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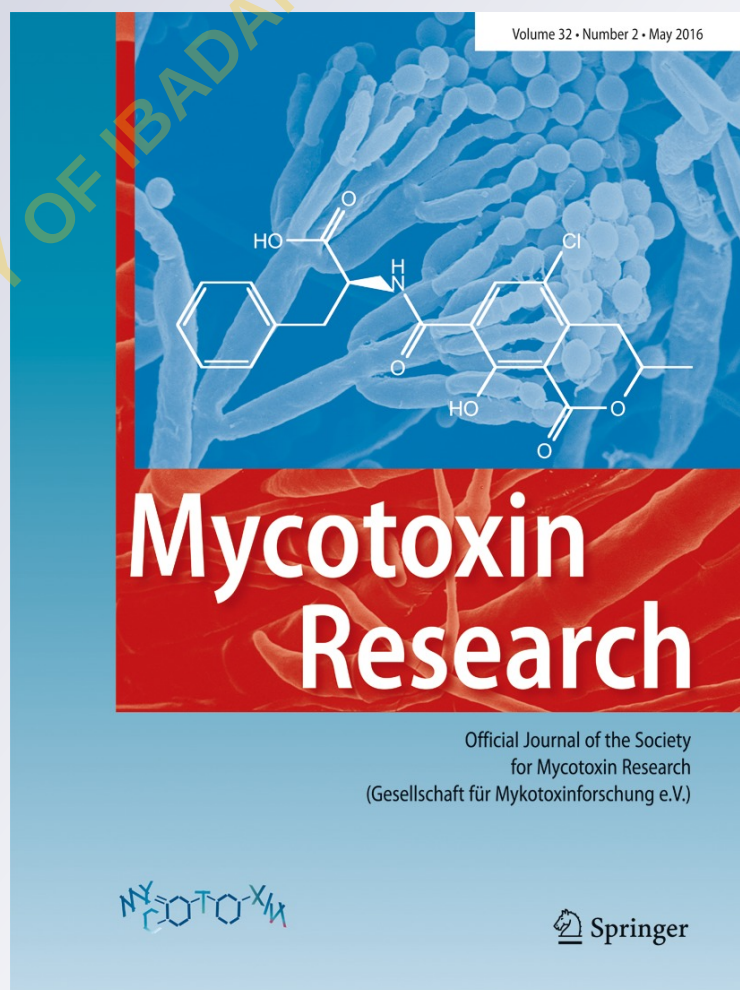
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Changes in serum cytokine levels, hepatic and intestinal morphology in aflatoxin B1-induced injury: modulatory roles of melatonin and flavonoid-rich fractions from *Chromolena odorata*

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Abstract Aflatoxins are known to produce chronic carcinogenic, mutagenic, and teratogenic effects, as well as acute inflammatory effects, especially in the gastrointestinal tract. The potentials of the flavonoid-rich extract from *Chromolena odorata* (FCO) and melatonin (a standard anti-oxidant and anti-inflammatory agent) against aflatoxin B1 (AFB1)-induced alterations in pro-inflammatory cytokine levels and morphology of liver and small intestines were evaluated in this study. We utilized Wistar albino rats (200–230 g) randomly divided into five groups made up of group A, control rats; group B, rats given AFB1 (2.5 mg/kg, intraperitoneal) twice on days 5 and 7; rats in groups C, D, and E were treated with melatonin (10 mg/kg, intraperitoneal) or oral doses of FCO1 (50 mg/kg) and FCO2 (100 mg/kg) for 7 days, respectively, along with AFB1 injection on days 5 and 7. Serum levels of interleukin 1 beta (IL-1 β) and tumor necrosis factor alpha (TNF- α) were determined using commercial ELISA kits and histopathological evaluation of the liver, duodenum, and ileum were also carried out. We observed significant elevation ($p < 0.05$) in serum IL-1 β correlating with hemorrhages and leucocytic and lymphocytic infiltration in the liver and intestines as evidences of an acute inflammatory response to AFB1 administration. All treatments yielded significant reduction ($p < 0.05$) in IL-1 β levels, although TNF- α

