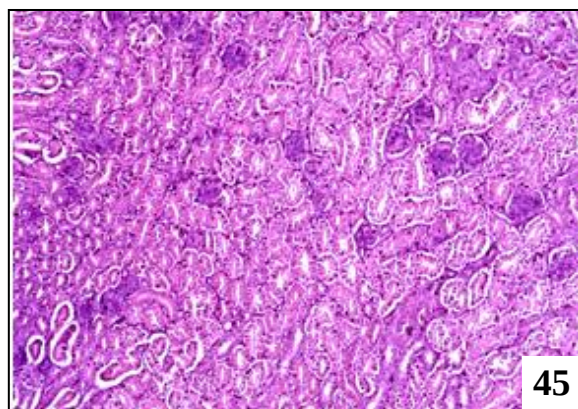
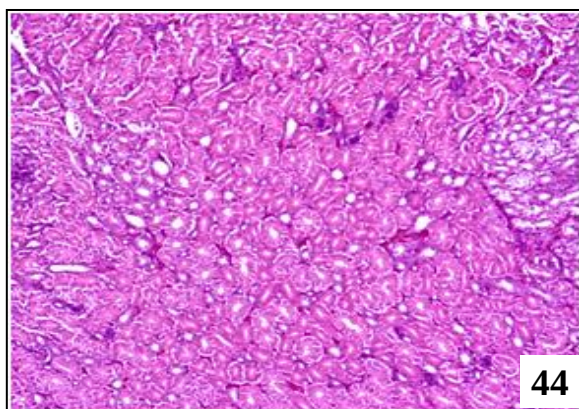


Plate 52: Section of pancreas from bird fed with 1 ppm AF showing pancreatic duct epithelial hyperplasia and moderate degeneration of acinar cells 35th day of the experiment. (H & E x 200)

Plate 53: Section of pancreas from bird fed with 0.5 ppm AF showing moderate vacuolar degeneration and disintegration of acinar cells on 35th day of the experiment. (H & E x 100)

Plate 54: Section of pancreas from bird fed with 1 ppm AF and 800 mg DAE/kg feed showing marked improvement in acinar cells on 35th day of the experiment. (H & E x 200)

Plate 55: Section of intestine from bird fed with 1 ppm AF showing sub-epithelial lymphoid aggregation and severe villus destruction on 35th day of the experiment. (H & E x 100)

Plate 56: Section of intestine from bird fed with 0.5 ppm AF showing severe villus degeneration and sub-epithelial lymphoid aggregation on 35th day of the experiment. (H & E x 200)

Plate 57: Section of intestine from bird fed with 1 ppm AF and 400 mg DAE/kg feed showing mild degree of degeneration of villus epithelium on 28th day of the experiment. (H & E x 100)

Plate 58: Section of intestine from bird fed with 1 ppm AF and 800 mg DAE/kg feed showing marked improvement in villi and minimal infiltration on 35th day of the experiment (H & E x 400)

Plate 59: Section of thymus from bird fed with 0.5 ppm AF showing moderate haemorrhages and depletion and lysis of lymphocytes on 14th day of the experiment (H & E x 200)

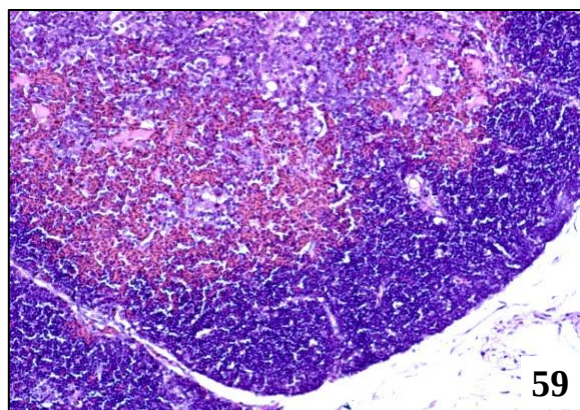
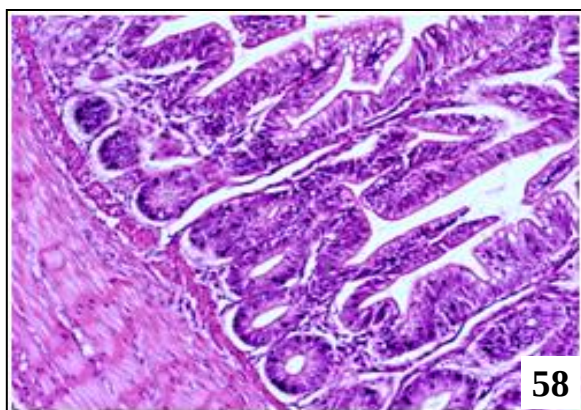
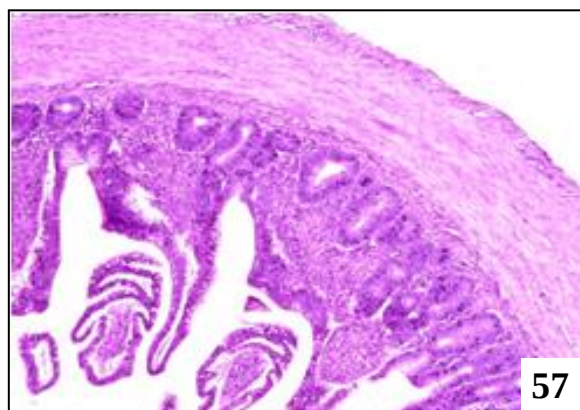
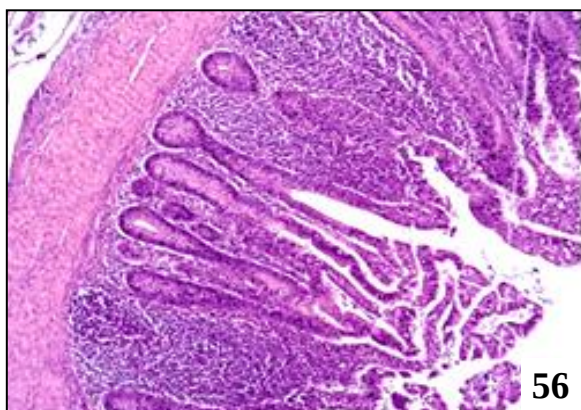
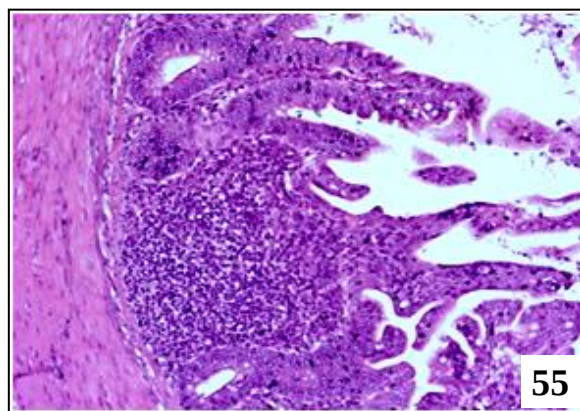
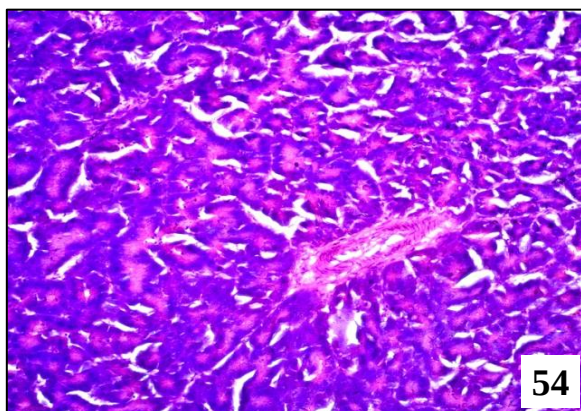
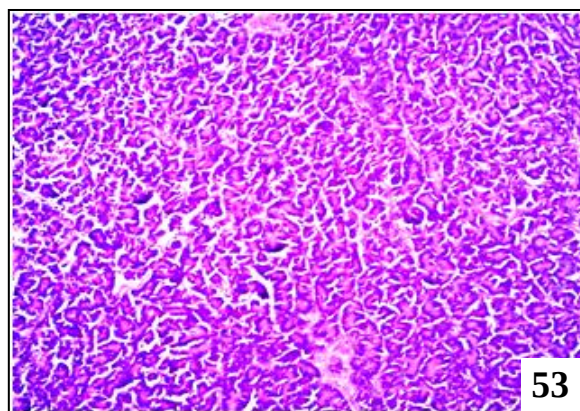
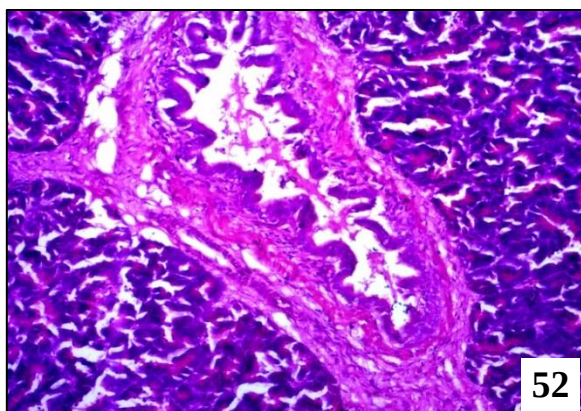


Plate 60: Section of thymus from bird fed with 1 ppm AF showing severe haemorrhages, depletion/lysis of lymphocytes with no differentiation of cortex and medulla on 28th day of the experiment. (H & E x 200)

Plate 61: Section of thymus from bird fed with 1 ppm AF showing depletion/lysis of lymphocytes on 35th day of the experiment. (H & E x 200)

Plate 62: Section of thymus from bird fed with 0.5 ppm AF showing haemorrhages and lymphocytolysis on 28th day of the experiment. (H&E x 200)

Plate 63: Section of thymus from bird fed with 1 ppm AF and 400 mg of DAE/kg feed showing mild congestion, presence of normal lymphocytes population and, differentiation of cortex and medulla on 14th day of the experiment. (H & E x 100)

Plate 64: Section of thymus from bird fed with 0.5 ppm AF and 400 mg DAE/kg feed showing mild congestion, presence of normal lymphocytes population and differentiation of cortex and medulla on 35th day of the experiment. (H & E x 100)

Plate 65: Section of thymus from bird fed with 1 ppm AF and 800 mg DAE/kg feed showing mild congestion and presence of normal lymphocytes population on 35th day of the experiment (H & E x 100)

Plate 66: Section of spleen from bird fed with 1 ppm AF showing marked lymphocytolysis and depletion of lymphocytes on 35th day of the experiment. (H & E x 100)

Plate 67: Section of spleen from bird fed with 0.5 ppm AF showing lysis and depletion of lymphocytes on 35th day of the experiment. (H & E x 100)

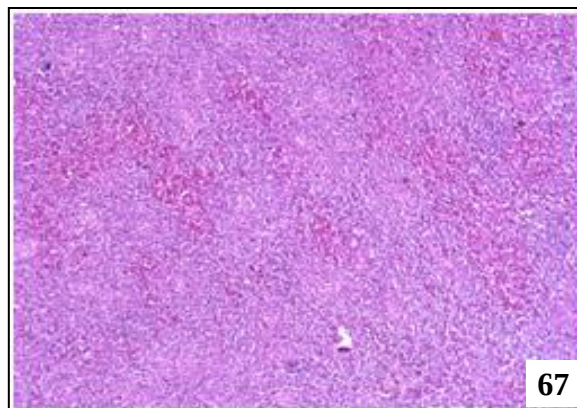
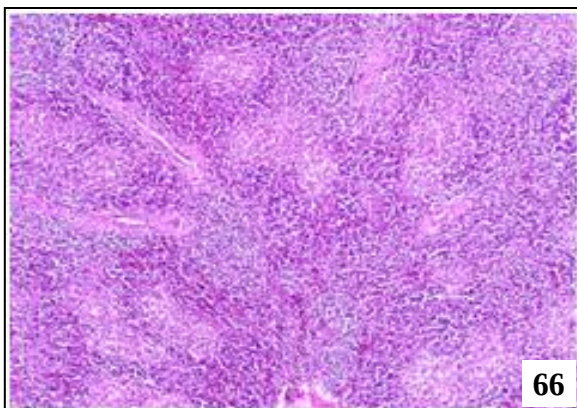
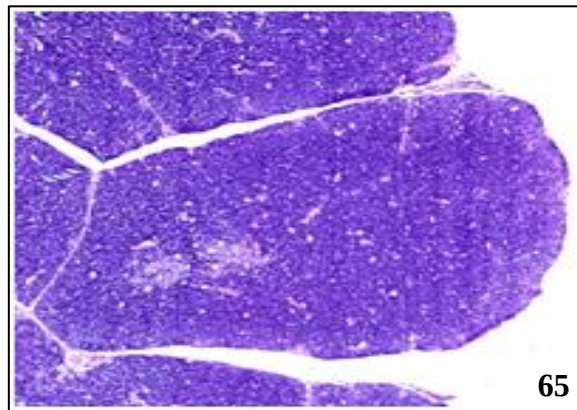
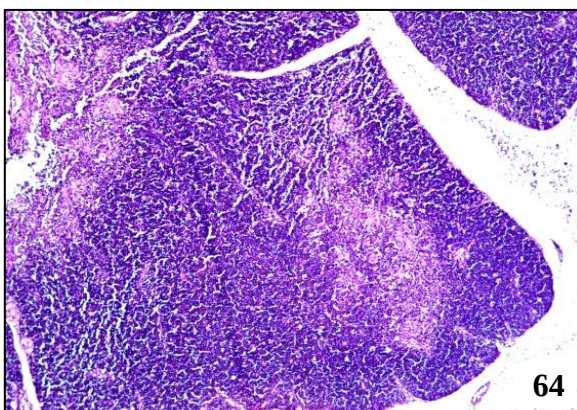
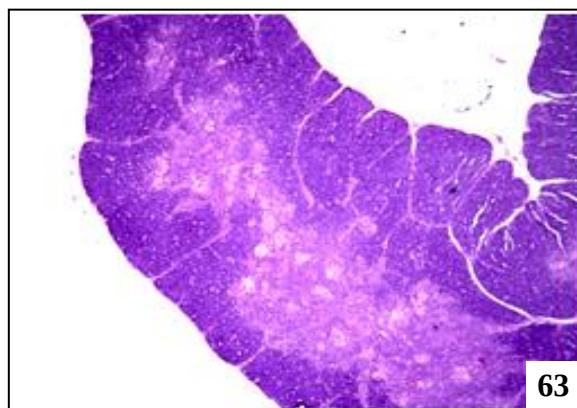
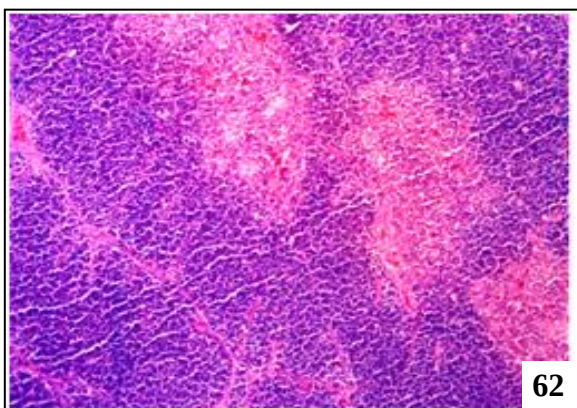
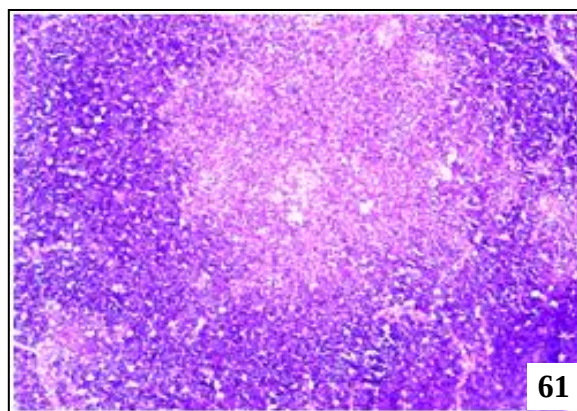
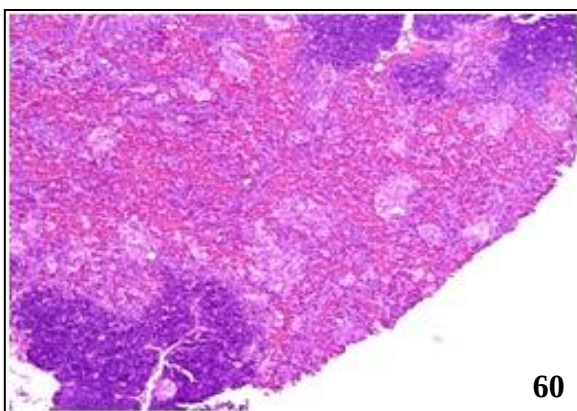


