Ameliorative efficacy of citrus oil in aflatoxin induced changes in lymphoid organs in broilers-A pathomorphological evaluation

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ABSTRACT
A total of 160 day-old broiler chicks were divided randomly into four groups with two replicates of 20 birds each. The birds were fed with basal diet, basal diet with CFO, basal diet with aflatoxin, basal diet with CFO and AF. Serum globulin level was significantly reduced in birds fed with AF only on 14th day of treatment. Microscopically, mild degree of congestion, histiocytosis and cellular sparcity of the periarteriolar lymphoid component were observed in the spleen whereas lymphoid cell depletion in thymus and bursal follicles were prominent. Additionally, thymus revealed moderate degree of congestion and haemorrhages in widened medulla, histiocytosis and degeneration of Hassall’s corpuscles. In CFO and AF fed birds, the extent and degree of lesions were lesser with compact arrangement of lymphoid cell with widening of cortex was observed mainly during later days of experiment. There was widening of lymphoid component of caecal tonsils up to mucosal layer in CFO fed birds.

Key words: Aflatoxicosis, Amelioration, Broilers, Citrus fruit oil, Immunotoxicity.

INTRODUCTION
Aflatoxins (AF) are heterocyclic compounds produced by Aspergillus flavus and A. parasiticus, are of major concern as they are carcinogenic, mutagenic, teratogenic and have growth inhibitory effects (Oguz et al., 2002). Avian species are more sensitive to aflatoxicity and chickens are selected as experimental model due to its availability and easy maintenance. Contamination of AF in feed produces clinical signs in birds like lethargy, ruffled feathers, listlessness, anemia, edema, inappetance, poor growth rate, diarrhoea, polydipsia and mortality (Asplin and Carnaghan, 1961), anaemia (Huff et al., 1988; Kececi et al., 1998), suppression of immune functions such as the decrease in humoral and cellular immune responses (Oguz et al., 2003). A dose and time dependent depletion of lymphoid cells in spleen, thymus and bursa of Fabricius (Ortatatli and Oguz, 2001). Lymphoid organ toxicity makes the birds susceptible to various infections which lead to death or lowered performance.

Fungal contamination of agricultural products is often unavoidable in tropical countries. Mycotoxins are one of the major factors affecting poultry productivity and product quality. Aflatoxin residues in the poultry product often detected (Herzallah et al., 2009) and poses major public health hazard in developing countries. A review (Friedman et al., 2013) suggests that food or feed compatible natural compounds and plant extract can be used to reduce the AFB1 content of food and its adverse in vivo effects. Among the several methods adopted, the most effective is the neutralization of mycotoxins present in feed by inclusion of inert adsorbents or toxin binders that prevent absorption of the toxin from the intestine. Conversely, toxin binders may also affect the nutrients availability. Besides detoxification, herbal extracts can also be used to enhance hepatic antioxidants. Polyherbal extracts of Operculina turpethum, Piper nigrum, Emblica ribes, Curcuma longa, Phyllanthus emblica, Piper officinarum, Zingiber officinale, Piper longum, Cedrus deodara etc. (Kalorey et al., 2005) and extracts of artichoke are included in animal feeds to detoxify the absorbed toxins and to prevent damage to the liver by enhancing the action of hepatic antioxidants (Stoev et al., 2004).

Microsomal cytochrome P-450 enzymes are responsible for bio-activation of AFB1 into epoxide form which favors the formation of aflatoxin-DNA adduct in liver of chicken and quail. Hence, liver is the target organ for AF toxicity (Diaz et al., 2010 and Murcia et al., 2011). D-limonene, a monoterpenoid constituent of citrus fruit oil (CFO) has inhibitory effects on cytochrome P450 enzymes, p-nitrophenol hydroxylase (pNP) and 7-ethoxyresorufin O-deethylase (EROD) activity in-vitro in liver microsomes, prevents bioactivation of procarcinogens and blocks tumour induction by chemical carcinogens (Reicks and Crankshaw, 1993). Citrus fruit oil (active ingredient d-limonene) also has some detoxification and antioxidant property by increasing the level of Glutathione.
–S- transferase (van Lieshout et al., 1998). Essential oils of *Citrus reticulata* and *Cymbopogon citratus* at inclusion level of 750 and 500 mg Kg⁻¹, respectively, inhibit AFB1 production by *A. flavus* (Singh et al., 2010). Based on these evidences on detoxification and inhibitory effects on microsomal enzymes, gross and microscopic changes of lymphoid organs during experimental aflatoxicosis (1 mg Kg⁻¹ in diet) was evaluated to determine possible preventive role of dietary CFO on pathological changes.

