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Effect of dietary supplementation of *Eclitpa alba* on oxidative stress and liver damage induced by aflatoxin in broilers

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ABSTRACT

The primary aim of the present study was to evaluate the efficacy of *Eclipta alba* leaf powder to ameliorate the oxidative stress and liver damage induced by aflatoxin B1 (AFB1) in broilers. Day-old male broilers were fed with basal diet (control), basal diet supplemented with *E. alba* (1.0%), basal diet with 1.0 ppm AFB1 or AFB1 (1.0 ppm) with *E. alba* (1.0%) for 42 days. Birds fed AFB1 showed significantly higher serum AST and ALT activities, and decreased concentrations of total protein, cholesterol and albumin. AFB1 feeding significantly increased hepatic lipid peroxidation products, decreased antioxidant enzymes and induced pathological changes in liver. The supplementation of *E. alba* leaf powder with AFB1 contaminated diet significantly improved serum biochemical profile, reduced the oxidative stress and pathological changes in liver. The present study revealed that supplementation of *E. alba* reduces the liver damage and oxidative stress induced by AFB1 and offer protection against aflatoxin toxicity in broilers.

Key words: Aflatoxin B1, Broilers, *Eclipta alba*, Liver damage, Oxidative stress

Mycotoxins are toxic metabolites of certain fungi and more than 25% of world's crops are reported to be contaminated with mycotoxins (Alshannaq and Yu 2017). Aflatoxins (AFs) are a major group of mycotoxins produced primarily on cereals, oilseeds and nuts by *Aspergillus parasiticus* and *Aspergillus flavus* fungi. A temperature between 25–32°C and moisture above 10% favours *Aspergillus* growth and aflatoxin contamination mainly occurs during transport and storage (Umaya *et al.* 2010, Fakruddin *et al*. 2015). Amongst different types of aflatoxins, Aflatoxin B1 (AFB1) is the most predominant and toxic. Consumption of AFB1 by poultry leads to hepatotoxicity (Umaya *et al*. 2012, Ma *et al*. 2015), nephrotoxicity, immunosuppression (Peng *et al.* 2015), reduced performance (Manafi *et al.* 2014) and carryover of the toxic metabolites of AFB1 into the poultry products, meat and egg (El-Yazeed *et al.* 2015). Oxidative stress is known to be one of the major mechanisms by which AFB1 induces liver damage and other toxic effects in poultry (Rawal *et al.* 2010). Contamination of crops and animal and human health loss due to aflatoxin leads to huge economic losses that accounts to millions of dollars annually (Kumar *et al.* 2016).

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Drying of aflatoxin-contaminated feed in sunlight reduces its concentration in feed (Gowda *et al.* 2006a). Further, supplementation of diet with clay materials like hydrated sodium calcium aluminosilicate (Gowda *et al.* 2006b) and clinoptilolite (Oguz *et al.* 2002) is reported to counteract the toxic effects of aflatoxin in livestock. Despite several research efforts, aflatoxin is still a major threat to food and agricultural commodities worldwide. In a recent study, aflatoxin contamination emerged as a major issue in maize in colder regions of Europe where they were not prevalent earlier, being the climate change effect (Baranyi *et al.* 2015).

In recent past, more emphasis is on use of phytoproducts as feed supplements in poultry, as they are considered as safe, less toxic and appropriate for food animal production. However, studies on use on phytoproducts against aflatoxin toxicity in broilers are limited. The plant *Eclipta alba* (Family: Asteraceae) is a weed that grows well in moist places and is commonly distributed throughout India. Ayurveda, one of the three major forms of traditional systems of medicine of India refers *E. alba* as *Karisalaankanni*. The Ayurvedic Pharmacopoeia of India considers *E. alba* as hepatoprotective agent (Khare 2007). The leaves of *E. alba* possess antioxidant (Thirumalai *et al*. 2011), antimicrobial (Majumdar *et al*. 2010) and anticancer effects (Arya *et al.* 2015). The aim of the study presented here was to explore the potential of *E. alba* leaves to counteract the oxidative stress and liver damage induced by aflatoxin in broilers.