Plate 68: Section of spleen from bird fed with 1 ppm AF and 400 mg DAE/kg feed showing mild congestion on 35th day of the experiment. (H & E x 100)

Plate 69: Section of spleen from bird fed with 0.5 ppm AF and 800 mg DAE/kg feed showing marked depletion of lymphocytes and hyperplastic changes and formation of secondary lymphoid follicles on 35th day of the experiment. (H & E x 200)

Plate 70: Section of bursa of Fabricius from bird fed with 1 ppm AF showing depletion of lymphocytes on 21th day of the experiment. (H & E x 400)

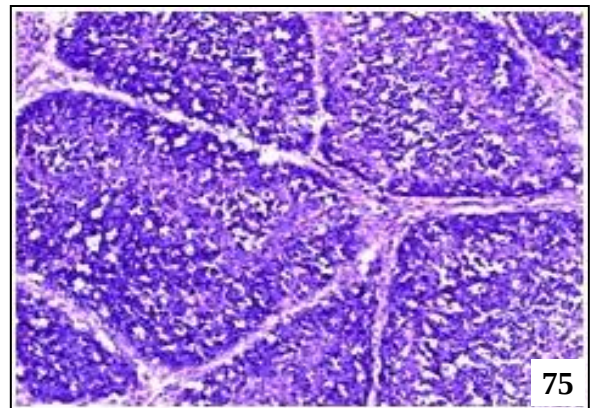
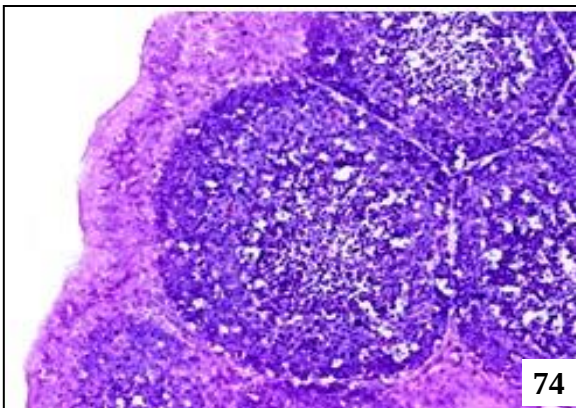
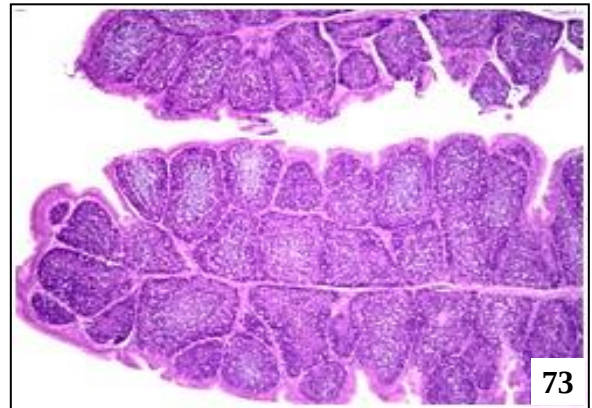
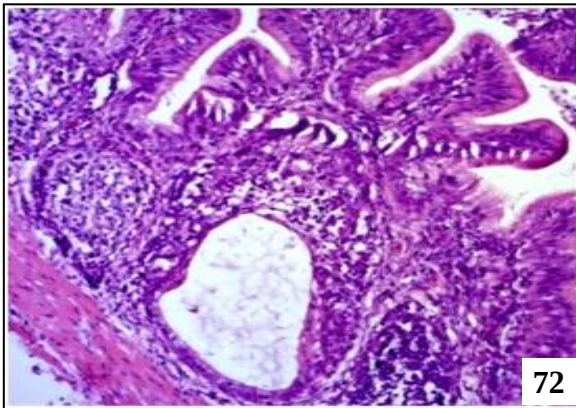
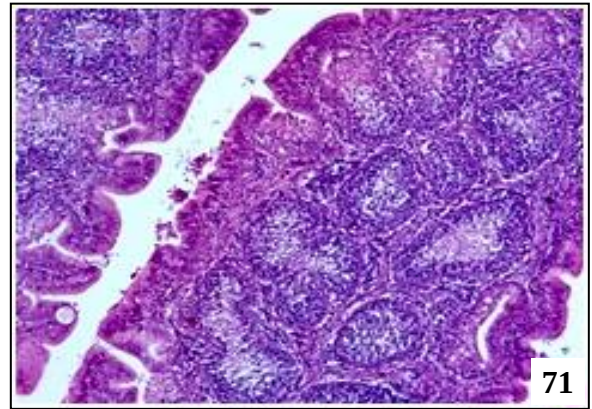
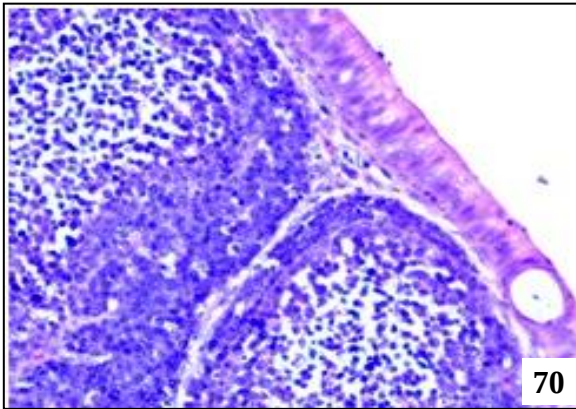
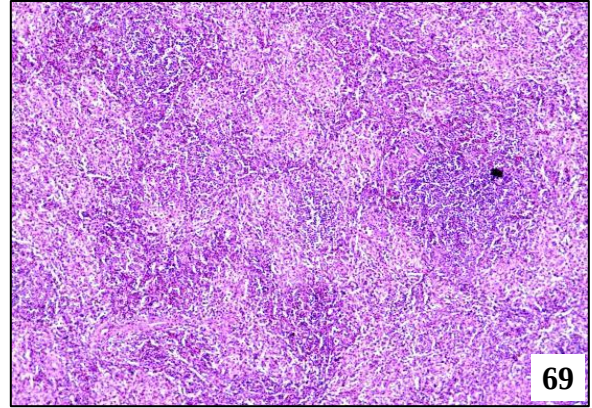
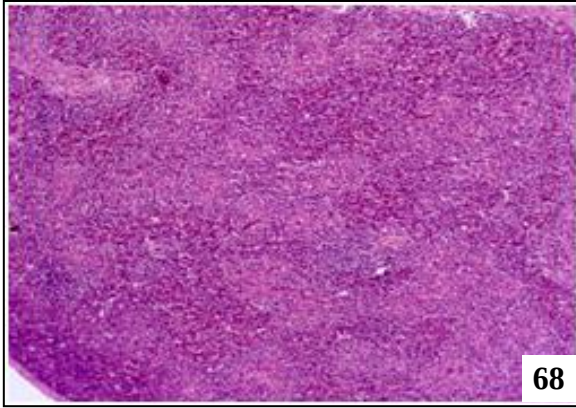
Plate 71: Section of bursa of Fabricius from bird fed with 1 ppm AF showing marked depletion of lymphocytes on 35th day of the experiment. (H & E x 100)

Plate 72: Section of bursa of Fabricius from bird fed with 1 ppm AF showing lymphocyte depletion, lymphocytolysis, well defined microcyst within the follicle and corrugation of plical epithelium on 35th days of the experiment. (H & E x 400)

Plate 73: Section of bursa of Fabricius from bird fed with 0.5 ppm AF showing mild depletion of lymphocyte population on 14th day of the experiment. (H & E x 100)

Plate 74: Section of bursa of Fabricius from bird fed with 1 ppm AF and 800 mg DAE/kg feed showing normal lymphocyte population within the follicles on 35th day of the experiment. (H & E x 200)

Plate 75: Section of bursa of Fabricius from bird fed with 0.5 ppm AF supplemented with 800 mg DAE/kg feed showing normal lymphocyte population within the follicles on 35th day of the experiment. (H & E x 200)



Discussion



V. DISCUSSION

The present study was undertaken to evaluate the efficacy of diatomaceous earth in ameliorating the toxic effects of aflatoxin in broilers to assess the performance parameters, serum biochemistry, relative organ weight, immune response through CD4+, CD8+ cell count, antibody titre against NDV and IBDV, and gross and histopathological changes in various organs.

Aflatoxins are a group of closely related frequently occurring mycotoxin produced by the toxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus*. These are considered to be unavoidable natural contaminant of food and feed causing serious health effects on human, animals and birds (Oguz and Kurtoglu, 2000; Anjum *et al.*, 2012). The degree of aflatoxicosis depend on the dose of aflatoxin ingested, duration of exposure and a variety of other factors including physiological, environmental and disease factors that has impact on the uptake, biotransformation, deposition and excretion of aflatoxin (Bryden, 2012). Aflatoxins exhibit a wide array of biological effects and can be mutagenic, carcinogenic, embryotoxic, nephrotoxic, estrogenic and immunosuppressive even at low concentrations (Jayabarathi and Parveen, 2010; Mohmand *et al.*, 2010). Like other animals, poultry is also sensitive to aflatoxins and their susceptibility is only, after ducks and turkeys (Arafa *et al.*, 1981; Dalvi, 1986; Leeson *et al.*, 1995). Aflatoxin has a special significance in tropical areas of India, where environmental conditions as well as pre- and post-harvest practices of grains are suitable for mould growth and toxin contamination in poultry feeds (Devegowda *et al.*, 1994).

Aflatoxin contaminated diet may cause huge economic losses to poultry producers due to reduced weight gain, interference with reproductive performances, impaired feed

conversion and increased mortality (Oguz, 2012). Several studies have been carried out to reduce the toxicity of AF in broilers and many commercial products have been investigated to bind as well as absorb AF in gastrointestinal tract (Schell *et al.*, 1993; Ramos and Hernandez, 1997). Adsorbents have been proven to be the most promising and economical approach of preventing aflatoxicosis in poultry.

In the present study, diatomaceous earth was used to study the ameliorating effect on aflatoxin in diet. Diatomaceous earth refers to a naturally-formed sedimentary mineral coming from the remains of what were once oceanic unicellular shells and algae known as diatoms. Diatoms, the ocean's "spiny honeycombs," are over 30 million years old and are formed when these microscopic algae-like plants died and remained compounded in the earth's surface as skeletal remains (Grant and Phillips, 1998). These organisms, much like a mollusc, emit lime-carbonate, and had the ability to emit silica. These clay-like, chalky remains are referred to as diatomite (Eralsan *et al.*, 2006). Diatomaceous earth, a clay mineral of the smectite group, is formed of highly colloidal material composed of mainly montmorillonite and is produced by *in-situ* diversification of volcanic ash. It has the unique characteristic of swelling to several times its original volume when placed in water and of forming thixotropic gels with water even though the amount of clay is relatively less. DAE possesses higher adsorptive capacity due to its structural composition, which can be responsible for the binding to mycotoxins in the feed materials.

In the earlier reports, different concentrations of DAE ranging from 500 mg to 5 g/kg feed have been used to study the amelioration of aflatoxicosis in broilers (Modirsanei *et al.*, 2008; Denli *et al.*, 2009; Silambarasan *et al.*, 2013). In the light of the above, in the present study DAE was used at concentrations of 400 and 800 mg/kg feed.

5.1 FUNGUS CULTURE

Aflatoxin used in this study was produced by inoculating the rice with culture of *Aspergillus parasiticus* (MTCC 2796). This technique had been widely used to produce aflatoxin for experimental purposes (Mohmand, 2010, Yalagod, 2014). The growth characteristics, toxin producing ability of this toxigenic fungi and toxin yield correlated with the reported literature (Shotwell *et al.*, 1966, Narendrakumar and Dhanapani, 2011; Yalagod, 2014). The fungal culture on rice yielded 25 ppm of aflatoxin in the present study.

5.2 TOXICITY TRIAL

5.2.1 GROWTH PERFORMANCE

5.2.1.1 BODY WEIGHT

In the present study, the performance of the birds was not affected by the addition of DAE alone in the diet indicating the inert and non-toxic property of diatomaceous earth. Body weight gain decreased in the birds fed diet containing AF in comparison with control group. Within the toxin fed birds, the weight gain was lower in 1 ppm in comparison to 0.5 ppm of AF indicating the dose dependent effect of aflatoxin on the body weight. These findings are in agreement with the results of previous reports on aflatoxicosis in poultry (Philips, 1999; Raju and Devegowda, 2000; Shi *et al.*, 2006; Modirsanei *et al.*, 2008; Denli *et al.*, 2009; Wafaa *et al.*, 2013; Kumar Dhanapal *et al.*, 2014, Yalagod, 2014).

The depression in body weight gain upon feeding of aflatoxin could be attributed to anorexia, restlessness, reduced protein and lipid synthesis (Thaxtone *et al.*, 1974);

reduced protein synthesis as reported by Verma *et al.* (2002); increased lipid excretion in dropping, impaired nutrient absorption and reduced pancreatic digestive enzyme production (Osborne and Hamilton, 1981) and reduced appetite as suggested by Sharline *et al.* (1980). Hassan *et al.* (2000) also opined that the toxicity of aflatoxin was characterised by reduction in body weight gain as aflatoxin interfere with metabolic pathway through the inhibition of protein synthesis and enzyme system that is involved in carbohydrate and energy release. Nelson *et al.* (1982) postulated that aflatoxin reduces the ability of birds to digest dry matter and amino acids and utilize energy from aflatoxin contaminated ration.

Dietary inclusion of DAE improved body weight gain of aflatoxin fed birds. The basic mechanism for protective effect of DAE against toxicity of AF appears to be sequestration of AF in the gastrointestinal tract and chemisorption to the adsorbent (similar to other adsorbent such as hydrated sodium calcium aluminosilicate), which reduces the bioavailability of aflatoxin (Philips *et al.*, 1988; Kubena *et al.*, 1990; Ledoux *et al.*, 1999).

Silambarasan *et al.* (2013) studied the efficacy of diatomaceous earth, sodium bentonite and zeolite alone or in combination as aflatoxin adsorbents in the diets of broiler chickens containing 300 ppb aflatoxin for a period of 42 days and reported increased body weight gain following incorporation of DAE in AF mixed diet. Further, the study revealed that DAE was least effective in ameliorating the toxic effects of AF in comparison to the other binders.

Contrary to the findings of the present study, Denli and Okan, (2006) reported that addition of diatomite to diets containing AFB₁ did not ameliorate toxic effects. These

differences in results are possibly due to the differences in the cationic compounds or chemical content of the adsorbents tested.

5.2.1.2 FEED CONVERSION RATIO

The conversion of feed to live weight is a complex process and the cause of poor or high FCR is usually multi-factorial involving various aspects of management, health and nutrition. The quality of ingredients of feed is the foremost factor from nutrition point of view in the broilers. The use of ingredients infested with mycotoxin(s) may cause adverse effect on the FCR in birds (Service Bulletin, 2011).

The feed conversion ratio of aflatoxin fed birds in the present study was significantly higher in comparison with that of control groups. The increased FCR could be attributed to the liver damage, inhibition of protein synthesis and lipogenesis by aflatoxin in birds as reported by earlier workers (Miazzo *et al.*, 2000; Rosa *et al.*, 2001; Santhosh Kumar, 2003; Shi *et al.*, 2006; Mohmand, 2010; Yunus *et al.*, 2011; Pourelmi, 2013; Yalagod 2014). Johri *et al.* (1996) recorded decreased amino acid and dry matter digestibility, nutrient and energy utilization in broiler fed mycotoxins contaminated diet.

In the present study, dietary inclusion of DAE improved the FCR of aflatoxin fed birds indicating the role of DAE in amelioration of toxin, as reported earlier (Modirsanei *et al.*, 2008; Silambarasan *et al.*, 2013; Manafi and Khosravinia, 2013).