**MATERIALS AND METHODS**

**Aflatoxin production:** Aflatoxin was produced on rice using *Aspergillus parasiticus*, NRRL 2999 culture by the method of Shotwell et al. (1966). Fermented rice was autoclaved and ground to a fine powder. The AF content in rice powder was analyzed by thin layer chromatography (TLC) (AOAC, 1980). The concentration of AFs was calculated from developed plates by densitometry at 366 nm using a fluorodensitometer (Camag II, Basal, Switzerland) using known standard (Sigma Chemicals Ltd, USA). The total quantity of AF in the culture material was found to be 317 mg kg⁻¹, consisting of 266 mg kg⁻¹ AFB1, 19.9 mg kg⁻¹ AFB2, 24.7 mg kg⁻¹ AFG1 and 6.2 mg kg⁻¹ AFG2. The rice powder was added to the basal diet to provide the final concentration of 1mg Kg⁻¹. Aflatoxin and citrus fruit oil (CFO) were added in feed wherever required and fed to birds from day 7 to 42 of age

**Chickens and feed:** One hundred and sixty (n=160) day-old Ross 308 broiler chicks of both sexes were procured from a commercial hatchery (Suguna Poultry Farm Pvt Ltd) and reared in battery cage system in experimental sheds with average temperature ranging from 27 to 31°C and relative humidity of between 59 to 62% with 16: 8 ±1hr L: D cycle of intensity 10 to 20 lux. Individually chicks were weighed and divided into 4 groups randomly with two replicates of 20 chicks each as group A, B, C and D after acclimatization for 7 days. The starter and grower basal diets were given to birds as recommended by National Research Council (NRC, 1994). All chicks were vaccinated on 7th and 11th day of age with Lasota strain of Newcastle disease virus and infectious bursal disease (Intermediate strain) respectively.

**Experimental design:** A 2 x 2 factorial design was used in which experimental diets were tested and these include basal diet (group a), basal diet + citrus fruit oil 2.5g kg⁻¹ (Group B), basal diet + 1 mg Kg⁻¹ aflatoxin (Group C), 1mg Kg⁻¹ Aflatoxin + basal diet + citrus fruit oil 2.5g kg⁻¹ (Group D). Citrus fruit oil containing volatile oils of citrus fruits were obtained from M/s Tetragon Chemie, Bengaluru, India.

Experimental protocol used was approved by the Institutional Animal Ethics Committee, Veterinary College, Bengaluru. Dose of aflatoxin was selected based on previous reports (Oguz et al., 2002). Citrus fruit oil dose selected was based on report by Kumar et al.,(2015) and it was found that 2.5g kg⁻¹ of broiler feed for duration of 35 days is suitable.

Six birds selected randomly from each group were weighed individually and sacrificed on 7th, 14th, 21st, 28th and 35th day of post treatment. Blood was collected from each bird at each interval from wing veins using syringe and needle for serum biochemical estimation and stored at -20°C. A detailed necropsy was conducted. Liver, kidneys, lymphoid organs viz., spleen, Bursa of Fabricius, thymus and caecal tonsils were removed and weighed. The relative lymphoid organ weights (weight of organ / live body weights) were calculated (Table 1). Individual serum samples were analyzed for total globulin using standard kits (Spam diagnostics Pvt Ltd, Surat, Gujarat, India) by automatic analyser (Fig. 1).

Representative tissue samples were collected in 10% neutral buffered formalin for sequential pathology at weekly intervals. After fixation, samples were dehydrated in alcohol, cleared and embedded in paraffin wax. Sections were cut at 3-5 microns thickness and stained with haematoxylin and cosin (Luna, 1968).