MATERIALS AND METHODS

Production of aflatoxin: The *Aspergillus parasiticus* (IMTECH 2797) was procured from Institute of Microbial Technology, Chandigarh, India and subcultured in potato dextrose agar media for 5 days. The culture material was collected, inoculated into broken rice and aflatoxin was produced by solid substrate fermentation (Shotwell *et al.* 1966). The aflatoxin content of rice material was determined by the method of Romer (1975). Appropriate quantity of rice material was incorporated into the diet to obtain the required experimental dose of aflatoxin in feed.

E. alba *leaf powder:* The leaves of *E. alba* were obtained from Tamil Nadu, India, identified, shade dried without direct sun exposure, finely powdered and stored at room temperature for further use.

Experimental birds: The present study was conducted at the experimental livestock unit of ICAR-National Institute of Animal Nutrition and Physiology, Bangalore, India after obtaining necessary approval from the Institutional Animal Ethics Committee (No:NIANP/IAEC/3/2012). Eighty day old (Vencobb) broiler chicks (weight: 43±10 g) were purchased from a commercial hatchery (Venkateshwara hatcheries) and housed in cages fitted with bulbs for heating arrangements, separate waterers, feeders and dropping trays. The cages were placed in a well-ventilated open-sided house and all the birds were maintained under standard management conditions.

Experimental diet: A maize soybean meal-based starter diet (1–21 days) and finisher diet (22–42 days) were prepared and used as the basal diet for the birds to meet the nutritional requirements (ICAR-NIANP 2013, Sridhar *et al.* 2016). The ingredient and nutrient composition of basal diet (starter and finisher diets) offered to the birds is given in Table 1. Trace mineral and vitamin premixes were added to the basal diet. The feed ingredients, maize and soybean and the starter and finisher diets were tested for the presence of aflatoxin and were free of aflatoxin contamination. At weekly intervals, appropriate amounts of *E. alba* leaf powder was mixed with respective diets, stored at room temperature and fed to birds. No synthetic antioxidants and liver stimulants were added to the diet. Day old birds were divided into four groups having four replicates per group with five birds in each replicate. The groups and their diets were: Control: basal diet; *E. alba* (1%): basal diet supplemented with *E. alba* (1.0%); AFB1 (1.0 ppm): basal diet + AFB1 (1.0 ppm) and AFB1 + *E. alba:* AFB1 (1.0 ppm) + *E. alba* (1.0%). The experiment was conducted for 42 days.

Sampling: At the end of 42 days, eight birds from each group were selected randomly. Blood samples were collected from jugular vein in non-heparin-coated and the birds were sacrificed by cervical dislocation. Blood samples were centrifuged (1400g at 4°C for 30 min), serum was separated and immediately transferred to 2.0 ml screw cap self-standing microcentrifuge tubes and stored at –20°C until analysis. The serum samples were used for the determination of aspartate aminotransferase (AST, EC2.6.1.1), alanine aminotransferase (ALT; EC 2.6.1.2), total protein, albumin and cholesterol concentrations. AST and ALT were quantified by the method of Reitman and Frankel (1957) and total protein and total cholesterol were determined according to the methods described by Lowry *et al.* (1951) and Zlatkis *et al.* (1953) respectively.

Table 1. Ingredient and nutrient composition (%) of starter and finisher diets (basal diet) used in the experiment.

Ingredient (%)	Starter diet (0–3 weeks)	Finisher diet (4–6 weeks)
Maize	58.6	61.5
Soybean	36.0	32.5
Fat/Oil (Refined sunflower oil)	2.00	2.5
Limestone	1.00	1.00
DCP	1.75	1.50
Salt	0.35	0.35
Lysine	0.20	0.10
Methionine	0.10	0.10
Nutrient composition (%)		
ME (kcal/kg)*	3008	3076
CP	22.15	20.72
Lysine	1.32	1.13
Methionine	0.48	0.45
Ca	1.07	1.06
P	0.45	0.44

Additionally, choline chloride @ 50g/100kg; Vitamin AB_2D_3EK premix @ 12g/q; B complex vitamin premix @ 25g/100kg and trace mineral mix @ 100g/100kg feed: Mg, 300; Mn, 55; I, 0.4; Fe, 56; Zn, 30; Cu, 4, were added. Basal diet: Aflatoxin not detected. *E. alba* @ 1% or AFB1 @ 1.0 ppm were added to respective diets.