levels were not significantly altered in all rats that received AFB1, irrespective of the treatments. Melatonin and FCO2 produced considerable protection of hepatic tissues, although melatonin was not quite effective in protecting the intestinal lesions. Our findings suggest a modulation of cytokine expression that may, in part, be responsible for the abilities of *C. odorata* or melatonin in amelioration of hepatic and intestinal lesions associated with aflatoxin B1 injury.

Keywords Aflatoxin B1 · Inflammation · *Chromolena odorata* · Melatonin · Liver · Gut

Introduction

Human and animal diets are often contaminated by toxic metabolic products of fungi called mycotoxins. Aflatoxins are highly potent mycotoxins produced by *Aspergillus* species. The most predominantly encountered aflatoxin is aflatoxin B1 (AFB1), which has been classified as a group 1 carcinogen (Eaton et al. 1994). Aflatoxin exposures occur mainly via ingestion of contaminated foodstuffs, in which case the absorption takes place through the gastrointestinal epithelium. However, blood-time concentration profiles of AFB1 in experimental animals have been obtained via intraperitoneal and intravenous routes (Hsieh and Wong 1994).

Absorbed AFB1 is translocated from the site of exposure to distant organs and tissues, the rate of organ perfusion being the primary factor determining the concentrations of the toxin reached in different tissues. Consequently, parenterally administered aflatoxin can reach substantial levels in highly perfused organs such as the liver. Bile produced in the liver is normally transported to the upper small intestine, where bile acids assist in digestion of lipids in the diet. In effect, xenobiotic metabolites that are secreted into bile can reach the small intestine,

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where enterocytes are exposed to the toxic actions (Ito et al. 2001; Botta et al. 2004; Shibamoto and Bjeldanes 2009). Re-absorption and re-cycling of AFB1 metabolites via entero-hepatic circulation also greatly contribute to a greater exposure and retention, and therefore, likelihood of toxicity of AFB1 to the intestinal epithelium (Grenier and Applegate 2013).

Much research on aflatoxin B1 transformation has focused on the chronic effects caused by formation of the toxic intermediate, exo-AFB1-8,9-epoxide (Gross-Steinmeyer and Eaton 2012), and other metabolites which can bind covalently to tissue macromolecules, forming lysyl adducts with proteins and guanine adducts with DNA (Bedard and Massey 2006). Biotransformation of AFB1 to this reactive epoxide occurs largely in the liver and also to some extent in extra-hepatic tissues (Sergent et al. 2008). Well characterized effects include the development of mutations of p53 and hepatocellular carcinoma (Ravinayagam et al. 2012). A number of reports in humans (Peraica et al. 1999) and animals (Gupta 2011) have, however, indicated symptoms of acute exposures to aflatoxins, including nausea, vomiting, diarrhea, abdominal pain, gastrointestinal erosions, etc. The exact mechanism by which AFB1 exerts acute effects on tissues, particularly the gastrointestinal tract is still largely unanswered. Effects on the cholinergic apparatus are thought to be implicated (Cometa et al. 2005).

More recently, the participation of inflammatory mediators in mycotoxin exposures have been evaluated. Available reports have suggested that low levels of mycotoxin exposure caused elevation in levels of some pro-inflammatory cytokines, while high concentrations induced down-regulation of some other cytokines (Grenier et al. 2012; Grenier and Applegate 2013; Singh et al. 2015).