Study conducted on supplementation of AflaDetox (a natural product obtained from diatomaceous earth extracted from a quarry) @ 1, 2, and 5 g of AflaDetox/kg feed to 1 ppm of AFB₁ contaminated feed, significantly improved the feed intake, body weight gain, and feed conversion rate in AFB₁ fed broilers. The improvement in the performance

of the broilers suggested the ameliorating effect of DAE on AF fed broiler birds and could be attributed to the presence of highly porous surface of DAE with high cationic interchange capacity that yields a higher *in-vitro* adsorption of aflatoxin (Denli *et al.*, 2009).

Manafi and Khosravinia (2013) reported that herbal mycotoxin binder (HMB) comprising of combination extra purified clay containing diatomaceous earth, antioxidants (curcuminoids extracted from turmeric) and enzymes (Epoxidases and Esterases) can cause the abatement of AFB₁ deteriorative effect in broiler chicken by improvement in feed consumption and concluded that presence of diatomaceous earth in the binder might be responsible for prevention of aflatoxin adsorption in the gut.

5.2.2 CLINICAL SIGNS

In the present study, the clinical signs of inappetance, reduced growth, lethargy, dullness, dropped wings, huddling, ruffled feathers, poor growth and diarrhoea were observed in AF fed groups (Group IV and V). Similar findings were consistently reported in aflatoxicosis by several workers (Devegowda, 1994; Prakash 2001; Ortatatli *et al.*, 2005; Zahid Hussain *et al.*, 2008; Nathanael and Vardhani, 2011; Yalagod, 2014).

The reduced severity of the clinical manifestation of aflatoxicosis and the improvement in the feather quality was observed in the DAE fed birds and perhaps indicated the beneficial effect of DAE in the toxin fed birds. Modirsanei *et al.* (2008) and Denli *et al.* (2009) also reported the beneficial effects of DAE in the aflatoxin fed birds.

5.2.3 RELATIVE ORGAN WEIGHTS

Organ weight is one of the most sensitive parameter to study the toxic effect of an experimental compound, as significant differences in organ weight between treatment and untreated (control) groups may occur in the absence of any morphological changes. The comparison of the organ weights of treated with untreated groups is often complicated by differences in body weights between groups. Therefore, other parameters that are commonly used for analysis of organ weight are the ratio of the organ weight to body weight (to account for differences in body weight). These ratios are generally described as relative organ weights (Bailey *et al.*, 2004). Most of the mycotoxins at varying dose levels are known to cause alterations in relative organ weights. A significant increase in the relative organ weight of various organ systems is one of the important toxic effects of aflatoxicosis in chicken.

In the present study, the birds fed with aflatoxin (Group IV and Group V) revealed increased relative weight of liver and kidneys. These findings are in accordance with those of earlier workers (Kubena *et al.*, 1995; Ortatatli and Oguz, 2001; Rosa *et al.*, 2001; Santhosh Kumar, 2003; Shivappa, 2005; Pradeep Kumar, 2007 and Senthil Kumar, 2007; Mohmand, 2010; Yalagod, 2014).

The increase in the relative weight of liver in aflatoxin alone treated birds could be attributed to the aflatoxin induced impaired fat metabolism in the liver with an increase in the fat content of the hepatocytes (Hsieh, 1979). The hepatic lipodosis is primarily mediated through inhibition of phospholipids synthesis and cholesterol. This in-turn affects the transportation of lipid from the liver (Manegar *et al.*, 2010). In addition, vascular, degenerative and inflammatory changes incited by AF in visceral organs might

be responsible for increased weights and this observation drew support from the histopathological lesions of severe fatty change and lipidosis recorded during the different interval in the present study.

Increase in the relative weight of kidney in the present study could be due to renal damage with development of vacuolar degeneration during aflatoxicosis. (McKenzi *et al.*, 1998; Prakash, 2001; Santhosh Kumar, 2003; Shivappa, 2005; Pradeep Kumar, 2007; Senthil Kumar, 2007; Yalagod, 2014).

Increase in the relative weight of heart in the present study corroborated with the earlier findings of enlargement of heart in aflatoxicosis in broiler birds (Huff *et al.*, 1992; Santurio *et al.*, 1999; Abousadi *et al.*, 2007; Sakhare *et al.*, 2007; Sharghi and Manafi, 2011).

Contrary to the findings in the present study, Kumar Dhanapal *et al.* (2014) reported no significant change in the relative weight of heart following feeding of 1 ppm of aflatoxin for a period of 35 days. However, Modirsanei *et al.* (2008) reported decreased relative weight of the heart following feeding of aflatoxin @ 1 ppm in diet for a period of 42 days and no reasonable explanation was provided for the decreased weight of heart. In the present study, increase in the relative weight of heart could possibly be attributed to the vascular and inflammatory reaction observed histologically in aflatoxin fed birds.

Increase in the relative weights of pancreas in the present study could be due to the damage to pancreas as evident by histopathological changes characterised by severe vascular congestion, degeneration of the acinar cells and infiltration of mononuclear cells

as also observed and documented by Valchev *et al.* (2013b); Marchioro *et al.* (2013) in broilers and Valchev *et al.* (2013c) in mulard ducks with experimental aflatoxicosis.

In the present study, the weights of thymus, spleen and bursa of Fabricius were significantly decreased in aflatoxin fed birds. This could be attributed to the toxic effect of AF on lymphoid component evident as of lymphocytolysis, which accounted for a numerical decrease in lymphocytes within the follicles. Similar changes in lymphoid organs was also documented by Kubena *et al.* (1990); Ortatatli and Oguz, (2001); Santhosh Kumar, (2003); Shivappa, (2005); Pradeep Kumar, (2007); Senthil Kumar, (2007) and Yalagod, (2014).

The microscopic findings such as lymphoid necrosis and depletion observed in all the lymphoid organs of toxin fed birds in the present study substantiate the regression recorded in their relative weights.

Contrary to the findings of the present study, significant increases in the relative weight of spleen in broilers exposed to aflatoxin-contaminated diets have also been reported by Bailey *et al.* (2006) and Shi *et al.* (2006). The variations in relative weights of spleen could be due to various factors like dosage, concentration and duration of exposure of AF, strains of broilers and other nutritional as well as environmental factors.

The birds fed with combination of AF and DAE showed gradual reduction in relative weight of liver, kidney, heart, pancreas and significant increase in relative weight of thymus, spleen, bursa of Fabricius as compared to aflatoxin alone fed birds. These findings could be related to possible role of DAE in ameliorating adverse effects of aflatoxicosis. The present study showed significant increase in peripheral blood CD4₊

and CD8⁺ cell population as well as higher HI titers against NDV and IBDV in DAE supplemented birds, which amply supports the increase in the relative weight of lymphoid organ. Similar findings have also been reported by earlier workers (Shi *et al.*, 2006; Bailey *et al.*, 2006; Denli *et al.*, 2009, Eckhardt *et al.*, 2014). However, Modirsanei *et al.*, (2008) reported no change in relative weight of liver, kidney, spleen and bursa of Fabricius in response to supplementation of DAE in aflatoxin mixed feed of broiler birds.

5.2.4 SERUM BIOCHEMISTRY

Blood as well as serum act as a pathological reflector of the status of exposed animals to toxicant (Olafedehan *et al.*, 2010). The examination of blood/serum provides the opportunity to investigate the presence of several metabolites and other constituents in the body of animals (Doyle, 2006). Mycotoxins or their metabolites in the blood stream are transported by plasma, plasma proteins or cellular elements and elicits direct adverse effects on the constituents and enzymes system. The toxic effects observed in the present study, have been discussed as here under.

5.2.4.1 SERUM ENZYME ACTIVITY OF ALT, AST AND ALP

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are enzymes involved in the catabolism of amino acids. The measurement of serum levels of these two enzymes is a standard feature of clinical chemistry investigations in toxicity studies (Dufour *et al.*, 2000). An increase in the serum level of ALT and/or AST activity is accepted as an indication of hepatic toxicity in animals and birds (damage to hepatocytes results in leakage of these enzymes into blood). ALT plays an important role in amino acid metabolism and gluconeogenesis, since it catalyzes the reductive transfer

of an amino group from alanine to α -ketoglutarate to yield glutamate and pyruvate. ALT is present in many tissues, but its greatest concentration in most species is within hepatocytes (Hall, 2001). AST is another liver enzyme that aids in producing proteins. It catalyzes the reductive transfer of an amino group from aspartate to α -ketoglutarate to yield oxaloacetate and glutamate. Besides liver, it is also found in other organs like heart, muscle, brain and kidney. Injury to any of these tissues can cause an elevated blood level. Serum AST is much less liver specific than serum ALT because of high concentrations in other tissues, especially muscle (including heart muscle). The ratio of serum AST to ALT can be used to differentiate liver damage from other organ damage (Nathwani *et al.*, 2005).

Alkaline phosphatase (ALP) is a hydrolase enzyme that is eliminated in the bile to cause hydrolysis of monophosphates at an alkaline pH. ALP is particularly present in the cells which line the biliary ducts of the liver. Hepatotoxicity leads congestion or obstruction of the biliary tract causing inability of liver to drain this enzyme into the duodenum leading to elevation of ALP in serum. Increase in alkaline phosphatase with minimal or no increase in ALT is primarily a biomarker of hepatobiliary effects and cholestasis (Ramaiah, 2007).

In the present study, a significantly ($P \leq 0.05$) elevated levels of serum ALT, AST and ALP were observed in birds fed with 0.5 and 1 ppm of aflatoxin (Group IV and V) compared to control birds from third week onwards in the present study. Similar findings of elevated serum enzyme activity due to aflatoxicosis were also recorded earlier by many workers (Rehman and Shakoori 2000; Sandhya and Vardhani 2007; Denil *et al.*, 2009; Madhuri *et al.*, 2009; Nathanael and Vardhani 2011; Sawarker *et al.*, 2011;

Yogeswari *et al.*, 2012; Singh and Mandal, 2013; Singh *et al.*, 2013; Dumari *et al.*, 2014; Yalagod, 2014; Li *et al.*, 2014).

On the contrary to the present study, the decreased level of ALT and AST in aflatoxin fed groups was also reported by Valdivia *et al.* (2001) at dose upto 3 ppm, for 21 days, Tedesco *et al.* (2004) observed at dose upto 0.8 ppm for a period of 14 to 49 days, and Zhao *et al.* (2010) who reported at dose upto 2 ppm AF for 21 days.

However, Raju and Devegowda (2000) recorded no difference in ALT and AST levels in birds fed aflatoxin upto 0.3 ppm for 21 days. Indresh *et al.* (2013) reported no alteration in activity of serum ALT in broilers fed aflatoxin @ level of 0.5 ppm for 35 days. The variations in serum enzymes activity may be influenced by various factors, viz, concentration and duration of exposure of AF, strain and sex of birds, health and nutritional as well as other environmental factors.

But in general, the hepatic damage associated with aflatoxicosis is primarily responsible for the derangement of serum enzymes. The increase in serum AST, ALT and ALP in present study amply draws the support of previous findings. The elevation in these enzymes could be due to degeneration and necrosis of hepatocytes resulting in leakage of enzymes into the circulation. The histopathological changes in liver following aflatoxicosis in the present study uphold this assumption. However, the discrepancies in enzyme profile as reported by several researchers indicate that AST and ALT levels may not suggest the extent of liver damage or could be a true indicator during aflatoxicosis (Waafa *et al.*, 2013).

Improvement in liver enzymes (AST, ALT and ALP) after treatment of AF contaminated diet with diatomaceous earth in the present study was also reported by Denli *et al.* (2009).

Shi *et al.* (2006) reported that reduced growth rate and serum biochemical changes associated with AFB₁ contamination (@ 0.1mg/kg) could be ameliorated by the supplementation of a modified montmorillonitenano composite (a compound comprising of DAE) at doses of 3 g/kg. Similarly, Bailey *et al.* (2006) reported that montmorillonite clay in broiler diets provided protection on growth performance, serum biochemistry and the relative organ weights from over 4 mg of AFB₁/kg diet.

Data from the present experiment show that there can be great variability in the efficacy of adsorbents *in-vivo*, even though the compounds may show great potential for binding to the toxins *in vitro*. Most of the adsorbents that have been shown to be effective against the adverse health effects of AF in chicks are quite specific for that mycotoxin and have been shown to be ineffective against other mycotoxins. Work reported by Davidson *et al.* (1987) and Phillips *et al.* (1990) identified the gastrointestinal tract as the *in-vivo* site of the detoxification action between AF and the adsorbent. Evidently, within this physiological environment, the toxin undergoes a tight chemical binding (chemisorption) to the adsorbent, thus inhibiting the toxin's availability for absorption through the intestinal tract following incorporation of DAE to the toxin mixed diet. A protection against liver damage noticed in the Groups VI to IX could be construed as a reason for modulation in serum levels of AST, ALT and ALP enzymes.

Further, Phillips *et al.* (1990) and Sarr *et al.* (1990) proposed that this *in-vivo* chemisorption involves the formation of a complex by the B-carbonyl system of the AF

with the uncoordinated “edge site” aluminium ions in HSCAS, a compound similar to diatomaceous earth. On the other hand, a few authors have not observed protective effects on the biochemical parameters when DAE was included in the AFB₁ contaminated diet of broilers (Denli and Okan, 2006; Modirsanei *et al.*, 2008). Differences among studies could be explained by different levels of adsorbents and their chemical nature or the aflatoxin levels in the diet might have influenced on biochemical alterations.

5.2.4.2 TOTAL PROTEIN AND ALBUMIN

The estimation of total proteins in the body is helpful in differentiating between a normal and damaged liver function as the majority of plasma proteins like albumin and globulins are produced in the liver. A decrease in serum total protein may reflect decreased protein synthesis or increased protein loss. Nearly all proteins are synthesized in the liver; hence, hepatic failure is a cause of decreased serum protein. Total protein is often reduced slightly but the albumin to globulin ratio shows a sharp decline during hepatocellular injury. (Dufour *et al*, 2000).

Albumin is the most abundant protein in serum, which constitutes approximately half of all serum proteins. Albumin is an anionic protein, containing an abundance of aspartate and glutamate residues; it is not functionally modified with carbohydrates; among all serum proteins. Albumin helps to maintain osmotic balance between intravascular and interstitial spaces; therefore, a deficiency in albumin ordinarily results in edema as water is redistributed to tissues (Bertholf, 2014). Hepatotoxicity leads to decrease in albumin production (Dufour *et al.*, 2000).

In the present study, a significant decrease in serum total protein and albumin was recorded following feeding of AF to the broilers @ 0.5 and 1 ppm throughout the experimental period. Similar findings of reduction in total protein and albumin were reported in birds fed AFB₁ ranging from 0.3 to 5 ppm by different workers (Devegouda, 1994; Kubena *et al.*, 1998; Ledoux *et al.*, 1999; Gowda and Ledoux, 2008; Zhao *et al.*, 2010; Yogeswari *et al.*, 2012 and Yalagod, 2014).

The hypoproteinemia and hypoalbuminaemia observed in AF treated groups could be attributed to the reduction in feed consumption and inactivation of biosynthetic pathway enzymes leading to impairment of protein synthesis by aflatoxin (Bunner and Morris, 1988). As the liver being the main organ of protein synthesis (Kaneko *et al.*, 1997), the hepatic damage observed in the present study amply supports reduced serum levels of total protein and albumin.

In the present study, dietary inclusion of DAE in aflatoxin mixed diet significantly elevated serum levels of total proteins and albumin of AF fed birds. This indicated the hepatoprotective effect of diatomaceous earth against aflatoxin adsorption of toxin during digestive process which renders most of the toxins unavailable for absorption from the gastro-intestinal tract. Improved serum levels of total proteins and albumin in broilers fed AF with clay materials was also supported by the results of Kubena *et al.* (1998); Santurio *et al.* (1999) and Rosa *et al.* (2001); Shi *et al.* (2006); Denli *et al.* (2009).

5.2.4.3 CHOLESTEROL AND TRIGLYCERIDE

Cholesterol and triglycerides, like many other essential components of the body, attract clinical attention when present in abnormal concentrations. Increased or decreased

levels usually occur because of abnormalities in the synthesis, degradation, and transport of their associated lipoprotein particles. In severe liver disorders and necrotic changes, the total serum cholesterol values are usually low with reduction in percentage of esterified cholesterol.