Immediately after sacrifice, liver tissues (n=8/group) were removed, rinsed with ice-cold phosphate buffered saline and blotted dry. Liver tissue/bird (4–6 g) was then homogenized in ice-cold phosphate buffer, centrifuged (10,000 g, 4°C, 15 min) and the clear supernatant obtained was preserved at –80°C for determination of parameters related to oxidative stress and antioxidant status. The malondialdehyde (MDA) concentration of liver sample was quantified by the method of Placer *et al.* (1966). The antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT) activities in liver samples were determined by the methods of Shaw *et al.* (2002) and Venturino *et al.* (2011) respectively. Units of SOD and CAT activities were expressed as the activity of enzyme per milligram of protein.

For histopathology studies, liver tissue samples were collected in 10% buffered formalin, processed as per the procedure of Bancroft and Stevens (1996) with some modifications, viewed under light microscope (Nikon, Japan) and the observations were recorded.

Statistical analyses of data: Data generated from biochemical and antioxidant assays were analyzed using the Statistical Package for Social Sciences (SPSS, version

18.0) by one-way analysis of variance (ANOVA) and comparison of means was done using Duncan's multiple range posthoc tests. The effects were considered significant at P≤0.05. All the experimental results are presented as means±SE.

RESULTS AND DISCUSSION

The AST and ALT levels were significantly (P<0.05) increased, while total protein, albumin and total cholesterol levels were significantly (P<0.05) decreased in birds fed AFB1 alone (1.0 ppm) as compared to the control. Supplementation of 1% *E. alba* with AFB1 diet significantly reduced AST and AST levels and improved total protein, albumin and total cholesterol levels compared to birds fed AFB1 alone (Table 2).

The concentrations of hepatic lipid peroxidation products and antioxidant enzymes in broilers fed basal diet, *E. alba*, AFB1 and AFB1 with *E. alba* are given in Table 3. Compared to birds fed basal diet (controls), a significant (P<0.001) increment in the levels of hepatic tissue MDA and decrease in liver SOD and catalase activities were recorded in birds fed AFB1. Supplementation of *E. alba* with AFB1 significantly reduced the MDA levels and elevated the antioxidant enzymes in liver (Table 3).

Table 2. Serum biochemical indices in broilers fed basal diet, *E. alba*, AFB1 and AFB1 with *E. alba*

Parameter	Group			
	Control (basal diet)	*E. alba* (1%)	AFB1 (1.0 ppm)	AFB1 + *E. alba*
AST (units/ml)	25.1±0.5^{c}	24.24±0.5^{c}	35.52±0.7^{a}	28.0±0.4^{b}
ALT (units/ml)	77.7±1.7^{c}	74.31±1.6^{c}	106.0±2.1^{a}	85.2±1.3^{b}
Total protein (g/dl)	3.7±0.05^{a}	3.8±0.1^{a}	1.85±0.06^{c}	3.44±0.04^{b}
Albumin (g/dl)	1.36±0.03^{a}	1.37±0.03^{a}	0.74±0.02^{c}	1.01±0.02^{b}
Total cholesterol (mg/dl)	103±1.6^{a}	106±2.7^{a}	60±1.5^{c}	92±1.5^{b}

^{a,b,c}Means with different superscripts in a row differ significantly (P<0.001).

Table 3. Hepatic lipid peroxidation products and antioxidant enzymes in broilers fed basal diet, *E. alba*, AFB1 and AFB1 with *E. alba*

Group	Parameter		
	MDA (nmol/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)
Control (basal diet)	0.9±0.02^{b}	141±3.4^{a}	175±3.8^{a}
E. alba (1%)	0.9±0.01^{b,c}	148±3.2^{a}	171±3.9^{a,b}
AFB1 (1.0 ppm)	1.9±0.03^{a}	81±3.0^{c}	124±2.7^{c}
AFB1 + *E. alba*	0.8±0.02^{c}	110±2.8^{b}	163±3.5^{b}

^{a,b,c}Means with different superscripts in a column differ significantly (P<0.001).