**Table 1:** Effect of citrus oil on mean relative weight of immune organs aflatoxicated birds at weekly intervals.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Relative Bursa of Fabricius weight (g %)</th>
<th>Days post treatment</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
<th>35</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>0.28±0.01*</td>
<td>0.34±0.02*</td>
<td>0.32±0.06*</td>
<td>0.30±0.02*</td>
<td>0.23±0.04*</td>
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<tr>
<td>Group B</td>
<td>0.26±0.03*</td>
<td>0.35±0.05*</td>
<td>0.36±0.05*</td>
<td>0.30±0.03*</td>
<td>0.22±0.02*</td>
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<tr>
<td>Group C</td>
<td>0.32±0.06*</td>
<td>0.27±0.03*</td>
<td>0.22±0.03*</td>
<td>0.17±0.01*</td>
<td>0.13±0.04*</td>
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<tr>
<td>Group D</td>
<td>0.29±0.02*</td>
<td>0.27±0.03*</td>
<td>0.25±0.02*</td>
<td>0.21±0.04*</td>
<td>0.17±0.50*</td>
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<thead>
<tr>
<th>Groups</th>
<th>Relative Thymus weight (g %)</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
<th>35</th>
</tr>
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<tbody>
<tr>
<td>Group A</td>
<td>0.50±0.11</td>
<td>0.37±0.06</td>
<td>0.34±0.05</td>
<td>0.21±0.04</td>
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<tr>
<td>Group B</td>
<td>0.49±0.12</td>
<td>0.38±0.02</td>
<td>0.36±0.08</td>
<td>0.25±0.01</td>
<td>0.15±0.01</td>
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<tr>
<td>Group C</td>
<td>0.47±0.04*</td>
<td>0.23±0.02*</td>
<td>0.18±0.01*</td>
<td>0.17±0.02*</td>
<td>0.09±0.03*</td>
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<tr>
<td>Group D</td>
<td>0.66±0.08*</td>
<td>0.32±0.01*</td>
<td>0.33±0.03*</td>
<td>0.20±0.02*</td>
<td>0.14±0.01*</td>
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<thead>
<tr>
<th>Groups</th>
<th>Relative Spleen weight (g %)</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
<th>35</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>0.07±0.02</td>
<td>0.10±0.002</td>
<td>0.10±0.00</td>
<td>0.17±0.00</td>
<td>0.20±0.003</td>
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<tr>
<td>Group B</td>
<td>0.07±0.02</td>
<td>0.10±0.01</td>
<td>0.11±0.00</td>
<td>0.17±0.00</td>
<td>0.20±0.00</td>
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<tr>
<td>Group C</td>
<td>0.11±0.02</td>
<td>0.11±0.01</td>
<td>0.14±0.02</td>
<td>0.19±0.02</td>
<td>0.15±0.01</td>
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<tr>
<td>Group D</td>
<td>0.09±0.01</td>
<td>0.06±0.02</td>
<td>0.11±0.01</td>
<td>0.18±0.02</td>
<td>0.19±0.06</td>
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*Mean ± SEM values with different superscripts within a row differ significantly (P<0.05)
**RESULTS AND DISCUSSION**

The data presented in Table 1 reveal the effect of feeding CFO to AF intoxicated birds on relative immune organ weights of broiler chicks. The present study shows that birds fed with diets containing AF (group C) had lesser relative weight of lymphoid organs than other treatment groups. Mean relative weights of thymus was significantly (P<0.05) decreased on 14 and 21 days of treatment as compared control birds (group A) whereas, relative weights of spleen and bursa of Fabricius showed only numerical decrease among the treatment groups of study. Supplementation of CFO along with AF in the diet (group D) significantly (P<0.05) improved the relative weights of thymus on 21 days of treatment. The effects of CFO and AF on serum total globulin are presented in Fig 1. Significant (P<0.05) reduction in total globulin levels was clearly seen in AF intoxicated birds on 14th day of treatment. Incorporation of CFO in the diet containing AF significantly (P<0.05) increased the level on 14th day of treatment.

Gross lesions exhibited by aflatoxin fed birds (Group C) were enlarged, yellowish or pale coloured, soft and friable livers with hematoma (Kumar et al., 2015); pale and slightly enlarged kidneys and marginal atrophy of bursa of Fabricius, spleen and thymus with hemorrhages. The liver lesions recorded from 7 days of AF inclusion while lesions in kidneys and lymphoid organs were observed only from 14th day of treatment onwards. CFO addition in AF contaminated diet marginally reduced the severity and magnitude of the lesions. Caecal tonsils had normal appearance on all the days of observations, irrespective of dietary treatments. The birds fed with basal diet (Group A) and addition of CFO in basal diet (Group B) did not show any lesions throughout the study.