In the present study there was significant reduction in serum cholesterol and triglyceride due to feeding aflatoxin to the broilers. Similar trend was also reported earlier by Kalorey *et al.* (2005); Sakhare *et al.* (2007); Shabani *et al.* (2010); Khadem *et al.* (2012); Sadeghi *et al.* (2014) and Yalagod, (2014).

Reductions of plasma cholesterol and triglyceride concentrations are consistent with the general decrease of lipogenesis and damaged lipid transport in broilers and prevention of hepatic cholesterol biosynthesis. The decreased level of triglycerides reported in the current study suggested the impairment of the release of hepatic triglycerides or to the accumulation of large lipid droplets in liver tissue (Basha *et al.*, 2007).

The addition of DAE to aflatoxin contaminated diet ameliorated the alterations in serum cholesterol and triglyceride levels of the birds in Group VI to IX. Similarly, decrease in relative weight of liver and the less severe intensity of histological changes in liver of combined treatment groups in comparison to only aflatoxin fed groups adequately supports these observations.

In support of the present findings, Modirsanei *et al.* (2008) also reported increase in serum levels of cholesterol, and triglycerides in broilers following supplementation of DAE to the AF (@ 1ppm) mixed diet. Denli *et al.* (2008) also reported the effectiveness

of Ocratox (a modified product of diatomaceous earth) in ameliorating the negative effects of Ochratoxin on serum concentrations of cholesterol, and triglycerides in chicken.

5.2.4.4 CREATININE AND BLOOD UREA NITROGEN

Creatinine is the final metabolite of creatine conversion and a major marker of kidney function. When the kidney is functioning normally, creatinine is rapidly excreted. Increased blood urea nitrogen concentrations along with increased urea and creatinine suggest impaired kidney function in birds (Hochleithner, 1994).

In the present study, higher serum levels of creatinine and BUN was recorded in the AF fed birds, which can be attributed to aflatoxin induced nephrotoxicity as suggested by Kubena *et al.* (1998). Umar *et al.* (2012) reported increased blood urea and creatinine in broiler chickens fed with 3 ppm of AF from 2 to 6 weeks of age and these alterations were attributed to inflammatory and degenerative processes in renal tubules as suggested by Benjamin (1978). It is assumed that higher urea and creatinine levels are due to disturbed transportation function of epithelial cells in collecting tubules and diffuse impairment of proximal tubules function (Ortatatli *et al.*, 2005; Umar *et al.*, 2012). In the present study, degenerative and necrotic changes were clearly evident in the renal parenchyma supporting the elevated levels of serum creatinine and BUN. According to other researchers, the changes in blood urea and creatinine could be secondary to necrotic changes in renal parenchyma (Pier, 1987; ManseldGrunert and Kautna, 1989; Jindal *et al.*, 1994; Fung and Clark, 2004; Hanif *et al.*, 2006; Sakhare *et al.*, 2007; Mohamed and Mohamed, 2009; Valchev *et al.*, 2014b). Yalagod, (2014) also demonstrated comparable

results in broiler chickens after dietary treatment with aflatoxin for a period of 28 days of age.

Addition of DAE to the aflatoxin mixed diet could alleviate the adverse effects of aflatoxin on renal functions in broiler as indicated by the significantly lower serum levels of urea and creatinine in the combined treatment Groups VI to IX. It has been reported that if these serum parameters do not increase two fold higher as compared to their normal values, no kidney damage could be expected (Tremlett and Oger, 2004). Therefore, it could be construed that incorporation of DAE in the aflatoxin mixed diet might have prevented adverse effect on kidney function. The microscopic observations of kidney sections in Groups VI to IX revealed apparently normal architecture, which also supports these findings.

Similar observation of decrease in serum levels of urea in chicken following co-treatment with ochratoxin and diatomaceous earth also supports the protective effect of DAE against the toxic effects of mycotoxins (Denli *et al.*, 2008)

5.2.5 IMMUNOLOGICAL STUDIES

A healthy immune system is important for the growing poultry, especially in an industry setting where the chickens are often challenged by many different pathogens. The impact of the immune system in modern poultry production needs no emphasis. The various factors affecting avian immune-competence have received substantial attention (McCorkle, 1998; Saif and Swayne, 1998) and nutritional modulation of resistance to infectious diseases in poultry is an important aspect for profitable production (Klasing, 1998).

Poultry are susceptible to mycotoxins, especially the aflatoxin and the effects are variable. The sensitivity of the avian immune system to aflatoxin induced immunosuppression arises from the vulnerability of the continually proliferating and differentiating cells that participate in immune mediated activities and regulate the complex communication network between cellular and humoral components. Mycotoxin induced immunosuppression may manifest as depressed T- or B-lymphocyte activity, suppressed antibody production and impaired macrophage/neutrophil-effector functions (Oswald *et al.*, 2005).

In the light of the above, effect of aflatoxin on humoral and cell mediated immunity has been discussed as here under.

5.2.5.1 HUMORAL IMMUNE RESPONSE:

Determination of serum titers against NDV and IBDV after regular vaccination is a method, which is often, used to evaluate the antibody response (Girish and Devegowda, 2006). Epidemiological data also indicate high correlation between outbreaks of Newcastle disease as well as Infectious bursal disease and aflatoxin contamination of broiler rations (Mahajan *et al.*, 2002; Yunus *et al.*, 2008). Aflatoxin targets the innate and cell mediated immunity, while humoral immune response is largely unaffected, although this varies with species and dosage of aflatoxin (Meissonnier *et al.*, 2006).

In the present study, a significant ($P \leq 0.05$) decrease in antibody titres against NDV and IBDV was observed in birds fed 0.5 and 1 ppm of aflatoxin and these results concurred with earlier findings of Venkatram *et al.*, (1988); Viridi *et al.*, (1989); Mangat *et al.*, (1999); Qureshi *et al.*, (1998); Gabal and Azzam (1998); Shivachandra *et al.*,

(2003); Manegar *et al.* (2010); Mahmood *et al.*, (2011); Indresh *et al.*, (2013); Hedayati *et al.*, (2014b); Yalagod (2014) in chicken fed with aflatoxin.

The lowered titer against the NDV and IBDV following aflatoxin feeding in the present study may be attributed to the regression in size and weight of bursa of Fabricius as well as lymphocytolysis and lymphoid depletion observed in lymphoid organs. This demonstrated immunosuppressive effect of aflatoxin (Dafalla *et al.*, 1987) which may be due to inhibition of RNA polymerase *in-vivo* and subsequently limitation of protein synthesis (Lafarge and Frayssinet, 1970). In addition, the immune suppression is also attributed to functional inhibition of reticulo-endothelial system and bursa of Fabricius which is the efferent limb of the immunological system in chicken for antibody production (Co-Oper *et al.*, 1965). Aflatoxin increases the specific activity of lysosomal enzymes in the liver and muscles causing enhanced degradation of antibodies (Tung *et al.*, 1975).

In the present experiment, a dose-dependent increase of IL-6 was also observed in the serum levels of birds exposed to AF. Recent studies demonstrated that IL-6 maintains dendritic cells in an immature state following aflatoxicosis (Takahashi and Kobayashi, 2003; Park *et al.*, 2004). Thus, the high IL-6 levels observed in the present study following feeding of aflatoxin could maintain this cell population in an immature state and thereby impair T cell activation. Earlier studies indicated that an elevated level of IL-6 reduces the ovalbumin specific lymphocyte proliferation following vaccination (Meissonnier *et al.*, 2008). These data suggest that IL-6 interferes with antigen presentation to lymphocytes and reduces the antigen-specific immune response. These alterations of immune status can participate in failure of vaccination protocols and increased susceptibility to infections described in birds exposed to aflatoxin.

Contrary to the findings of the present study, feeding of AF @ 600 ppb upto 6 weeks to the broilers significantly increased the antibody titre against NDV (Manafi *et al.*, 2014b). However, Li *et al.* (2014) reported no change in humoral immunity following feeding of 74 µg/kg upto 3 weeks to the broilers and opined that low dose of AF does not cause significant negative modulation of the humoral immune response as also suggested by Meissonnier *et al.* (2008). These variations can be explained by the differences in the dosage and duration of exposure to AF as well as other nutritional and environmental factors.

Enhancement of the humoral immune response after addition of DAE to the aflatoxin mixed feed in this investigation is in line with the earlier study on efficacy of clay comprising of diatomaceous earth in ameliorating the negative effect of aflatoxin on the percentage and mean of phagocytosis and HI-titre in chicks vaccinated against ND, IB and AIV (Hedayati *et al.*, 2014b). The possible mechanism of the counteraction of AF by minerals from clays could be due to binding of aflatoxin in the intestine leading to excretion of AF without affecting the immune system. Improvement of antibody titres against IBD and ND diseases on clay-product comprising of diatomaceous earth was earlier reported by Kubena *et al.* (1997) and Rosa *et al.* (2001).

5.2.5.2 CELL MEDIATED IMMUNITY (CMI)

The assessment of CMI was performed following estimation of per cent of CD4+ and CD8+ cells in peripheral blood of birds of various treatment groups with the help of flow cytometric fluorescent activated cell sorting machine.

Among the White blood cells, lymphocytes are more sensitive indicator of immunotoxic effects than spleen lymphocytes and have been assessed in many immunotoxicological studies of avian species. Peripheral blood sampling has so far been the main provider of information on immune responses against toxicants since it is the only readily accessible source of leukocytes (Grasman, 2002). With the advancements in immunophenotyping in conjunction with flow cytometry, the studies on the identification, evaluation and estimation of subsets of lymphocytes have been made easier, accurate and reliable (De Boever *et al.*, 2010).

Flow cytometry has provided immense value to the study of cell biology since its inception. Cells can be identified and characterized within a heterogeneous population without the time-consuming and often cumbersome process of physical separation (Massaro, 1997). By assessing the relative population of these cells, including the T cells, B cells, NK cells and onocytes/macrophages, a generalised view of the immune status can be obtained in animals and birds. Amongst all lymphocyte types, T cells or T lymphocytes plays a central role in cell-mediated immunity. T cells are produced in the thymus migrate to the spleen to proliferate and then migrate to the peripheral blood and lymphatic tissues (Gisela *et al.*, 1998). T cells are further classified according to their expression of cell surface proteins. CD3 molecule is the surface marker of mature T cells, most CD4⁺ T cells are helper T cells, and CD8⁺ T cells are cytotoxic T cells (Pavelka and Roth, 2005). Studies on farm animals and avian models have given evidence of the ability of AFB₁ to induce thymic aplasia, reduce T-lymphocyte function and number and suppress phagocytic and complement activity (Bondy and Pestka, 2000; Kataria *et al.*, 2005).

In the present study, the flow cytometric fluorescent activated cell sorting revealed significantly lowered per cent of CD4⁺ and CD8⁺ cells in peripheral blood of birds fed with 0.5 and 1 ppm aflatoxin in comparison to controls. Similar findings were also recorded by Kamalavenkatesh *et al.* (2005) who reported feeding of 1 ppm AFB₁ to broiler chicken for four weeks resulted in significant reduction of thymic and splenic per cent of CD4⁺ and CD8⁺ cells and Chen *et al.*, (2014) also reported similar findings of decreased per cent of CD4⁺ and CD8⁺ cells in AFB₁ fed birds.

Similar to the present findings, Quist *et al.* (2000) reported dose dependent decrease in percentage of CD4⁺ and CD8⁺ cells in birds fed with 100 and 200 µg/kg AFB₁.

Studies confirmed the role of aflatoxin in causing selective mitochondrial damage (Rainbow *et al.*, 1994; Alpsoy *et al.*, 2009), disturbing the integrity of cell membranes in lymphocytes (Amstad *et al.*, 1984), reducing the proliferation of T cells by decreasing DNA and RNA synthesis (Reddy and Sharma, 1989; Groopman and Kensler, 1999) and inducing splenocyte apoptosis (Wang *et al.*, 2013). In addition, the congestion in red pulp of spleen observed in the present study can be associated with the decreased percentages of CD3⁺, CD3⁺ CD4⁺ and CD3⁺ CD8⁺ T cells as suggested by Chen *et al.* (2014). Studies conducted earlier showed that hypoxia, caused by congestion, markedly diminished the proliferation of T cells (Conforti, 2003; Robbins *et al.*, 2005). In addition, the atrophy of lymphoid organs due to lymphocytolysis and lymphoid depletion and DNA fragmentation observed in all lymphoid organs of toxin fed groups (IV and V) further supports the observations made in the present study.

In the present study, birds supplemented with diatomaceous earth (Group VI, VII, VIII and IX) showed significant increase in per cent CD4⁺ and CD8⁺ cells in comparison to toxin fed birds (Group IV and V). Literature revealed paucity of information on the role of diatomaceous earth in ameliorating the immunotoxic effect of aflatoxin with reference to the lymphocytes subtypes (CD4⁺ and CD8⁺ cells) in poultry. Thus, it can be construed that the ameliorative effect of DAE might be responsible for reducing cytotoxic effect of aflatoxin on different organs and systems involved in immunological responses. Furthermore, higher relative thymic weights and distinctly larger thymic lobes with uniform sheet of thickly populated lymphoid cells observed microscopically substantiate the increase in per cent of CD4⁺ and CD8⁺ cells in peripheral blood of birds following co-treatment with DAE and aflatoxin.

5.2.6 CYTOKINE ESTIMATION AND mRNA EXPRESSION

Cytokines are secreted glycoproteins that regulate the nature of immune responses by affecting growth, differentiation, and activation of cells. These are involved in almost all stages of cell survival, immunity, inflammation and hematopoiesis. The cytokine production is induced by a variety of stimuli such as bacterial, viral or parasitic infections, toxins, cancer, inflammation, apoptosis, necrosis, fibrosis and the interaction between T cells and antigens. The cytokine group is highly heterogeneous and consists of different types of molecules; such as the interleukins, the tumor necrosis factor family, the interferons, the chemokines, the transforming growth factor and others. The biological actions of cytokines are produced when they act as ligands and bind to their high-affinity receptors. Cytokines can act by an autocrine manner, affecting the cell that releases the cytokine, or by paracrine manner, affecting nearby cells, or by an endocrine

manner, affecting distant cells after their distribution via the blood circulation. Cytokines which are active in picomolar to nanomolar concentrations, control the type and duration of the immune response (Prescott *et al.*, 2002; Borish and Rosenwasser, 2003; Larrubia *et al.*, 2009). The liver is a major organ in the production of cytokines and is involved in physiologic and pathologic processes occurring in the liver. They play an important role in liver growth and regeneration, as well as in inflammatory processes including toxic liver disease, in liver fibrosis and cirrhosis (Tangkijvanich *et al.*, 2000). Cytokines can regulate the immunological and inflammatory responses and tissue damage mechanisms. Changes in various cytokine activities have been reported during liver diseases, while an imbalance of proinflammatory and anti-inflammatory cytokine production influence their immunopathogenesis (Meissonnier *et al.*, 2007)

Chickens are considered as one of the good model to study the effectiveness of cytokine as diagnostic, therapeutic agent and vaccine adjuvant. When compared to mammal, chickens contain a few number of cytokine family genes (Kalaiyarasu *et al.*, 2013). They play a physiological role in inflammation and any imbalance in cytokine production and dysregulation of a cytokine process could result in various pathological disorders (Tayal and Kalra, 2007).

Detecting cytokines in avian species was considered to be limited due to the lack of availability of specific antibodies (Swaggerty *et al.*, 2008), however a few bioassays like ELISA have been developed for chicken cytokines and molecular techniques like qRT-PCR is commonly used to study the cytokine response in various pathological conditions and diseases (Kalaiyarasu *et al.*, 2013). Estimation of cytokines by EISA is considered to be ideal by most laboratories as they are simple to perform, need little

specialized equipment and are relatively cost effective. In addition, the expression of mRNA of cytokine in cells or tissues can be detected by RT-PCR in which the mRNA transcript is converted to a cDNA by enzyme reverse transcriptase. In determining cytokine levels in the chicken, relative expression of mRNA is considered as the most appropriate technique (Li *et al.*, 2014).

Earlier studies demonstrated that pro- and anti-inflammatory cytokines are up- or down regulated in association with inflammatory processes and diseases in chicken (Wigley and Kaiser, 2003; Shini and Kaiser, 2009). Amongst various cytokines, IL-2, IL-6, IL-8 and TNF α are considered most important to play major role in the immunopathological conditions. These circulating cytokines are involved in generation of inflammation as well as tissue damage and hence called as proinflammatory cytokines. IL-6, IL-8 and TNF α are proinflammatory cytokines that are released from a variety of cells including peripheral blood leucocytes (predominantly mononuclear cells) as part of induced innate immune response.