Natural products from medicinal plants have been widely reported for their anti-fungal and anti-aflatoxigenic properties (Joseph et al. 2005; Kumar et al. 2007; Abdulmajeed 2011). *Chromola odorata*, of the Asteraceae family, is a common perennial shrub that grows in many parts of the humid tropics and has been largely reported for its potent medicinal use due to its anti-inflammatory, anti-microbial, and cyto-protective properties (Owoyele et al. 2005; Nur Jannah et al. 2006; Ling et al. 2007; Hanh et al. 2011). A recent comprehensive review of the medicinal potentials of this plant is available (Omokhua et al. 2015). Phenolics from the plant have been found to exhibit potent protection against damage to cultured skin cells, with implications for cutaneous wound healing, a process with significant inflammatory component (Phan et al. 2001). We have also previously reported benefits of the methanol extracts of *C. odorata* in attenuation of intestinal injury in a model of ischemia-reperfusion injury in a previous study (Akinrinmade et al. 2015).

Aflatoxin-induced acute symptoms may involve inflammatory processes which may be related to the occurrence of some

gastrointestinal symptoms earlier mentioned. In this study, we sought to investigate alterations in serum levels of the pro-inflammatory cytokines, tumor necrosis factor alpha (TNF- α), and interleukin 1 beta (IL-1 β) following AFB1 administration in rats with a view to correlating these with lesions, if any, in the liver and intestinal tissues. As *C. odorata* has been reported to possess potent anti-inflammatory activities (Owoyele et al. 2005), we also investigated the effects of flavonoid-rich fractions from the plant on modulation of inflammatory status of the liver and intestines in rats exposed to AFB1.

Materials and methods

Chemicals and kits

Aflatoxin B1 was generously provided by Dr. Joseph Atehnkeng of the Aflasafe Laboratory at the International Institute of Tropical Agriculture, Ibadan, Nigeria. Melatonin (5-methoxy-*N*-acetyltryptamine), TNF- α , and IL-1 β kits were obtained from Abcam Biochemicals (Cambridge, UK). All other chemicals and solvents were of analytical grade and were purchased commercially.

Collection of plant materials and flavonoid extraction

Fresh *C. odorata* leaves were collected from the University of Ibadan campus. They were authenticated at the Department of Botany, University of Ibadan (voucher number UIH-22385). The leaves were air-dried at room temperature, after which they were milled into coarse powder with an electric blender. The plant materials were first de-fatted with *n*-hexane after which the flavonoid fraction was extracted using an ultrasonic method previously reported by Zhu et al. (2010), with slight modifications. A quantity of the de-fatted material was suspended in 70 % ethanol in the ratio of 1 g in 50 ml. This was placed in an ultrasonic bath (Supersonic X-3, Model DSD80A5QS) for flavonoid extraction for 1 h at room temperature (25 °C). The sample obtained was filtered, and the filtrate was concentrated using a rotary evaporator. The concentrated extract was suspended in distilled water for administration to animals.

Animals and experimental design

Thirty-five male Wistar rats (200–230 g) were obtained from the Experimental Animal Unit, Faculty of Veterinary Medicine, University of Ibadan. They were kept in plastic cages in a well-ventilated animal house and maintained on standard rat chow purchased commercially and clean water ad libitum. All animals received humane care in compliance with guidelines prepared by the National Academy of Science and published by the National Institutes of Health.

Institutional approval was obtained from the Animal Care and Use Research Ethics Committee of the University of Ibadan (Approval number: UI-ACUREC/App/2015/006). The animals were initially acclimatized for a period of 1 week after which they were divided randomly into five groups of seven rats each as follows: group A (control) included healthy rats given distilled water orally for 7 days; group B had rats twice administered intraperitoneally on days 5 and 7 with aflatoxin B1 (2.5 mg/kg); group C rats were treated with melatonin for 7 days (10 mg/kg) along with aflatoxin B1 on the 5th and 7th days; Rats in groups D and E were treated with the flavonoid fraction from *C. odorata* at either 50 mg/kg (FCO1) or 100 mg/kg (FCO2), respectively, together with aflatoxin B1 on the 5th and 7th days. All animals were killed on the 8th day, about 24 h after the last treatment.