The effect of AF feeding in the diet was recorded microscopically in spleen as mild degree of congestion, histiocytosis and cellular sparcity of the periarteriolar lymphoid component from 7th Day of feeding (Fig. 2A). The changes were mild with occasional secondary follicle formation was visualized on 35th day of treatment. In those birds, lymphoid cell depletion in cortical and medullary areas of both thymus and bursal follicles were prominent from 14th day of feeding (Fig. 2B). Additionally, thymus revealed mild to moderate degree of congestion and haemorrhages in widened medulla, increase in the number of histiocytes containing cellular debris in cortex from 14th day onwards and degeneration of Hassall’s corpuscles visualized as eosinophilic mass on Day 28 and 35 of treatment (Fig. 2C).

Ameliorative effect of CFO on lymphoid organs was appreciated as widening of periarteriolar lymphoid component with formation of occasional secondary lymphoid follicles from 14th day of treatment till the end of the experiment in spleen (Fig. 2D). The widening of lymphoid component and increase in number of secondary follicle progressed with days of treatment. Bursa of Fabricius showed formation of large follicles with numerous mitotic cells in the sheet of compactly arranged lymphoid cells and increase in mitotic cells progressed during later days of experiment (Fig. 2E). In thymus, mild to moderate congestion of medulla with histiocytosis from 21st day of treatment to till the end of the experiment. There was widening of cortex with compact arrangement of lymphoid cell during later days of experiment (Fig. 2F). Microscopically, caecal tonsils revealed no lesions in any of the groups throughout the period of study. There was widening of lymphoid component extended up to mucosal layer from 14th day onwards. The birds fed with AF free diet (Group A) and with CFO in AF free diet (Group B) did not exhibit any lesion at any point of time during the study.

Aflatoxin has many adverse effects, of which immunotoxicity is one of the important toxicity reported in birds (Giambrone et al., 1985, Celik et al., 2000). Aflatoxin...
affects the immune organs mainly due to formation of DNA adduct in fast dividing lymphoid cells which leads to profound immunodeficiency by hampering the development and maturation of lymphoid organs (Dietert et al., 1985; Neldon-Ortiz and Qureshi, 1992). Amelioration of the negative effects of aflatoxicity was attempted using herbal preparations by previous workers (Stoev et al., 2004; Kalorey et al., 2005). Citrus fruit oil with active ingredient D-limonene has inhibitory effect on Cytochrome P 450 enzymes (Reicks and Crankshaw, 1993) which converts AF to AFB1 - 8, 9 epoxide which ultimately forms DNA adducts and produces toxicity. CFO also possesses antioxidant activity by enhancing the level of Glutathione –S- Transferase (van Lie Shout et al., 1998). Based on these evidences, an attempt has been made to study the ameliorative effect of CFO during AF exposure on relative organ weight, serum total globulin levels and pathology of lymphoid organs.

The relative weight (RW) of all studied lymphoid organs marginally decreased in AF fed birds. Mean relative weights of thymus was significantly (P<0.05) decreased on 14 and 21 days of treatment as compared to control birds, could be attributed to the effect of AFB1 on lymphoid component in the form of lymphocytolysis, which was clearly visualized in histopathology and also documented by Venkataram et al. (1988), Kubena et al. (1990), and Ortatatli and Oguz (2001). Birds supplemented with CFO in AF containing diet showed significant increase in relative weight of thymus on 21 days post treatment with mild degree of histological lesions in immune organs. The relative weight of immune organs mainly thymus and reduced histological lesions clearly shows the ameliorative effects of CFO in AF fed birds. Similar ameliorative studies using various chemical compounds other than CFO have also been reported by earlier workers (Swamy and Devegowda, 1998; Phillips et al., 1999).

The mean (±SE) serum globulin values were significantly (P≤0.05) decreased in AF fed birds as compared to controls (Group A). Similar findings were also reported by Bakshi et al. (1997), Mckenzie et al. (1998), Ledoux et al. (1999 and Rosa et al. 2001), lending support to the present findings. The reduction in globulin level in AF fed birds could be due to degeneration of endoplasmic reticulum.

Fig 2A, 2B, 2C: Moderate lymphocytolysis in periarteriolar lymphoid sheath of spleen, moderate histiocytosis in bursa of Fabricius, haemorrhages in the medulla also note eosinophilic oedematous fluid (arrow) in aflatoxin alone (Group C) fed birds. Fig. 2D, 2E, 2F: multiple lymphoid follicle formation in spleen, compact and regular arrangement of lymphoid cells in the follicles of bursa of Fabricius, medulla of thymus in aflatoxin and citrus oil (Group D) treated birds.
in hepatocytes and covalent binding of AF metabolites to template RNA and in turn reduction in protein synthesis (Tung et al. 1975 and Hilton et al. 1989). Supplementation of CFO along with AF in the diet increased the levels marginally could be attributed to partial alleviation of toxic effects. The findings of the present study were in agreement with those of Devegowda et al. (1994) and Raju and Devegowda (2000). The improved body weight and feed conversion ratio (Data not presented) observed in this study further supported this assumption.