IL-2, discovered in 1985 is a single polypeptide chain of 133 amino acid residues produced principally by T cells which are immune regulatory cells. When a helper T cell binds to an APC with co-stimulants CD28 and B7, the activated T cell produces IL-2. This cytokine supports the proliferation and differentiation of any cell that has high-affinity IL-2 receptors. Resting T lymphocytes (unstimulated) belonging to either the CD4⁺ or the CD8⁺ subsets possess few high-affinity IL-2 receptors, but following stimulation with specific antigen, a substantial increase in their number occurs. Though interleukin-2 is primarily secreted by activated T-helper lymphocytes, the interleukin-2 receptor (IL-2R) is widely expressed among many leukocytes (Nelson *et al.*, 1986). An

increased expression in peripheral lymphocytes suggests positive correlation with the degree of inflammatory and necrotic changes in hepatic tissue (Malek and Castro, 2010; Khan *et al.*, 2011)

IL-6, discovered in 1986 is a pleiotrophic proinflammatory cytokine, which is a member of the family of cytokines termed as “the IL-6 type cytokines”. IL-6 affects various processes including the immune response, reproduction, bone metabolism and aging. It is synthesized by mononuclear phagocytes, vascular endothelial cells, fibroblasts and other cells in response to toxicity, trauma, burns, tissue damage, inflammation, IL-1 and, to a lesser extent, TNF α . Pathogen-associated molecular patterns (PAMPs) binding to the TLRs present on macrophage result in the release of IL-6. This cytokine is also synthesized by some activated T cells as well. Elevated levels of IL-6 have been associated with morbidity and disease activity in a variety of chronic diseases (Gabay, 2006; Cronstein, 2007) as well as in chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (Tangkijvanich *et al.*, 2000)

Tumor Necrosis Factor- α (TNF- α) is the most potent proinflammatory cytokine, first isolated in 1975, and its name is misleading in that it does not cause the necrosis of all tumors. As a matter of fact in contrary, may stimulate the growth of some tumors. The major cell source of TNF α is the macrophage, specifically the endotoxin-activated mononuclear phagocyte. Other sources include endothelial cells after tissue damage, antigen-stimulated T cells, activated NK cells and mast cells. TNF- α is a mediator of both natural and acquired immunity. TNF α induces expression of adhesion molecules in vascular endothelial cells. Thus accounts for increased adhesion of neutrophils to endothelial cells and facilitates the effector functions of neutrophils. TNF-

α induces the production of IL-1, IL-6, TNF itself and chemokines via stimulation of macrophages. In liver physiology, TNF- α has a dual effect due to its capacity to induce both hepatocyte cell death and hepatocyte proliferation (Schwabe and Brenner, 2006).

IL-8 (CXCL8) is one of the important members of the CXC chemokine family. The CXC chemokine IL-8 (CXCL8) has long been identified as a major factor of acute inflammation, acting as a potent chemoattractant and activator of polymorphonuclear leucocytes (Kobayashi, 2008). In avian species, it mediates the heterophil influx at the site of inflammation and induces a further increase in oxidant stress mediators, making it a key parameter in localized inflammation (Jarosinski *et al.*, 2005). High IL-8 levels in liver circulation have been found to be associated with neutrophil-mediated tissue damage and acute liver injury (Jaeschke and Hasegawa, 2006). Likewise, IL-8 signalling also has been linked to damage to pancreas and lung tissues (Motoo *et al.*, 2004). IL-8 also induces phagocytosis and is also known to be a potent promoter of angiogenesis. In target cells, IL-8 incites a series of physiological responses required for migration and phagocytosis, such as increases in intracellular Ca²⁺, exocytosis (e.g. histamine release), and the respiratory burst.

In the present study, serum levels of cytokines IL-6 and TNF α were estimated to understand the influence of proinflammatory cytokines on occurrence of inflammation in experimental aflatoxicosis and the serum levels of IL-2 was quantified to correlate its impact on immune status as well as on CD4⁺ and CD8⁺ cells in peripheral blood during aflatoxin induced toxicity.

The estimation of IL-6 and TNF α in the present study by ELISA revealed a significant increase in the aflatoxin fed birds at both 0.5 and 1 ppm (Group IV and V).

The IL-6 levels in serum of 0.5 and 1 ppm AF fed birds were 172.13 ± 5.84 and 217.98 ± 9.11 pg/ml, respectively at the end of 35th day of experiment as against 56.1 ± 1.57 pg/ml in the control birds (Group I). Similarly the serum levels of TNF α in 0.5 and 1 ppm AF fed birds were 92.8 ± 1.46 and 101.83 ± 1.67 pg/ml, respectively in comparison to 44.13 ± 0.67 pg/ml in the control birds (Group I) at the end of 35th day of the experiment.

The production of cytokine is influenced by several mycotoxins (Huttunen *et al.*, 2004; Al-Anati and Petzinger, 2006) but most prominently by aflatoxins because of its immunomodulatory effect (Oswald *et al.*, 2005). Aflatoxicosis, which is a highly toxic condition, induces in general hepatic lipidosis and necrosis initially followed by biliary hyperplasia, fibrosis, heterophilic and lymphocytic infiltration in response to necrosis in the later stages (Ahmed *et al.*, 2009; Ramdas *et al.*, 2013; Valchev *et al.*, 2014a; Yalagod, 2014, TheophilusAnand Kumar and Balachandran, 2014).

The increase in the serum levels of IL-6 and TNF α in the present study following experimental aflatoxicosis is well correlated with the occurrence of inflammatory changes observed during later intervals of microscopical observations. Hinton *et al.* (2003) observed association between induction of inflammatory response and increase in the production of proinflammatory cytokines in AF induced liver injury in mice. Similarly, Chaytor *et al.* (2011) and Abbès *et al.* (2008) reported a significant increase in circulating TNF- α following feeding of AFs contaminated diet and suggested increased mRNA levels in response to acute- phase response to inflammation induced by the toxic effects of AF responsible for the altered levels of these cytokines. Modification of

inflammation-related gene expression by aflatoxin has been confirmed in the liver of rats (Ellinger-Ziegelbauer *et al.*, 2004).

Contrary to the findings of the present study, suppressive effects of inflammatory cytokines were observed in rats/mice during respiratory aflatoxicosis (Jakab *et al.*, 1994) or after oral intoxication (Dugyala and Sharma, 1996). In addition, Marin *et al.* (2002) also reported that AFs could decrease proinflammatory (IL-1 and TNF) and increase anti-inflammatory (IL-10) mRNA expression in phytohemagglutinin-stimulated blood cells. Ahmad *et al.* (2011) reported significant reduction in serum levels of TNF α in mice following feeding of aflatoxin to mice for a period of 21 days. These variations in levels of interleukins in earlier studies may be due to differences in dosage and duration of exposure of AF, species and strains as well as health, nutritional and environmental factors.

The IL-6 and TNF α levels in serum in the present study were reduced in combined treatment groups (VI to IX) fed with aflatoxin and DAE and were comparable with those of control birds (Group I, II and III). The higher dosage of DAE @ 800 mg/kg feed in both 0.5 and 1 ppm levels showed a significant reduction in levels of IL-6 and TNF α in comparison to that of DAE at 400 ppm levels (Group VI and VIII) at the end of fifth week. The observation of the present study indicated that there was improvement in the combined treatment groups fed with AF and DAE in clinical, biochemical and morphological aspects of the toxic conditions and also indicated the efficacy of toxin binders in alleviating the toxic effects of aflatoxicosis in birds. These findings were further substantiated by the significant reduction in the serum levels of proinflammatory cytokines indicating the lowered inflammatory reaction. This observation was well

supported by microscopical findings, which revealed almost normal architecture of liver in combined treatment groups. Similar to the results of the present study, (Abbès *et al.*, 2010) observed that following co-treatment with Tunisian Montmorillonite (TM) clay (a compound similar to DAE) plus aflatoxin caused significant decrease in the elevated levels of TNF- α and IL-1 which resulted from aflatoxin feeding to the rats. The study concluded that Tunisian Montmorillonite clay was effective in the protection against AF and prevented its toxic effects. The protective effects of DAE may be explained by the ability of this clay mineral to bind aflatoxin in the gastrointestinal tract and reduce the toxin bioavailability to different organs and systems.

In the present investigation, the serum levels of IL-2 were estimated by ELISA to evaluate the efficacy of DAE in regulating the immune response following aflatoxicosis. In the toxin fed birds (Group IV and V), the mean serum levels of IL-2 in the birds at the end of 35th day of age were 0.63 ± 0.02 and 0.72 ± 0.01 pg/ml, respectively in comparison to 1.5 ± 0.03 pg/ml in the control birds.

IL-2 is an important signalling molecule in the immune system, which regulate the activities of white blood cells, often lymphocytes that are responsible to elicit the immune response. Aflatoxin has been shown to induce thymic aplasia (Pier, 1986), reduce T-lymphocyte function and number, suppress phagocytic activity, and reduce complement activity (Pier, 1986; Reddy *et al.*, 1987). The decrease in serum levels of IL-2 in AF fed birds in the present study could be directly on an account of aflatoxin induced immunosuppression. Many studies conducted in poultry, pigs, and rats showed that exposure to aflatoxin in contaminated feed results in suppression of the cell-mediated immune responses (Ali *et al.*, 1994; Pestka and Bondy, 1994; Silvotti *et al.*, 1997).

Decrease in splenic CD4⁺ (helper T) cell numbers and IL-2 productions decreased significantly in mice treated with AFB at a dose of 0.75 mg/kg (Hatori *et al.*, 1991). Impairment of cellular function by aflatoxin seems to be due its effects on the production of lymphokines and antigen processing by macrophages (Pier *et al.*, 1985), as well as a decrease in or lack of the heat-stable serum factors involved in phagocytosis (Richard and Thurston, 1975).

The decrease in levels of IL-2 following aflatoxicosis can also be correlated with the reduction in the size and weight as well as lymphocytolysis and lymphoid depletion observed in lymphoid organ especially the thymus of AF fed groups (IV and V). In addition, the flow cytometry results of lowered CD4⁺ and CD8⁺ cells in peripheral blood adequately supports the previous finding of suppression of IL-2 production following aflatoxicosis in broilers. DNA fragmentation observed invariably in all lymphoid organs of toxin fed groups further justify the observations made in the present study. Aflatoxin is known to cause down regulation of IL-2 (Dugyala and Sharma (1996; Han *et al.*, 1999). The decreased T cell proliferation could be the result of the AF mediated down regulation of MHC II surface expression which in turn affecting the antigen presentation by the dendritic cells (Mehrzaad *et al.*, 2014).

The current investigation revealed an improvement in the serum level of IL-2 in co-treatment groups (VI to IX) following supplementation with DAE @ 400 and 800 mg/kg feed in feed along with AF @ 0.5 and 1 ppm. Though the serum levels of IL-2 in combined treatment groups increased in comparison to the toxin fed birds, but this increased level could not reach the serum levels of IL-2 in the control groups at the end of fifth week. The DAE control birds (Group III) recorded highest serum level of IL-2

value of 1.9 ± 0.04 , which indicated the immunostimulatory effect of diatomaceous earth. Perusal through the available literature did not reveal any report on immunomodulatory effect of DAE and the present study appears to be first to demonstrate the same.

The increase in the level of IL-2 was comparatively higher in the birds supplemented with 800 mg DAE/kg feed than that of 400 mg DAE/kg feed in the aflatoxin mixed feed. The results of increased levels of IL-2 in serum can be correlated with correspondingly higher size and weight of thymus, $CD4^+$ and $CD8^+$ cells in peripheral blood and reduced or almost nil DNA fragmentation observed in the co-treatment groups (VI to IX) in comparison to the toxin fed birds (Groups IV and V). In addition, the histological changes of improvement in the liver architecture towards normalcy coupled with infiltration of lymphocytic cells in combined treatment groups also substantiate the findings on improvement in the levels of IL-2 following supplementation of DAE to the aflatoxin mixed diet. The improvement in the morphology of thymus, $CD4^+$ and $CD8^+$ cells in peripheral blood and DNA laddering assay was comparatively higher in the birds supplemented with 800 mg DAE/kg feed than that of 400 mg DAE/kg feed in the aflatoxin mixed feed demonstrated the dosage dependent protection of DAE for mitigation of aflatoxicosis and immunostimulatory effects in broilers.

In the present study, an attempt was made to determine the level of expression of IL-8 gene in peripheral lymphocytes by RT-PCR as a support to the estimation of other proinflammatory cytokines such as IL-6 and $TNF\alpha$ by ELISA.

IL-8 being an important CXC chemokine is known to mediate acute inflammation, chemoattractant and activator of heterophils in chicken. In the current

investigation, at the end of fifth week the IL-8 gene expression ranged between 144.34 ± 0.21 to 291.92 ± 1 fold in control groups and was drastically upregulated in AF alone fed birds (4002.38 ± 1.23 and 3490.92 ± 0.62 in Group IV and V, respectively).

Literature search revealed a paucity of information as well as conflicting reports on mRNA expression of proinflammatory cytokines especially, IL-8 in chicken. Aflatoxins can stimulate the synthesis of IL-8 in response to inflammation and cellular damage (García *et al.*, 2007). The higher IL-8 expression could be correlated with the higher intensity and magnitude of inflammation and tissue damage in liver, infiltration of heterophils in multifocal areas consequent to aflatoxin induced necrosis. The moderate decrease in IL-8 expression in co-treatment groups (VI to IX) could be directly related to the DAE induced toxin binding and amelioration of toxic effects of AF, which was well substantiated by the improved microscopical findings.

Based on results of significantly higher level IL-6 and TNF α in serum and up regulation of IL-8 mRNA expression in peripheral lymphocytes and its correlation with the histopathological evaluation of the liver following aflatoxicosis in the present study, it can be construed that these cytokines have positive correlation with an acute phase reaction in response to inflammation followed by progressive liver injury due to the toxic effects, as also suggested by Zimmermann and Tacke, (2011); Chaytor *et al.* (2011) and Li *et al.* (2014). The lowered serum levels IL-2 is indicative of suppressive effect of aflatoxin especially on T lymphocytes. The present study emphasised that increased levels of cytokine protein estimated in serum samples may not equate to mRNA expression in peripheral blood lymphocytes.

The current results revealed that co-treatment with DAE and aflatoxin resulted in a significant improvement in the tested parameters as indicated by the significant decline of the serum pro-inflammatory cytokine level and IL-8 mRNA expression in peripheral lymphocytes as well as increased serum levels of IL-2. These findings can be correlated with the decreased inflammatory reaction and hepatic tissue damage in the combined treatment groups (VI to IX). The aflatoxin induced inflammatory response significantly varied with the dosage of DAE supplementation to the broilers. The inflammatory response in liver was comparatively lower in the birds supplemented with 800 mg DAE/kg feed than that of 400 mg DAE/kg feed to the aflatoxin mixed feed. Hence, the correspondingly lower level of IL-6, TNF α and IL-8 expression recorded in the co-treatment groups (VI to IX) indicated the hepatoprotective effect of DAE.

Similar to the present investigation, (Abbès *et al.*, 2010) observed that following Co-treatment with Tunisian Montmorillonite (TM) clay (a compound similar to DAE) plus aflatoxin caused significant decrease in the elevated levels of TNF- α and IL-1 which resulted from aflatoxin feeding to the rats. The study concluded that TM clay was effective in the protection against AF and prevents its toxic effects. The protective effects of DAE may be explained by the ability of this clay mineral to bind AFs in the gastrointestinal tract and reduce the toxin bioavailability to different organs and systems.

In addition, the possible mechanism for the removal of the aflatoxin by DAE may be due to its higher absorption through high affinity of mineral channel and thus it is readily encountered by ions (i.e Ca²⁺, Mg²⁺, Fe²⁺, Na⁺, K⁺...etc) provided by diatomaceous earth in the gastrointestinal tract by cationic exchange. The free ions can effectively compete with residual aflatoxin and prevent the latter from entering intestinal

cells and also inhibit the aflatoxin absorption into blood and to accelerate its elimination from the gastrointestinal tract (Abbès *et al.*, 2010).