The histopathological changes in liver tissue of experimental birds are presented in Fig. 1. The liver tissues of birds fed basal diet and basal diet with *E. alba* showed normal hepatocyte architecture. The hepatocytes of birds fed only AFB1 showed vacuolar degeneration, focal infiltration of mononuclear cells and bile duct hyperplasia. The hepatocytes of birds supplemented *E. alba* with AFB1 demonstrated hepatocytes returning to normal architecture.

Aflatoxin contamination of Indian poultry feeds and feed ingredients (Umaya *et al.* 2010). Poultry are highly sensitive to aflatoxin and liver is the major target organ in broilers. Since oxidative stress and liver damage are hallmarks of aflatoxin toxicity, phytoproducts with antioxidant and hepatoprotective effect could be potential candidates against aflatoxin toxicity in broilers (Umaya *et al.* 2012). Antioxidants or bioactive compounds are best acquired through consumption of whole foods rather than from costly dietary supplements (Liu 2004). Hence, in the present study, the *E. alba* leaf powder was tested for its efficacy to counteract aflatoxin toxicity in broilers.

Liver is the major source of most of the serum proteins, the only site of albumin synthesis and the main organ of synthesis and lipid transport. Stress on liver function is reflected in terms of biochemical changes and alterations in hepatic marker enzymes activities in serum (Hamzawy *et al.* 2013). The elevated levels of serum AST and ALT in AFB1 fed birds reflect the loss of liver cell integrity and

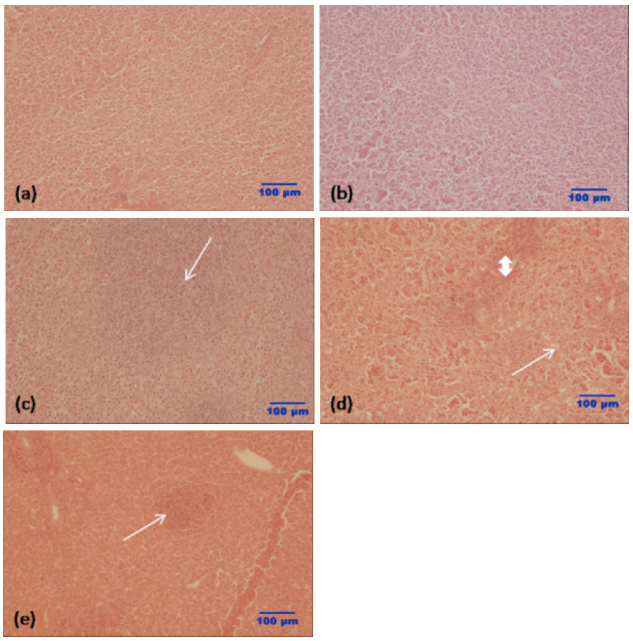

Fig. 1 (a-e). Histopathological changes in liver of birds fed basal diet, *E. alba*, AFB1 and AFB1 with *E. alba*.(a) Hepatocytes showed normal architecture in birds fed basal diet. (b) Hepatocytes showed normal architecture in birds fed basal diet with *E. alba*. (c) Hepatocytes showed infiltration of mononuclear cells in the liver parenchyma. (d) Vacuolar degeneration and mononuclear cell infiltration in cells in birds fed AFB1 alone. (e) Hepatocytes observed to return to normal architecture.

the liver damage induced by AFB1. Similar observations were elsewhere recorded during aflatoxicosis (Umaya *et al.* 2012). Aflatoxin ingested through contaminated diet is metabolized by the liver cytochrome P450 enzymes to a highly reactive compound, aflatoxin B1-8, 9-epoxide (AFBO). This AFBO readily binds nucleic acids and proteins and forms adducts and alter their functions (Rawal *et al.* 2010). Aflatoxin has also been reported to inhibit the synthesis of structural and secretory proteins that are important components of the plasma membrane, gap junctions and intercellular matrix and produce toxic effects (Singh *et al.* 2006). Consistent with previous findings (Oguz *et al.* 2002), the reduced levels of serum total protein and albumin presented in the study ascertains the inhibitory effect of aflatoxin on protein synthesis in liver (Yatim and Sachan 2001). The aflatoxin-related decrease in cholesterol observed by feeding AFB1 observed could be probably related to reduced lipogenesis, impaired lipid transport and/or inhibition of hepatic cholesterol biosynthesis by AFB1 (Hussein and Brasel 2001).