Gross lesions observed in aflatoxicated birds were pale and slightly enlarged kidneys, marginal atrophy of bursa of Fabricius, spleen and thymus with haemorrhages. These findings were in accordance with those of earlier workers (Mohanaraju, 1990; Kubena et al., 1995; Ortatatli and Oguz, 2001; Rosa et al., 2001). Atrophy of lymphoid organs was considered as impact of AF on immune system during post embryonic developmental period (Neldon-Ortiz and Qureshi, 1992; Devegowda et al., 1998).

Microscopically in the present study, spleen revealed mild degree of congestion of red pulp, sparse cellularity in the periarteriolar sheath indicative of lymphocytolysis with mild histiocytosis from Day 7 to Day 35 post treatment, in birds treated with aflatoxin (Group C). However on Day 35 post treatment, the spleen also revealed formation of occasional secondary follicles. Aflatoxin which is a known immunosuppressant, as reported by earlier workers (Thaxton et al., 1974; Ortatatli and Oguz, 2001) could be the reason for the lesions in spleen.

Bursa of Fabricius and Thymus revealed cellular sparcity and increase in the number of histiocytes in AF fed birds (Group C). Thymus also showed mild congestion with multi focal haemorrhages and Necrosis of reticular cells with degeneration of Hassall’s corpuscles. This could be attributed to the effect of AFB1 on lymphoid component in the form of lymphocytolysis, which accounted for a numerical decrease in relative weight, as also observed and documented by Venkataram et al. (1988), Kubena et al. (1990), Ortatatli and Oguz (2001) and Ortatatli et al. (2005). The metabolism of aflatoxin involves elaboration of free radicals, (Santhosh Kumar, 2003) and rapidly proliferating cells of the immune system were reported to be prone for peroxidation damage of free radicals which could be the possible reason for the lesions noticed in the lymphoid organs of toxin alone treated group.

Supplementation of CFO in AF mixed diet reduced the incidence and severity of both gross and microscopic lesions. Histologically, spleen with very mild degree of lesions during early period of experimentation followed by formation of multiple follicles and widening of lymphoid component during later periods was observed. Bursa of Fabricius and Thymus revealed mild cellular sparsity and histiocytic infiltration indicating the effect of aflatoxin even after supplementation. In addition, bursa of Fabricius with widened occasional follicles with compactly arranged lymphoid cells and widening of lymphoid component of cortex in thymus were observed during later period of study. The widened follicles with compact lymphoid components indicated that CFO had immunostimulatory effect. The gross and microscopic examination of caecal tonsils of all birds irrespective of the group revealed normal appearance on all the days of observation. Surprisingly, CFO treated birds in Group B and D exhibited increased formation of secondary follicles, which could again be attributed to some immune stimulatory effect. A similar finding has been reported as development of secondary follicle with prominent lymphoid nodules and aggregates in the pancreas and intestinal mucosa in mice receiving the d-limonene (Evans et al., 1987). D-limonene may also exhibit immune-modulating properties. Proliferation of CON A induced rat splenocytes was observed in rats fed with d-limonene diet (Hamada et al., 2002) further supports the immune stimulatory effect of CFO.

In conclusion, during this study, it was observed that AF (1 mg Kg$^{-1}$) fed birds showed significant decrease in total globulin level at 14 day post treatment with moderate to severe histopathological lesions in all lymphoid organs. Apart from this, addition of CFO at 2500 mg Kg$^{-1}$ in the diet increased the globulin levels, reduced the severity of the histological lesions in the lymphoid organs by improving its relative weight and formation of secondary follicles in caecal tonsils indicates its immunostimulatory effect and partial alleviation of immunotoxic effects. No lesions were observed in CFO only supplemented birds indicating its non-toxicity at this level. Supplementation of CFO in the diet could be a solution to reduce the AF induced immunotoxicity in addition to other mycotoxin management practices.

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Conflict of Interest

Authors declare that there is no conflict of interest in publishing the results.

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