5.2.7 DNA FRAGMENTATION ASSAY

DNA fragmentation was first documented by Williamson in 1970 when he observed discrete oligomeric fragments occurring during cell death in primary neonatal liver cultures. He described the cytoplasmic DNA isolated from mouse liver cells after culture as characterized by DNA fragments with a molecular weight consisting of multiples of 135 kDa. This finding was consistent with the hypothesis that these DNA fragments were a specific degradation product of nuclear DNA (Williamson, 1970).

Genomic DNA constitutes the total genetic information of an organism. The genomic DNA in cells can be subjected to injury following interaction due to exposure to toxicants. Mycotoxins, especially the aflatoxins are known to cause interaction with the cellular total genomic DNA (Choy, 1993). This interaction can result in small to large changes in genomic DNA evident as DNA fragmentation (Kallio and Lahdetie 1997; Faridha *et al.*, 2006) as confirmed by findings in the present study.

The fragmentation assay of DNA extracted from heart, liver, kidneys, pancreas, thymus, spleen and bursa of birds fed with different dietary levels of aflatoxin in the present study revealed severe damage at 1 ppm in comparison to 0.5 ppm. Following aflatoxin treatment, severe damage caused to DNA of liver and lymphoid organs, followed by kidneys and the least damage was observed to DNA of heart and pancreas when compared with negative control groups.

Similar to the findings in the present study, Shebl *et al.*, (2010) reported the higher mean percentages of DNA fragmentation and increase in the frequency of micronucleated cells in liver cells following feeding of aflatoxin @211.88 µg / kg feed to the broiler in comparison to negative control groups and opined that AFB₁ was selective inhibitor of DNA synthesis in mammalian cells and can induce DNA adducts, induce mutations by intercalating to DNA by forming adduct with guanine moiety in the DNA. Similar findings were also reported earlier by Metcalfe and Neal, (1983); Smith *et al.* (1994); Smela and Curier (2001); Abbès *et al.* (2008) following mycotoxicosis.

Al-Terehi (2012) observed higher degree of DNA fragmentation in liver and spleen in comparison to kidney and blood in female albino rats following feeding of aflatoxin for a period of 2 weeks. The DNA damage observed in various organs could be attributed to AFB₁- DNA adduct formation at cellular level, as suggested by Tolliver and Robbins, (1991). In addition, oxidative stress to rats following exposure to AFB₁ can also induce DNA lyses by forming (8-oxo d G) in liver cells (Shen *et al.*, 1995).

Aflatoxin possess genotoxic potential through the mixed-function oxidase system to a number of hydroxylated metabolites and to aflatoxin 8, 9 epoxide which binds to DNA, forming covalent adducts (Busby *et al.*, 1984) and disturbs DNA replication causing genetic alteration (Preston and Williams, 2005). The adduct formation occurs preferably with guanine resulting in AFB₁-N-7 guanine adduct responsible for mutagenesis in AFB₁treated cells (Kallio and Lahdetie, 1997).

DNA fragmentation test in the present study clearly indicated that aflatoxin could not damage the nucleus of cells in different organs of DAE supplemented birds (Group VI to IX), which might be explained by the strong adsorption of AF to diatomaceous

earth and thereby preventing the entry of AF into circulation (Abbès *et al.*, 2008). Similar to the findings of present study, Shebl *et al.* (2010) also reported that the addition of clay material (DAE) either alone or in combination with aflatoxin reduced the mean percentages of DNA fragmentation in different organs.

5.2.8 PATHOLOGY

Evaluation of the pathological alterations induced in animals and birds by feed materials, drugs, chemical, feed supplements, biologicals and xenobiotics forms the cornerstone of safety assessment procedures. The gross and histopathological assessment is a critical part of the toxicological studies and is aimed to determine morphological changes to identify target organ toxicity (if any) due to the entry of the xenobiotic compound to satisfy both the regulatory demands for unbiased observations while facilitating the sensitive and efficient evaluation of large amounts of microscopic information. The effect of aflatoxin and their amelioration using DAE have been evaluated in great detail by both gross and histopathological examination and are discussed appropriately.

5.2.8.1 GROSS PATHOLOGY

In the present study, enlarged, swollen friable, yellowish pale livers with soft and friable texture were observed from second week onwards in Group IV (AF @ 1ppm) and third week onwards in Group V (AF @ 0.5 ppm). In addition, hematomas on liver lobes were a consistent feature during fourth and fifth week in groups fed with 1ppm of aflatoxin. Kidneys of Group IV revealed pale and moderate enlargement from third week onwards. In Group V, the kidney showed mild to moderate congestion and enlargement

during fourth week. Intestine revealed catarrhal enteritis with areas of congestion, haemorrhages and ulcers in the mucosa. A moderate atrophy of thymus with petechiae on lobes, atrophy of bursa of Fabricius and spleen towards the end of the experiment were also recorded. The changes observed in toxin fed group in liver, kidneys and lymphoid organs could be attributed to toxic effects of aflatoxin and the same was correlated with biochemical and histopathological changes.

Similar findings were also reported by Arshad *et al.*(1992); Miazzi *et al.*(2000); Rosa *et al.* (2001); Prakash (2001); Zahid Hussain *et al.* (2008); Kumar and Balachandran, 2009; Sawarkar *et al.* (2011); Ramdas *et al.* (2013); Yalagod, 2014, Theophilus Anand Kumar and Balachandran (2014) in aflatoxicosis of broiler chickens. However, Ahmed *et al.* (2009); Yalagod, (2014) also observed haemorrhagic spots and varying sized hematomas along with infarction of the liver.

The macroscopic changes in the birds of Group VI, VII, VIII and IX fed with DAE in combination with aflatoxin included those that were observed in aflatoxin alone fed birds, but of reduced magnitude and severity. The gradual improvement observed in the gross lesions following incorporation of DAE in aflatoxin mixed feed are in agreement with earlier reports (Shi *et al.*, 2006; Denli *et al.*, 2009) indicating a good binding capacity of DAE to aflatoxin and reduce the toxin bioavailability for absorption through intestinal tract.

5.2.8.2 HISTOPATHOLOGY

5.2.8.2.1 LIVER

Liver is the primary detoxification organ; it filters blood coming directly from the intestines and prepares toxins for excretion from the body. The liver is a target organ for AF toxicity as it is the site where aflatoxins undergo bioactivation to reactive 8,9-epoxide, which then binds to DNA and proteins (Miazzo *et al.*, 2005; Bailey *et al.*, 2006; Pasha *et al.*, 2007) causing wide spread damage to the hepatic tissue. The hepatotoxicity of AF entails disturbances in lipid, carbohydrate and protein metabolism (Kubena *et al.*, 1993; 1998; Ledoux *et al.*, 1999), as well as haematopoiesis (Oguz *et al.*, 2002).

In the present study, liver of aflatoxin fed birds showed vascular congestion, occasional areas of hemorrhages, varying degree of diffused fatty change, leukocytic infiltration, mild to moderate degree of biliary hyperplasia and periportal fibrosis throughout the experimental period. During the fourth and fifth week, bile duct epithelial hyperplasia, severe fatty change with loss of architecture was noticed in birds fed 1ppm of AF. Further, the arrangement of hepatocytes in glandular pattern was also a consistent feature of aflatoxicosis at the end of experiment. Similar histological observations were earlier recorded by many workers (Prakash 2001; Ortatatli *et al.*, 2005; ZahidHussain *et al.*, 2008; Ahmed *et al.*, 2009; Lafi *et al.*, 2010; Yogeswari *et al.*, 2012 ; Ramdas *et al.*, 2013; Valchev *et al.*, 2014a; Yalagod, 2014, TheophilusAnand Kumar and Balachandran, 2014).

The vascular dilation, congestion, and moderate haemorrhages in AF fed birds have also been reported in other studies (Dafalla *et al.*, 1987). Salim *et al.* (2011) also

reported dilated sinusoids and hepatocyte necrosis as a result of aflatoxicosis. Dilation of arterioles causes congestion due to increased blood to the tissue depending on inflammatory reaction (Unal, 2012).

Enlarged and swollen hepatocytes can arise from stored metabolism products as a result of slow metabolic activity within the cell (Nunez *et al.*, 1998). The aflatoxins undergo bioactivation to reactive 8, 9-epoxide in liver, which then binds to DNA and proteins (Miazzo *et al.*, 2005; Bailey *et al.*, 2006; Pasha *et al.*, 2007). Aflatoxin is cytotoxic for hepatocytes and inhibits their proliferation (AbdelWahhab *et al.*, 2002; 2007). The degenerative and necrotic changes in the liver result from altered primary macromolecules (lipids, proteins and DNA) provoked by oxidative stress-induced damage of DNA and lipid peroxidation (Mohamed and Mohamed, 2009). The observed vacuolar degeneration of hepatocytes could be due to impaired lipid transport rather than increased lipid biosynthesis (McLean and Dutton, 1995). On the other side, the accumulation of calcium in hepatocytes cause mitochondrial dysfunction and reduced adenosine triphosphate synthesis, which can also cause alterations in liver morphology (Quezada *et al.*, 2002; Fatemi *et al.*, 2006). The hyperplasia of bile duct epithelium, which is one of the characteristic lesion may be due to direct effect of aflatoxins on biliary epithelial cells or the excessive production of prostaglandins due to AF-induced lipid peroxidation (Quist *et al.*, 2000; Saif *et al.*, 2003; Mohamed and Mohamed, 2009). The inflammatory cellular infiltration within the parenchyma comprised of mononuclear cells and heterophils occur in response to degenerated hepatocytes (Hinton *et al.*, 2003).

DAE supplementation to aflatoxin mixed diet in Group VI to IX caused significant reduction in the magnitude and severity of hepatic lesions throughout the

experimental period, which is indicative of the protective role of diatomaceous earth against aflatoxicosis through its adsorptive property.

Study on liver of birds fed with 1 ppm of aflatoxin in diet revealed vacuolar degeneration of hepatocytes, perilobular inflammation (mainly mononuclear cells), bile duct hyperplasia, and hypertrophy. Following supplementation of AflaDetox, a compound similar to DAE (@1, 2, and 5 g/kg) to AF mixed diet significantly reduced the lesions of aflatoxicosis and confirmed the protective effects of AflaDetox (Denli *et al.*, 2009).

On the other hand, several other authors have not observed protective effects on the histopathological changes in liver sections when clay material (@ 5 and 3 g/kg of feed) was included in the diet mixed with aflatoxin (Santurio *et al.*, 1999; Rosa *et al.*, 2001). These differences among studies could be explained by different nature and levels of adsorbents or the AF dose and duration of exposure in the earlier studies (Denli *et al.*, 2009).

5.2.8.2.2 KIDNEY

Kidneys are the major excretory organs and about 20–25% of the total amount of circulating toxins, including mycotoxins, reach them (Harriet, 2003). The demands of kidneys for nutrients and oxygen are high because of their functional load. The different parts of nephrons are exposed to the toxic effect of AF and its metabolites before being excreted, resulting in nephrotoxicity (Coulombe Jr, 1994; Sharma *et al.*, 2011). In birds exposed to the toxic effect of AFB, haemorrhagic and fatty kidney syndrome, thickening of glomerular membranes, degenerative changes in renal epithelial cells and congestion

of renal parenchyma are reported (Mollenhauer *et al.*, 1989; Coulombe Jr, 1994; Hussain *et al.*, 2008).

The kidneys of aflatoxin fed birds revealed progressive changes characterised by mild to moderate degree of congestion and haemorrhages in occasional areas, mild to moderate degree of tubular epithelial degeneration, desquamation and necrosis, collections of mononuclear cells along with heterophils in interstitial spaces, varying degree of glomerular hypercellularity, degeneration and hyaline changes. These findings are very well supported by the observations of previous workers (Prakash, 2001; Santosh Kumar, 2003; Ortatatli *et al.*, 2005; Zahid Hussain *et al.*, 2008; Ahmed *et al.*, 2009; Kumar and Balachandran, 2009; Ramdas *et al.*, 2013; Valchev *et al.*, 2013a; Yalgod, 2014; Valchev *et al.*, 2014b).

The AFB induced generation of reactive oxygen species (ROS) occurs mainly in the mitochondria of hepatocytes and renal epithelial cells and results in damage of important biomolecules such as DNA, proteins and lipids (Hwang and Kim, 2007). Aflatoxins increase lipid peroxidation in liver and kidney tissues and induce cellular damage causing impaired morphology of the organs (Verma and Chakraborty, 2008; Darwish *et al.*, 2011). Despite that AFB is eliminated mainly through the kidneys, the accumulation of a relatively high concentration of toxin impairs the excretory function and leads to congestion with subsequent patho-morphological alterations (Glahn *et al.*, 1991). Low activity of glutathione-S-transferases involved in conjugation, detoxication and excretion of aflatoxins has also been reported in birds. The deficiency or the total lack of functional activity of these enzymes with affinity for binding to the major AF metabolite in birds is the mechanism of renal toxic effect of aflatoxins during their

excretion (Klein, 2000). Aflatoxins are known to inhibit the protein synthesis, forming adducts with DNA, RNA and proteins, inhibit RNA synthesis via binding DNA-dependent RNA polymerase, degranulate endoplasmic reticulum and thus, cause a variety of alterations in many organs namely liver, kidneys, heart and skeletal muscles (Wangikar *et al.*, 2005; Mohammed and Metwallyn, 2009; Sharma *et al.*, 2011). Aflatoxin induced nephrotoxicity is also assumed to be due to interference with transport function in collecting tubule cells together with diffused impairment of the proximal tubules function (Ortatatli *et al.*, 2005).

Supplementation with DAE could reduce the severity of lesions compared to toxin which indicate the protective role of DAE. Further, birds of Group VI and VIII showed less severe lesions than Group IV and V indicating the protective role of DAE. However, the birds in Group VII and IX revealed lesion with less intensity, which could be attributed to binding by DAE at higher doses as suggested by Shi *et al.* (2006) and Denli *et al.* (2009). The improvement in kidney function tests observed in the Groups VI to IX were also in tandem with these findings thereby affirming the beneficial effect of DAE against toxic effects of aflatoxin.

5.2.8.2.3 HEART

The findings of mild to moderate degree of congestion, haemorrhage, infiltration of mononuclear cells and myocardial degeneration observed microscopically in aflatoxin fed birds of Group IV and V corresponded with earlier reports following aflatoxicosis in birds (Kiran *et al.*, 1998; Ramdas *et al.*, 2013; Yalagod, 2014) and is indicative toxic effects on cardiac tissue (Motawe *et al.*, 2014).

In co-treatment groups (VI to IX) fed with AF and DAE, the heart remained morphologically normal and could be compared with the controls.

5.2.8.2.4 INTESTINES

Gastrointestinal tract is the first organ coming into contact with aflatoxin of dietary origin and should be expected to be affected by AF with greater potency as compared to other organs. Aflatoxins induce intestinal mucosal injury by changing their morphology (Applegate *et al.*, 2009). In broilers, it appears that the unit absorptive surface of small intestine would deteriorate during the exposure to AF even at low levels (Yunus *et al.*, 2011). Aflatoxin is widely believed to result in malabsorption syndrome and also in reduced activity of digestive enzymes (Devegowda and Murthy, 2005). Osborne and Hamilton (1981) reported that aflatoxicosis caused poor absorption of nutrients from the diet, steatorrhea, reduction of biliary acids, and reduction of the activity of lipase, trypsin, amylase, and RNase in broilers exposed to 1.25 and 2.5 mg AFB1/kg diet leading to adverse effect on intestinal functions.

In addition, aflatoxin adversely affects the innate immune system and intestinal intraepithelial cells of intestine leading to disturbance in structural integrity of the intestine. At levels of 1 ppm of AF in diet, Kumar and Balachandran, (2009) observed catarrhal enteritis with lymphocytic or mononuclear cell infiltrations in the intestine of broilers fed on the toxin contaminated ration for 4 weeks.