Determination of serum TNF- α and IL-1 β

Blood samples were collected in plain sample bottles and later centrifuged at 3000 $\times$ g for 10 min. Serum was collected as the resultant supernatant and was used in the assay of TNF- α and IL-1 β by means of kits supplied by Abcam Biochemicals (Cambridge, UK) using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions.

Histopathological analysis

The liver and small intestines were carefully dissected out, and small portions of different lobes of the liver were removed, while small portions of the proximal and distal areas of the duodenum and ileum were cut and opened along their lengths. The collected tissues were rinsed in normal saline to remove traces of blood and were immediately fixed by suspending in 10 % formal saline prior to histological processing. The fixed tissues were initially sectioned at a thickness of 4 mm, then embedded in paraffin wax and microtome sections at 4 μ m were finally obtained. The sections were stained with hematoxylin and eosin (H&E) and examination was done by light microscopy by pathologists who were blind to the treatments in the experimental groups. The histological criteria for the assessment of lesions are as stated in Tables 1 and 2. The severity of lesions were graded as absent (–), mild (+), moderate (++) or severe (+++).

Results

Changes in serum cytokine levels after aflatoxin B1 administration and treatment with melatonin and flavonoid fractions of *C. odorata*

The serum levels of IL-1 β and TNF- α are presented in Fig. 1. Animals exposed to AFB1 alone demonstrated a significant

Table 1 Summary of severity of histopathological lesions induced by aflatoxin B1 in the liver

	Histopathological lesion	A	B	C	D	E
1	Hemorrhage	–	+++	–	–	–
2	Leukocyte infiltration	–	++	–	++	–
3	Sinusoidal dilatation	–	++	–	–	–
4	Portal tract congestion	–	++	–	–	–
5	Hepatocellular necrosis	–	–	–	–	–
6	Cytoplasmic vacuolation	–	–	–	–	–

A control, B AFB1 (2.5 mg/kg), C AFB1 (2.5 mg/kg) + melatonin (10 mg/kg), D AFB1 (2.5 mg/kg) + FCO (50 mg/kg), E AFB1 (2.5 mg/kg) + FCO (100 mg/kg), “–” absent (severity score), “+” mild (severity score), “++” moderate (severity score), “+++” severe (severity score)

increase ($p < 0.05$) in IL-1 β levels compared with the controls. Treatment with melatonin and the two doses of the flavonoid fractions from *C. odorata* produced significant reductions ($p < 0.05$) in IL-1 β levels. On the other hand, a significant reduction ($p < 0.05$) in TNF- α levels was observed in rats exposed to AFB1 alone when compared with control. However, neither melatonin nor the flavonoid fractions were able to raise TNF- α levels to those in the control.

Histopathology

Photomicrographs showing the histological appearance of the liver in the different groups are presented in Fig. 2. The

Table 2 Summary of severity of histopathological lesions induced by aflatoxin B1 in the duodenum and ileum

	Histopathological lesion	A	B	C	D	E
1	Hemorrhage	Duodenum	–	–	–	–
		Ileum	–	–	–	–
2	Sub-epithelial space	Duodenum	–	+++	+++	+
		Ileum	–	+++	+++	–
3	Villi degeneration	Duodenum	–	+	+	–
		Ileum	–	++	+	–
4	Loss of villi	Duodenum	–	–	–	–
		Ileum	–	–	–	–
5	Leucocyte infiltration	Duodenum	–	++	++	+
		Ileum	–	+	+	+
6	Lymphocyte infiltration	Duodenum	–	+	–	–
		Ileum	–	+	+	–
7	Hyperplasia of lamina propria	Duodenum	–	–	–	–
		Ileum	–	–	–	–

A control, B AFB1 (2.5 mg/kg), C AFB1 (2.5 mg/kg) + melatonin (10 mg/kg), D AFB1 (2.5 mg/kg) + FCO (50 mg/kg), E AFB1 (2.5 mg/kg) + FCO (100 mg/kg), “–” absent (severity scores), “+” mild (severity scores), “++” moderate (severity scores), “+++” severe (severity scores)