Aflatoxin is known to increase the production of reactive oxygen species (ROS), superoxide radicals, hydrogen peroxide and hydroxyl radicals in liver (Rawal *et al.* 2010). Free radicals are established to cause oxidative damage to a number of cellular biomolecules, including membranes-lipids, proteins and nucleic acids. In the present study, the liver damage might be due to oxidative stress that led to lipid peroxidation. Lipid peroxidation is a common manifestation of oxidative damage. The level of lipid peroxidation was assessed by measuring malondialdehyde (Ismail *et al.* 2015), a sensitive and reliable indicator of oxidative stress in cells and tissues (Ayala *et al.* 2014). The increased MDA levels observed in birds exposed to AFB1 indicates enhanced lipid peroxidation leading to tissue injury. Cells possess antioxidant enzymes such as superoxide dismutase and catalase and numerous studies have shown the importance of these enzymes in protecting cells against oxidative stress. The decreased activity of SOD and catalase in liver tissue of broilers fed AFB1 could be due to the oxidative inactivation of the enzymes by the excess superoxide radicals, hydrogen peroxide and hydroxyl radicals generated by AFB1 and its metabolites (Rawal *et al.* 2010, Hamzawy *et al.* 2013). The reduced levels of these enzymes might have led to the reduced antioxidant defense, increased oxidative stress and in turn liver damage. The histopathological changes observed in liver are similar to those reported in the literature (Sridhar *et al.* 2016) and ascertain the liver damage caused by aflatoxin.

Supplementation of *E. alba* leaves with AFB1 significantly reduced the levels of the ALT and AST in serum. A similar effect has been observed in the serum of rats during carbon tetrachloride induced liver injury (Thirumalai *et al.* 2011). The present study also revealed that *E. alba* supplementation could improve the concentration of serum total protein, albumin and cholesterol in birds fed AFB1. These changes could be attributed to the hepatoprotective effect of *E. alba* by regulating hepatic microsomal drug metabolizing enzyme levels (Saxena *et al.* 1993) and by stimulating liver cell regeneration (Wagner *et al.* 1986). *E. alba* leaves are rich sources of polyacetylenes, steroids, triterpenes and flavonoids that have the potential to improve serum albumin levels (Prakash *et al.* 2011). It is also possible that these compounds could have contributed to the improved albumin levels, however further research is needed. The reduction in lipid peroxidation products and improvement in antioxidant enzyme activities observed in birds supplemented *E. alba* with AFB1 suggest that *E. alba* stimulated the antioxidant system in the liver to reduce the oxidative damage caused by the toxin. The histopathological findings observed in our study are consistent with the serum biochemistry and liver antioxidant status and indicates the potential of *E. alba* to reduce liver damage induced by AFB1. Similar reduction in lipid peroxidation and increased antioxidant enzyme activity levels during *E. alba* supplementation had been reported during paracetamol induced hepatotoxicity in rats (Jahan *et al.* 2014). The antioxidant phenolic compounds (Majumdar *et al.* 2010), predominantly the coumestan derivatives, wedelolactone and demethylwedelolactone and the triterpenes and flavonoids of *E. alba* supports its hepatoprotective functions. Further, wedelolactone, one of the major component of *E. alba* is reported to scavenge superoxide and nitric oxide radcials *in vitro* (Unnikrishnan *et al.* 2007). A number of herbal preparations with *E. alba* as hepatoprotective drug are available for treatment of jaundice and viral hepatitis (Jahan *et al.* 2014). The above findings explain a mode of action of *E. alba* as an antioxidant to exhibit the hepatoprotective effect during AFB1 exposure.

In conclusion, the findings of the study suggest that supplementation of *E. alba* (1.0%) to a diet containing AFB1 (1.0 ppm), improved serum biochemistry, reduced lipid peroxidation with concomitant improvement in hepatic antioxidant status and offered significant protection against the oxidative stress and liver damage induced by AFB1 in broilers. Further research is needed to determine the efficacy of *E. alba* and its major antioxidant phytochemicals to protect against aflatoxin toxicity in other susceptible species.

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