The sections of intestines in the present study revealed mild to moderate congestion, haemorrhage, catarrhal changes, stunted and hyperplastic villus epithelium with marked basophilia, degeneration, desquamation and necrosis of villus epithelium

with increased goblet cell activity, mild to moderate sub-epithelial lymphoid aggregations and lymphoid depletion in caecal tonsils were observed in birds fed with 0.5 and 1ppm of aflatoxin. Similar observations were reported by Kana *et al.*, 2010; Yunus *et al.*, (2011) and Nathanael and Vardhani (2011); Yalagod (2014); Theophilus Anand Kumar and Balachandran, (2014). The irritating effect of AF on contact with alimentary organs is the cause for various lesions in the intestines (Abousadi *et al.*, 2007). This influence may have led to a reduction or lack of production of the hormone responsible for the release of digestive enzymes. Furthermore, according to Mallmann *et al.* (2006), in chronic aflatoxicosis outbreaks one of the most remarkable characteristics is the malabsorption of feed that is manifested by the presence of poorly digested feed particles in the excreta of the birds. The significantly lower body weight in toxin fed birds observed in this study can be related to reduce ability to digest the feed in presence of aflatoxin in the intestine.

On the contrary to the findings of the present study, Warren and Hamilton, (1980) observed no histopathological changes in the intestine of birds fed with AFB₁ @ 0.6, 1.2, 2.5, 5 and 10 mg AFB₁/kg diet. Similarly, Ledoux *et al.* (1999) also recorded no histological changes in duodenum, jejunum, caecum and ileum of the birds fed with 4mg AFB₁/kg diet for three weeks.

In the light of the above variations, it can presumed that the lesions in the intestine depends on factors such as dose, duration, age of birds and gut environment as these factors influence the occurrence of intestinal lesions following aflatoxicosis.

Intestine of birds in all the combined treatment groups (VI to IX) fed with aflatoxin and DAE appeared almost normal throughout the experiment. The normal villus height and epithelial cells, absence of inflammatory lesions in the intestinal mucosa and

presence of lymphoid cells in caecal tonsils can be attributed to dietary DAE supplementation and its protective effect against aflatoxin.

5.2.8.2.5 PANCREAS

The pancreas is a vital organ involved in the metabolism of carbohydrates, proteins and lipids through its enzymes and hormones. Disturbance in the function of this organ could directly influence the growth and production parameters in birds and animals (Simsek *et al.*, 2007).

Although, numerous studies demonstrate the deleterious effects of afltoxins on productive traits in domestic fowl (Huff *et al.*, 1986; Cheng *et al.*, 2001; Zaghini *et al.*, 2005) the studies on their effect on pancreas are rather scarce. One of the most significant complications in chickens fed aflatoxin contaminated feeds is the non-efficient utilisation of lipids and steatorrhea due to reduced lipase activity resultant to pancreatic damage (Matur *et al.*, 2010).

In the present study, pancreas showed periductular acinar cell degeneration, with mononuclear cells infiltration and periductular connective tissue proliferation in birds fed 0.5 and 1 ppm of aflatoxin.

Study on effect of aflatoxin fed to broilers at different levels (0.7, 1.7 and 2.8 mg/kg feed) revealed accumulation of lymphocytes degeneration of acinar cells with vacuolization and increase in the activity of endogenous enzymes namely, α -amylase, lipase and trypsin in the pancreas. The increase in digestive enzymes did not result in better performance, indicating that nutrient absorption, rather than digestion, is affected by the presence of aflatoxin in the feed (Marchioro *et al.*, 2013).

Studies (Agag, 2004; Jakhar and Sadana, 2004) have shown that aflatoxins cause an increase in size of pancreas as well as congestion, hemorrhage, and degeneration of pancreatic acinar cells in birds. Analysing the effect of different doses of aflatoxin in quails, Simsek *et al.* (2007) found that aflatoxin can affect the pancreas by decreasing the number and size of zymogen granules, formation of vacuoles in acinar cells, and loss of cell integrity and increase in the number of necrotic areas.

Microscopic evaluation of the pancreas showed moderate to severe vacuolar degeneration and necrotic changes and mononuclear infiltrations in exocrine pancreatic areas in mulard ducks fed with 2.5 and 5 mg/kg AF (Valchev *et al.*, 2013c).

Development of pancreatic lesion could be attributed to membrane damage and possible disruption of lysosome that could lead to release of lysosomal proteolytic enzymes which in turn activate pancreatic proenzymes, enhancing existing injury and acinar cell necrosis (Longnecker, 1982).

In the combination groups (VI to IX), the addition of DAE to the feed contaminated with aflatoxin could effectively alleviate the severity of aflatoxicosis-induced histological lesions of the pancreas, which can be attributed to binding of toxin within the intestinal tract and, thus reduce absorption of this mycotoxin.

5.2.8.2.6 LYMPHOID ORGANS

Avian immune system is capable of responding to numerous factors such as dietary, environmental, physiological, genetic and toxicological factors. Amongst all dietary factors, presence of mycotoxins or their metabolites in feed may be highly reactive and may destroy lymphoid tissues. Aflatoxins are one of best studied mycotoxins

due to their direct tropism for any of the primary or secondary lymphoid organs resulting into either the organ necrosis or atrophy or depletion of specific subpopulations of the lymphoid cellular components (Qureshi *et al.*, 1998).

In broiler chickens fed with 0.2 ppm of AF, Sakhare *et al.* (2007) demonstrated necrotic foci in germinal centres, central artery sclerosis, reduction in lymphocyte counts, hyperplasia of artery sclerosis, reduction in lymphocyte counts, hyperplasia of the bursa of Fabricius, cells in lymphoid follicles' core were less numerous. Similar changes with haemorrhagic foci were also observed in lymphoid follicles of the thymus. In broiler chickens receiving 4 mg/kg of aflatoxin with the feed, the lymphoid organs (thymus, bursa of Fabricius and spleen) exhibited atrophy, reduction of lymphoid cell counts, lymphoid follicles necrosis and haemorrhages (Ortatali and Oguz, 2001; Ortatali *et al.*, 2005; Mohamed and Mohamed, 2009).

In the present investigation with feeding of different levels of AF showed lymphocytolysis, depletion of lymphocytes and focal hemorrhages in the cortex of thymus, thinning of cortex and hemorrhages in medulla and cortex of bursa of Fabricius as well as atrophy of thymus and spleen of AF fed birds.

Atrophy of thymic lobes and a significant reduction in peripheral blood CD4⁺ and CD8⁺ cells could be correlated with the histopathological findings in aflatoxin fed birds in the present study.

A mild to moderate degree of lymphoid depletion, lymphophagocytic areas giving a starry sky appearance, atrophy of bursal follicles, multifocal cyst formation and hyperplastic changes in plical epithelium with corrugations were recorded consistently in

bursa of AF fed groups. Focal areas of congestion and haemorrhage, lymphoid cell necrosis and depletion around periarteriolar sheath were observed microscopically in the spleen of toxin fed birds.

Caecal tonsils exhibited mild to moderate degree of lymphocytolysis with prominent histiocytic activity. The lesions recorded in lymphoid organs were dependent on dose and duration of exposure to AF.

Similar observations were made earlier by several workers in lymphoid organs of birds fed AFB₁ (Ghosh *et al.*, (1989); Ortatatli and Oguz (2001); Prakash (2001); Santosh Kumar (2003); Shivachandra *et al.*, (2003); Sharif and Omar (2012); Ramdas *et al.*, (2013); Chen *et al.*, (2014); Valchev *et al.*, (2014a); Motawe *et al.*, (2014); Theophilus Anand Kumar and Balachandran, (2014).

Similarly, Sur and Celik, (2003) also reported regression of lymphoid organs and depletion of lymphoid cells following experimental aflatoxicosis in broilers. Other researchers observed mild to moderate depletion of lymphocytes, cystic degeneration and fibrous tissue proliferation in the bursa from 2 weeks old Japanese quails orally exposed to aflatoxin @ 1ppm for a period of 8 weeks (Parlata *et al.*, 2001). Dietary aflatoxin in chicken results in degeneration of follicles in bursa of Fabricius, destruction of thymic cortex and decrease of splenic T cells (Celik *et al.*, 2000; Wang *et al.*, 2013)

In the present study, aflatoxin (@ 0.5 and 1ppm) fed birds which received DAE supplementation (@ 400 and 800 mg/kg feed) showed hyperplastic changes in different lymphoid organs with normal population of lymphoid cells giving a histological appearance comparable to control birds. Based on the above findings it could be

construed that the improvement in lymphoid organs could be attributed to strong aflatoxin-binding/adsorptive capacity of DAE resulting in a decrease in the bioavailability of aflatoxin for absorption through gastrointestinal tract and subsequent reduction in the distribution of AF in different organs and systems.

Inclusion of new adsorbent products in the diet such as DAE can significantly ameliorate most of the adverse effects of AF. There are a few conflicting reports on efficacy of DAE in mitigation of toxic effects of aflatoxins in relation to some of the tested parameters. The differences among studies could be explained by different levels of adsorbents or the AF exposure dose tested. In the present study, effective protection against the AF was obtained by incorporation of 800 mg DAE/kg of feed in comparison to the 400 mg DAE/kg of broiler feed.

Based on the available scientific literature, the chemical complexity of mycotoxins means that the effectiveness of compound in sequestering one mycotoxin does not mean an equal ability to sequester other mycotoxins. Each of the mycotoxin has different functional groups; thus, the binding capacity of an adsorbent depends on its chemical and physical properties and its relation with the physical structure of the target mycotoxins. Thus, the physicochemical differences among the adsorbents used in the studies mentioned above could explain the higher or lower efficacy among them. However, the ability of the toxin binder to bind mycotoxins depends on other factors such as pH, molecular arrangement and its geographic region of origin (Vieira, 2003). Natour and Yousef (1998) reported significantly higher *in-vitro* adsorption ability of DAE to aflatoxin, which is directly proportional to the number of diatom valves. *In- vitro* study showed that DAE has high (94.71 %) ability to absorb AF from the feed at pH 6.5

(Soleimani *et al.*, 2011). The normal pH of the chicken intestinal tract contents is 5.7–6.0 in the duodenum/jejunum, 6.3–6.4 in the ileum/rectum and finally up to pH 7.0 or higher in the caecum (Denbow, 2000). Considering the correlation between the pH and ability of mycotoxin binder in *in-vitro* studies, higher binding ability of DAE to the aflatoxins can be expected at the pH of 6-7 in the intestinal tract of chicken to reduce the absorption and systemic availability of this mycotoxin.

In conclusion, the incorporation of DAE in the diet during the period of exposure to AF in the present study could prevent the toxic effects of aflatoxin. This result confirms the protective effects of DAE, which might be due to its capability of specific chemisorption of AF in gastrointestinal tract, which reduces aflatoxin bioavailability (Phillips *et al.*, 1990; Abdel-Wahhab *et al.*, 1999). Diatomaceous earth mineral is a powerful natural adsorbent and which might effectively adsorb the toxins through its polar ends (Gowda *et al.*, 2008). DAE has a small mass (0.5-0.8 g/cm³), high porosity and high content of silicon responsible for the high adsorption capacity (Whitlow, 2006). These properties and ability of DAE might possibly be responsible for the ameliorating action against afltoxin in broiler birds.

In respect of abundant availability, source and cost of DAE as well as ability of this absorbent in reducing the moisture content in the poultry litter (Kiaei *et al.*, 2002), it seems that using DAE at the level of 800 mg/kg feed could be effective and economic way of ameliorating adverse effect of AF.

At the same time it is well documented that clay, zeolite minerals and DAE are structurally and functionally diverse; they vary considerably from source to source and may not have equal affinities and capacities for binding of aflatoxin and other

mycotoxins. Thus these adsorbents should be rigorously tested one by one and thoroughly in *in-vivo* conditions, paying particular attention to their effectiveness and safety in sensitive animal/avian models and their potential for harmful interactions. Similarly, generalisations should be avoided for all potential mycotoxins detoxifying agents, as adsorbing compounds can differ in efficacy even within the same category.

Considering the results of present study and earlier work done on effect of different levels of DAE on aflatoxin, further studies employing the broader perspectives seems to be necessary to determine whether lower levels of DAE in broilers diet will be effective in controlling or preventing the occurrence of aflatoxicosis in chicken.

Summary



VI. SUMMARY

The present study was conducted to assess the biochemical, pathomorphological and immunotoxic effect following feeding of aflatoxins at 0.5 and 1 ppm from 1-35 days of age and also to evaluate the efficiency of diatomaceous earth (DAE) in ameliorating the toxic effect of aflatoxin in broiler chicken.

The aflatoxin was produced on rice using culture of *Aspergillus parasiticus* (MTCC-2796) and the toxin yield was 25 ppm.

A total of 360 day old commercial broiler chickens were equally divided into nine groups. The Group I birds served as control and were fed aflatoxin free diet. The birds in Groups II and III were fed with 400 and 800 mg of diatomaceous earth (DAE)/kg feed, respectively. The birds in Group IV and V were fed 0.5 ppm and 1ppm of aflatoxin toxin, respectively. The birds of co-treatment Group VI and VII were fed diet containing 1 ppm of AF toxin along with DAE at the rate of 400 and 800 mg/kg in feed, respectively. The birds of combined treatment Group VIII and IX were fed diet containing 0.5 ppm of AF toxin along with DAE at the rate of 400 and 800 mg/kg in feed, respectively. The birds were fed experimental diets upto 35 days of age.

The Group IV and V birds fed with 0.5 and 1 ppm of aflatoxin, respectively showed a significant reduction in body weight gain starting from 14th day onward and continued till the end 35th day when compared to the control birds. The birds in Group II and III recorded almost similar body weights compared to control birds at the end of fifth week. In the combined treatment Groups (VI to IX), a significant increase in the body weight was seen from 28th day of experiment as compared to only toxin fed birds of

Group IV and V. Among the various treatment groups, birds supplemented with DAE @ 800 mg/kg feed to the aflatoxin mixed feed (Group VII and IX) showed significant increase in body weight in comparison to toxin fed groups (IV and V) as well as Group VI and VIII (aflatoxin + DAE @ 400 mg/kg feed)

The FCR was significantly increased in toxin fed birds (Group IV and V) from second week onwards in comparison to control birds (Group I, II and III). The birds in Group I, II and III recorded almost similar FCR throughout the experiment. The birds in combined treatment groups (VI to IX) recorded significantly lower FCR compared to toxin fed birds (Group IV and V) at the end of 35th day of experiment.

Birds that received diet containing aflatoxin (Group IV and V) exhibited clinical signs such as dullness, poor growth, ruffled feathers and diarrhoea from 7th day onwards, which persisted till the end of the experiment. The birds fed with diet containing aflatoxin and supplemented with DAE (Group VI to IX) showed clinical manifestation of reduced severity similar to that of AF alone fed birds. The improved and lustrous feather pattern was evident in combined treatment groups.

Relative organ weights of liver, kidney, heart and pancreas were significantly higher from second week onwards in Group IV and V. However, significant reduction in the relative weight of spleen, bursa and thymus was observed from 21st day of the experiment. The birds fed with combination of AF and DAE showed gradual reduction in relative weight of liver, kidneys, heart and pancreas, and significant increase in relative weight of thymus, spleen and bursa of Fabricius as compared to aflatoxin alone fed birds.

Serum levels of AST, ALT and ALP were significantly increased in Group IV and V birds from 21st day onwards of toxicity trial when compared to the values in control group. A significant reduction in the total serum proteins levels were observed in AF fed birds from 7th day onwards and continued till the end of experiment. There was a significant reduction in serum albumin values in toxin fed birds from 21st day onwards in comparison to control birds. A significant increase in serum creatinine values was noticed in birds of Group IV and V from 28th day onwards, whereas the BUN levels in serum were significantly elevated from 14th day onwards in comparison to control groups (I, II and III). A significant decrease in serum levels of cholesterol and triglyceride were noticed birds fed with aflatoxin from 28th day onwards in comparison to control birds. In the control birds (Group I, II and III), the levels of serum constituents and enzymes were almost similar till the end of the experiment. The addition of DAE in toxin mixed diet could improve the adverse effects of AF on the serum biochemical values as evidenced by increase in the total protein, albumin, cholesterol and triglycerides levels. However, there was decrease in the serum levels of AST, ALT, ALP, BUN and creatinine in the co-treatment groups of VI to IX.

A significant decrease in HI and antibody titre (ELISA) against NDV and IBDV respectively were recorded in birds fed with 0.5 ppm and 1 ppm of aflatoxin in comparison to the control groups. Supplementation of DAE in AF mixed diet significantly improved the antibody titres against ND and IBD vaccines in comparison to the birds fed 0.5 and 1 ppm of AF alone. CD4⁺ and CD8⁺ cells in peripheral blood significantly decreased in birds of Group IV and V (toxin fed), whereas the birds in co-treatment groups (VI to IX) showed a significant increase in CD4⁺ and CD8⁺ cells in peripheral blood compared to toxin fed birds.