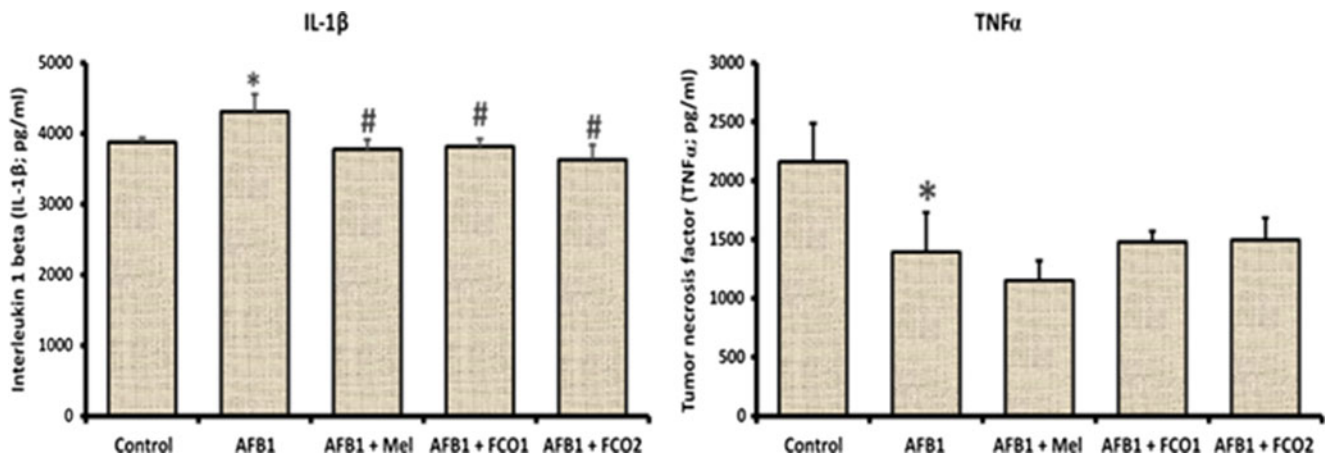


Fig. 1 Changes in serum tumor necrosis factor alpha ($TNF-\alpha$) AND interleukin 1 beta ($IL-1\beta$) levels after aflatoxin B1 administration. Values are expressed as mean $\pm$ standard deviation. Asterisk indicates significant difference ($p < 0.05$) compared with control; number sign

indicates significant differences compared with aflatoxin B1 group. AFB1 aflatoxin B1, Mel melatonin, FCO1 flavonoid fraction of *Chromola odorata* (50 mg/kg), FCO2 Flavonoid fraction of *C. odorata* (100 mg/kg)

severity of the lesions is as summarized in Table 1. Control rats had normal architecture of the liver with normal hepatocytes and clear sinusoids and portal tracts. Rats exposed to

aflatoxin B1 alone showed hemorrhages with dilated sinusoids and inflammatory cellular infiltration. The portal tracts also exhibited some degrees of congestion. Considerable protection of hepatic morphology was obtained in animals treated with melatonin and the higher dose of the flavonoid fraction of *C. odorata*. The lower dose of the latter, however, could not prevent inflammatory cell infiltration as was obtained with rats given aflatoxin B1 alone. In all the groups, the hepatocytes were not severely affected.

In the duodenum and ileum (Figs. 3 and 4, respectively), the major lesions observed with aflatoxin B1 exposure included the development of sub-epithelial space, with mild to moderate degrees of villi degeneration. Moderate degrees of leucocyte and lymphocyte infiltration of the lamina propria were also observed. These lesions were also observed in the rats treated with melatonin. However, treatment with flavonoids from *C. odorata* produced significant amelioration of the aforementioned lesions compared with the rats in the aflatoxin B1 group.

Discussion

Aflatoxin B1 has been shown to induce immune-inflammatory responses in different conditions (Li et al. 2014; Qian et al. 2014). The immunosuppressive effects of aflatoxins, such as toxicity to T cell populations have also been well documented (Meissonnier et al. 2008). However, the effects of aflatoxin B1 on cytokine expression so far, still requires harmonization as some conflicting results have been obtained from in vitro, ex vivo and in vivo experiments (Meissonnier et al. 2008). Inflammation, immune response, and repair are major events in the early response to tissue injury. Measurement of serum cytokines have been considered valuable biomarkers in routine toxicity studies to detect drug-

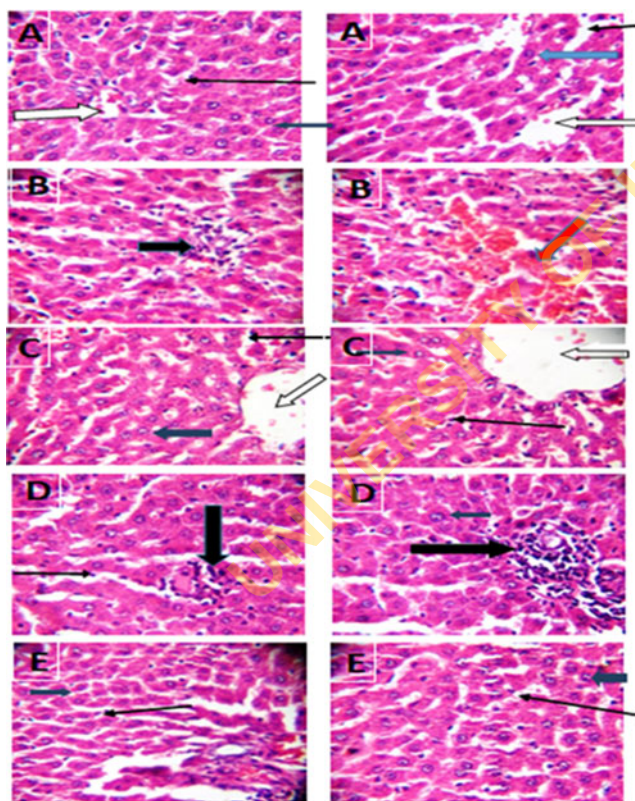


Fig. 2 Histological architecture of the liver ($\times 400$) following AFB1 administration. Photomicrographs show two different representative portions of the liver of each of **a** control rats, **b** rats given with AFB1 alone (2.5 mg/kg), **c** rats given AFB1 (2.5 mg/kg) and melatonin (10 mg/kg), **d** rats given AFB1 (2.5 mg/kg) with FCO1 (50 mg/kg), and **e** rats given AFB1 (2.5 mg/kg) with FCO2 (100 mg/kg). Blue arrows show normal hepatocytes; thin black arrows show normal sinusoids; white arrows show normal portal tracts; black arrows show inflammatory cell infiltration and red arrows show hemorrhages