A significantly higher serum levels of IL-6 and TNF α , and IL-8 mRNA expression in peripheral lymphocytes was observed in aflatoxin fed broilers in comparison to the control Groups I, II and III. However, the IL-2 level in serum of toxin fed groups (IV and V) showed a significant reduction in comparison to the control groups. Co-treatment with DAE and aflatoxin (Group VI to IX) resulted in a significant decline in the serum levels of pro-inflammatory cytokines (IL-2 and TNF α) and IL-8 mRNA expression in peripheral lymphocytes. A significant increase in the serum levels of IL-2 was also observed in combined treatment groups (VI to IX) in comparison to the aflatoxin alone fed birds.

The fragmentation of DNA was severe in hepatocytes and lymphoid cells in thymus, bursa of Fabricius and spleen followed by kidneys, heart and pancreas in the toxin fed birds. The damage was more pronounced at 1 ppm (Group IV) in comparison to 0.5 ppm (Group V) of dietary aflatoxin. The damage to DNA in most of the organs was strikingly reduced in birds of co-treatment groups (VI to IX) fed with varying dosage of aflatoxin and DAE in the diet.

Grossly, aflatoxin fed birds (Group IV and V) revealed enlarged, yellowish or pale, soft and friable livers with hematoma, pale and enlarged kidneys and catarrhal enteritis with focal ulcers in the mucosa. A moderate atrophy bursa of Fabricius, spleen and thymus with petechiae on lobes were also observed towards the end of the experiment.

Microscopically, liver revealed vascular congestion, haemorrhages, varying degree of vacuolar degeneration of hepatocytes along with necrosis especially around the bile ducts and perivascular region with stray infiltration of mononuclear cells, glandular

transformation of hepatic cords, hyperplastic epithelium of bile ducts and periductular fibro-vascular tissue proliferation in toxin fed birds. The fatty change was noticed in toxin fed groups from second week onwards till the end of toxicity trial, where as severe fatty changes and fatty cysts formation was noticed in Group IV (1 ppm AF). Predominant lesions in kidney of AF fed birds included vascular congestion, haemorrhages, vacuolar degeneration of tubular epithelium with focal infiltration of heterophilic and mononuclear cells. Birds of Group IV revealed prominent vascular and degenerative changes in cardiac musculature in comparison to Group V. Vascular congestion, acinar cell degeneration, infiltration of mononuclear cells and hyperplastic pancreatic ductular epithelium with fibrous tissue proliferation around pancreatic duct were observed in birds fed with AF. Intestines of toxin fed birds (Group IV and V) showed haemorrhage, congestion, catarrhal changes, increased goblet cell activity, degeneration as well as desquamation of villous epithelium and sub-epithelial lymphoid aggregation. The lymphoid organs such as thymus, spleen, caecal tonsils and bursa of Fabricius in toxin fed birds revealed congestion and haemorrhages, lymphocytolysis, lymphoid depletion and multifocal cyst formation within the bursal follicles.

Combined treatment with AF and DAE in Group VI to IX demonstrated gradual improvement in the visceral organs as compared to toxin fed birds. A prominent thymic, splenic and bursal enlargement and hyperplastic changes with formation of secondary lymphoid follicles in spleen and caecal tonsils were observed in birds of combined treatment groups (VI to IX) which could be compared with the control birds on 35th day of the experiment.

CONCLUSIONS

1. Feeding of aflatoxin @ 0.5 and 1 ppm produced alterations in clinico-biochemical parameters, decreased growth rate, increased relative weights of visceral organs and markedly decreased the weight of lymphoid organs.
2. Aflatoxin induced toxic effects in visceral organs especially in liver and kidneys included varying degree of vascular, inflammatory degenerative and necrotic changes.
3. The inflammatory changes in liver due to aflatoxin can be correlated to increased serum levels of cytokines IL-6 and TNF α and mRNA expression of IL-8 in peripheral blood lymphocytes.
4. Aflatoxin induced immunotoxic effect was evident by suppression of both humoral and cell mediated immune functions.
5. Aflatoxin induced varying degree of DNA fragmentation in liver, kidneys, pancreas, thymus spleen and bursa of fabricius.
6. Incorporation of diatomaceous earth at levels of 400 and 800 mg/kg feed of broiler feed showed no adverse effects on body weight, feed conversion ratio, relative organ weights, biochemical and immunological parameters as well as morphology of visceral and lymphoid organs indicating the non-toxic and inert nature of DAE.
7. Addition of diatomaceous earth to aflatoxin mixed feed showed improvement in growth, relative organ weight, serum biochemical and immunological parameters. In addition, decreased fragmentation of DNA, minimal hepatic and

renal lesions as well as regenerative changes especially in lymphoid organs were also observed.

8. The protective effect at 800 mg was higher than 400 mg of DAE/kg feed in abetting the toxic effects of aflatoxin.
9. The present investigation conclusively elucidated the ameliorative effect of DAE in enhancing the performance of the aflatoxin fed birds. However, it is suggested to carry out further studies to ascertain the benefits of DAE at different concentrations in broiler chickens.

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Abstract



VIII. ABSTRACT

The study was conducted to evaluate the toxic effect of dietary levels of aflatoxin (AF) and ameliorative effect of diatomaceous earth (DAE) in commercial broiler chicken for 35 days. 360 day old broiler chicks were divided into nine groups. First three groups were fed control diet, 400 mg DAE and 800 mg DAE/kg feed, respectively. Two groups were fed 0.5 and 1 ppm aflatoxin, respectively in feed. In the four combination groups, birds were supplemented with 400 and 800 mg DAE/kg feed along with 0.5 and 1 ppm of AF, respectively. Decreased body weight, increased feed conversion ratio, increased relative weights of liver, kidney, heart and pancreas and decrease in weights of thymus, spleen and bursa of Fabricius were recorded in toxin fed birds. DAE supplementation caused significant improvement in growth rate and organ weight. Feeding of AF alone significantly decreased serum total proteins, albumin, triglycerides and cholesterol levels. However, significant increase was found in AST, ALT, ALP, BUN and creatinine levels in AF fed broilers. DAE Supplementation ameliorated the adverse effect on serum biochemical parameters. Birds fed toxin showed lowered antibody titres against NDV and IBDV and decreased CD4⁺ and CD8⁺ cells. Aflatoxin induced DNA fragmentation in liver, kidneys and lymphoid organs. Birds fed with aflatoxin showed higher serum levels of IL-6, TNF α , IL-8 mRNA expression in peripheral lymphocyte and decreased serum level of IL-2. Pathomorphological lesions characteristic of aflatoxicosis were prominent in liver, kidneys and lymphoid organs and DAE supplementation reduced severity of lesions.

Key words: Aflatoxin, broiler chicken, diatomaceous earth, growth rate, immunopathology.

Appendices



IX. APPENDICES

I. For Quantification of CD4+ AND CD8+ Cells

1. FACS Tubes

Round bottom polypropylene tubes (5ml) with caps (M/s Becton Dickinson, UK)

2. Phosphate buffered saline with bovine serum albumin (PBS- azide-BSA)

Phosphate buffered saline 1000 ml

Sodium azide 5.0 g

BSA (Sigma) 10.0 g

The solution was freshly prepared and sterilized by filtration.

3. Wash buffer (PBS-Azide)

Sodium chloride 8.0 g

Disodium hydrogen phosphate 1.21 g

Potassium chloride 0.20 g

Potassium dihydrogen phosphate 0.20 g

Distilled water (DW) 1000 ml

Sodium azide 0.5 g

The solution was sterilized by autoclaving at 121°C for 15 min at 15 pounds pressure and stored at 4°C in aliquots of 100 ml.

4. Cell fixing buffer (Para Formaldehyde in PBS-Azide)

Phosphate buffered saline 1000 ml

Sodium azide 0.5 g

PFA (Sigma) 20.0 g

5. Lymphocyte separation medium

Lymphocyte separation medium (Histopaque®-1077) from Sigma Aldrich, Co., USA.

6. Antibodies

Mouse anti chicken CD4: FITC (Fluorochrome) and Mouse anti chicken CD8a: RPE (Fluorochrome) from M/s AbD Serotech, USA.

II. IL-2 Estimation kit

1. List of materials

Assay plate-	1
Standard -	2
Sample Diluent -	1 x 20 ml
Biotin-antibody Diluent -	1 x 10 ml
HRP-avidin Diluent	1 x 10 ml
Biotin-antibody	1 x 120µl
HRP-avidin	1 x 120µl
Wash Buffer	1 x 20 ml
(25×concentrated)	
TMB Substrate	1 x 10 ml
Stop Solution	1 x 10 ml

2. Detection Range

The range of detection of IL-2 in the serum sample was 0.19 pg/ml-12 pg/ml. The standard curve concentrations used for the ELISA are 12 pg/ml, 6 pg/ml, 3 pg/ml, 1.5 pg/ml, 0.75pg/ml, 0.38 pg/ml, 0.19 pg/ml.

3. Reagent preparation

All reagents were brought to room temperature before use.

1. Wash Buffer was warmed up to room temperature and mixed gently until the crystals dissolved completely. 20 ml of Wash Buffer concentrate was diluted into deionized water to prepare 500 ml of wash Buffer.
2. The standard vial was centrifuged at 6000-10000 rpm for 30s and reconstitute with 1.0 ml of sample diluent. This reconstitution produced a stock solution of 12 pg/ml. The standard was allowed to sit for a minimum of 15 minutes with gentle agitation prior to making serial dilutions. The undiluted standard served as the high standard (12 pg/ml). The sample diluent served as the zero standards (0 pg/ml).
3. The vial of Biotin-antibody was centrifuged before opening. For working concentration, the Biotin-antibody was diluted using Biotin-antibody diluent (1:100), respectively.
4. The vial of HRP-avidin was also centrifuged before opening. The working concentration of HRP-avidin was made by using HRP-avidin diluent (1:100), respectively.

III. IL-6 Estimation Kit

1. Detection Range

The detection range of IL-6 by the kit was 15.6 pg/ml-1000 pg/ml. The standard curve concentrations used for the ELISA was 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.2 pg/ml, 15.6 pg/ml.

2. List of Materials

Assay plate	1
Standard	2
Sample Diluent	1 x 20 ml
Biotin-antibody Diluent	1 x 10 ml
HRP-avidin Diluent	1 x 10 ml
Biotin-antibody	1 x 120 μ l
HRP-avidin	1 x 120 μ l
Wash Buffer	1 x 20 ml
(25 $\times$ concentrate)	
TMB Substrate	1 x 10 ml
Stop Solution	1 x 10 ml

3. Reagent Preparation

All reagents were thawed to room temperature before use.

1. Wash Buffer was warmed up to room temperature and mixed gently until the crystals completely dissolved. 20 ml of Wash Buffer concentrate was diluted into deionized or distilled water to prepare 500 ml of Wash Buffer.
2. Centrifugation of the standard vial at 6000-10000 rpm for 30s was performed. The Standard was reconstituted with 1.0 ml of sample diluent. This reconstitution produced a stock solution of 1000pg/ml. The standard was allowed to settle for a minimum of 15 minutes with gentle agitation prior to making serial dilutions. The undiluted standard serves as the high standard (1000 pg/ml).The Sample diluent served as the zero standard (0 pg/ml). All the solutions were used within 4 hours and discarded after use.

3. Biotin-antibody was centrifuged before opening the vial. The working concentration was diluted using Biotin-antibody diluents (1:100), respectively.
4. HRP-avidin was centrifuged before opening of the vial and diluted to the working concentration using HRP-avidin diluents (1:100), respectively.

IV. TNF- α Estimation Kit

1. Detection Range

The detection range of TNF- α by using the kit was 0.27 pg/ml to 200 pg/ml. The standard curve concentrations used for the ELISA were 100 pg/ml, 40 pg/ml, 24 pg/ml, 10 pg/ml, 4 pg/ml.

2. List of Materials

Assay plate	1
Standards (S1-S5)	5 x 0.5 ml
Antibody	1 x 6 ml
HRP-conjugate	1 x 6 ml
Wash Buffer	1 x 15 ml
(20 $\times$ concentrate)	
Substrate A	1 x 7 ml
Substrate B	1 x 7 ml
Stop Solution	1 x 7 ml

3. REAGENT PREPERATION

1. All reagents and plate were thawed to room temperature at least 30 minutes before use.

2. Wash buffer was warmed to room temperature and mixed gently until the crystals dissolved completely. 15 ml of Wash Buffer was diluted and the concentrate was also diluted into deionized water to prepare 300 ml of wash buffer.
3. To avoid cross-contamination, pipette tips were changed between additions of each standard level, between sample additions, and between reagent additions. In addition, separate reservoirs were used for each reagent.

V. CYTOKINE GENE EXPRESSION OF IL-8 IN mRNA IN LYMPHOCYTES

1. List of materials

Thermal cycler (Applied Biosystems Step One), 48 well real time PCR plates (Axygen), PCR film-self-adhesive (Eppendorf, Germany), Micropipettes, 1.5 ml micro-centrifuge tubes for preparing master mix, 2X Sybr Master mix (KapaBiosystems, SA) dNTP mixture (Biogene, USA), nuclease free water, gene specific primers (forward and reverse) (Sigma USA), housekeeping gene (GAPDH).

2. RT PCR components

PCR components	Volume (μ l)
Nuclease free water	8.0
2X Fast Start DNA Master SYBR Green	10.0
Primer FP (10picomoles/ μ l)	0.5
Primer RP (10picomoles/ μ l)	0.5
Template cDNA (50ng/ μ l)	1.0
Total volume	20.0

3. Temperature profile for the RT PCR

Initial denaturation	95°C for 10 min	
Denaturation	95°C for 10 s	} 45 cycles
Annealing	60°C for 5 s	
Extension	72°C for 30s	

4. Primer Sequences used in the study

Oligonucleotide		Sequences (5'- 3')	Length
GAPDH FP	Forward	GGT GGT GCT AAG CGT GTT AT	20
GAPDH RP	Reverse	ACC TCT GTC ATC TCT CCA CA	20
IL8 FP	Forward	GGC TTG CTA GGG GAA ATG A	19
IL8 RP	Reverse	GCT GAC TCT GAC TAG GAA ACT GT	23

VI STUDIES ON DNA FRAGMENTATION

1. List of Materials for DNA extraction

a. Microcentrifuge (Eppendorf, Germany)

b. Spectrophotometer

c. Micropipettes (Eppendorf, Germany)

d. Eppendorf tubes

QIAampMiniSpin Columns 50 Nos.

Collection tubes (2 ml) 150 Nos.

Buffer ATL (Tissue lysis buffer) 10 ml

Buffer AL (Lysis buffer) 12 ml

Buffer AW1 (Concentrate) 19 ml

Buffer AW2 (Concentrate) 13 ml

Buffer AE 22 ml

Proteinase K 1.25 ml

2. List of materials for DNA Confirmation and Fragmentation Study by Agarose Gel Electrophoresis

- a. Weighing balance (Shimadzu Philippines Manufacturing Inc., Japan)
- b. Horizontal electrophoresis apparatus with power pack (Bangalore GeNei, India)
- c. Microwave oven (M/s. BPL Pvt. Ltd.)
- d. UV transilluminator
- e. Gel documentation unit (Biorad, USA)
- f. Agarose (M/s. Sigma Aldrich, USA)
- g. Tris - Borate EDTA buffer (TBE buffer) (10X, pH 8.2)
- h. Tris base 108.0 g
- i. Boric acid 55.0 g
- j. EDTA disodium salt 8.3 g
- k. Double distilled water up to 1000.0 ml

The stock solution was sterilized by autoclaving and made to 0.5X before use.

- l. Gel loading dye (6X)
- m. Bromophenol blue 0.25% (w/v)
- n. Xylene cyanol 0.25% (w/v)
- o. Sucrose 40% (w/v) in distilled water

The dye was procured from M/s. Bangalore GeNei, Bangalore and stored at 4°C.

- p. Ethidium bromide (10 mg / ml) from M/s Bangalore GeNei
- q. Ethidium bromide (Bangalore GeNei) 100 mg
- r. Double distilled water 10 ml

The suspension was stirred to ensure that the dye had dissolved. The container was then wrapped in aluminium foil and stored at 40⁰ C until further use.