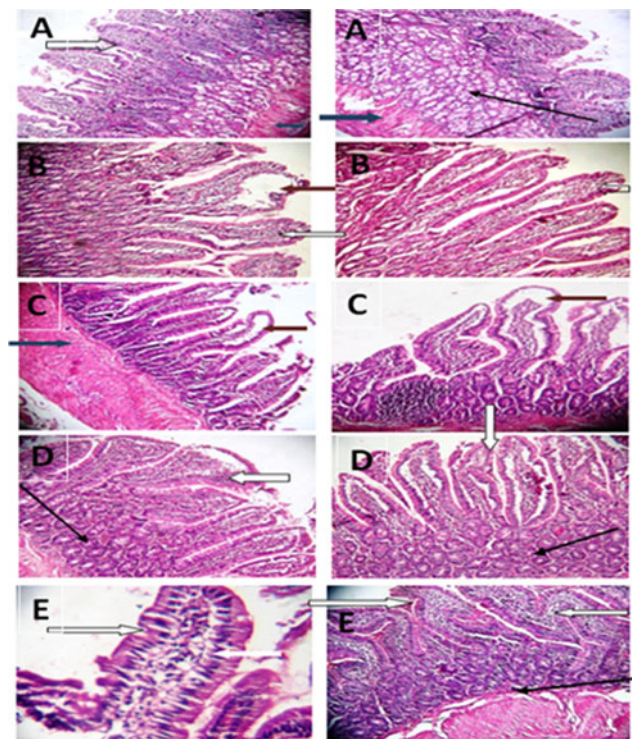


Fig. 3 Histological architecture of the duodenum ($\times 100$) following aflatoxin B1 administration. Photomicrographs show proximal and distal portions of the duodenum of each of **a** control rats, **b** rats given with AFB1 alone (2.5 mg/kg), **c** rats given AFB1 (2.5 mg/kg) and melatonin (10 mg/kg), **d** rats given AFB1 (2.5 mg/kg) with FCO1 (50 mg/kg), and **e** rats given AFB1 (2.5 mg/kg) with FCO2 (100 mg/kg). White arrows indicate normal villi; thin black arrows show intestinal glands; blue arrows indicate normal muscularis mucosa; red arrows show development of sub-epithelial space

induced toxicities to liver and vascular tissues (Lacour et al. 2005; Kerns et al. 2005). The accessibility to blood for serial quantitative monitoring of these cytokines in a patient with availability of assay platforms makes the utilization of cytokines as markers of inflammation highly useful in clinical medicine (Tarrant 2010).

In this study, we aimed to investigate whether changes in the morphology of the liver and intestines induced by aflatoxin B1 were accompanied by any significant alterations in serum cytokine levels. The reported anti-inflammatory potentials of melatonin and *C. odorata* were also investigated for their roles against AFB1-induced acute inflammation. We observed a significant increase in interleukin 1 beta levels in rats given AFB1 alone, while treatments with melatonin and the flavonoid fractions of *C. odorata* reversed this trend and returned IL-1 β levels to those of control rats. Elevation of this pro-inflammatory cytokine clearly indicates the occurrence of some degree of systemic inflammation (Safieh-Garabedian et al. 1995). Inflammation is basically a component of the body's response to many chemical insults that normally serves to protect tissue integrity, although excessive inflammatory responses can eventually lead to damage to normal tissues (Rostasy 2005).

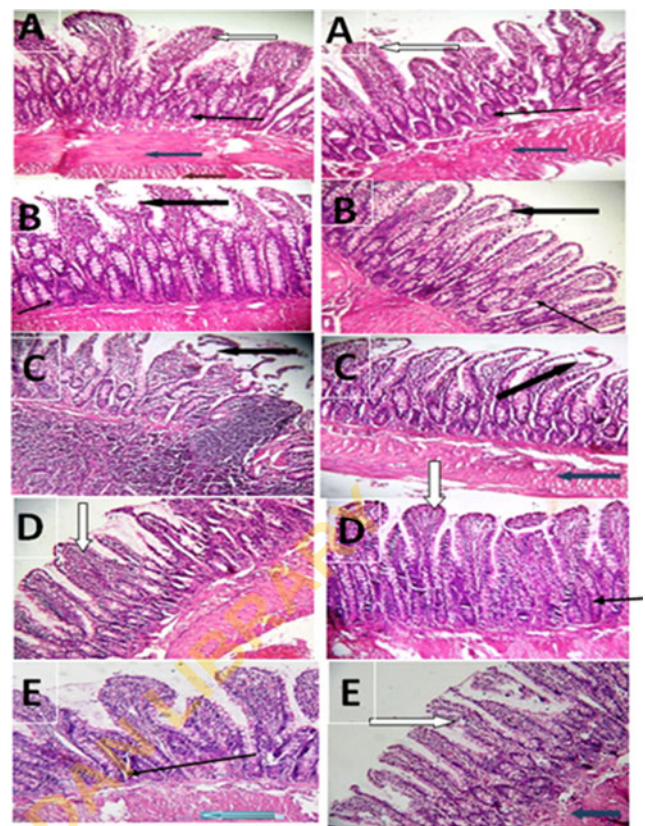


Fig. 4 Histological architecture of the ileum ($\times 100$) following aflatoxin B1 administration. Photomicrographs show proximal and distal portions of the duodenum of each of **a** control rats, **b** rats given with AFB1 alone (2.5 mg/kg), **c** rats given AFB1 (2.5 mg/kg) and melatonin (10 mg/kg), **d** rats given AFB1 (2.5 mg/kg) with FCO1 (50 mg/kg), and **e** rats given AFB1 (2.5 mg/kg) with FCO2 (100 mg/kg). White arrows indicate normal villi; thin black arrows show intestinal glands; blue arrows indicate normal muscularis mucosa; thick black arrows show development of sub-epithelial space

Contrary to the response obtained for IL-1 β , significant reduction in TNF- α was obtained in all the groups exposed to AFB1, compared with control rats, irrespective of treatments with either melatonin or *C. odorata*. AFB1 has been reported to inhibit the production of TNF- α and other cytokines in mice in vivo (Moon et al. 1999a) and in murine macrophages (Moon et al. 1999b) and lipopolysaccharide-activated human macrophages (Rossano et al. 1999) in vitro. The reduction in cytokine expression induced by aflatoxin B1 may be attributed to impairment in immune functions usually seen with aflatoxins (Bruneau et al. 2009). The lowering of TNF- α in all the groups exposed to AFB1, including the treated groups, may suggest a more selective effect towards TNF- α , rather than IL-1 β . Previous studies have also attributed the lowering of TNF- α production to a reduction in secretory molecules, such as CD14, which stimulate the secretion of TNF- α by macrophages (Takai et al. 1997). Melatonin has been previously reported to reduce the expression of TNF- α (Wu et al. 2001). The profound reduction in TNF- α obtained in the group of rats treated with melatonin in this

study may indicate a combination of the immunosuppressive effects of AFB1 and the anti-inflammatory effects of melatonin. The same reasoning could be responsible for the similar response obtained with *C. odorata*.

The liver has been identified as a major target for the toxicity of aflatoxin (Kumagai et al. 1998). There are intricate links between the liver and the intestines with regard to vascular supply and the composition of xenobiotic-metabolizing enzymes, which could be relevant in activation of AFB1 into the toxic metabolites (Sergent et al. 2008). The rise in serum pro-inflammatory IL-1 β seemed to be consistent with histologic evidence of inflammation observed in the liver and small intestines. The ability of other types of toxins administered intraperitoneally to cause toxicity in the gastrointestinal tract has also been reported elsewhere (Botta et al. 2004). Our study revealed histologic evidences of inflammation including hemorrhages, congestion and leucocytic infiltration around sinusoids and portal tracts in the liver, as well as leucocytic and lymphocytic infiltration of the lamina propria in the intestinal mucosa, in rats given aflatoxin B1. Histo-morphological examination is a recognized tool for assessing inflammatory-related changes in many animal models (Erben et al. 2014). Previously, Kumagai et al. (1998) reported similar findings of perivascular infiltration of leucocytes in hepatic connective tissues, when 2 mg/kg of AFB1 was administered orally to rats. Hemorrhages are consequences of alterations in hemodynamic processes which characterize inflammation (Palipoch et al. 2013). The occurrence of histological lesions of hemorrhages (Baker and Green 1987; Dafalla et al. 1987) and dilated sinusoids (Salim et al. 2011) has previously been reported in aflatoxicosis. The recruitment of leucocytes to inflammatory sites is mediated by chemokines secreted by monocytes and macrophages, as well as products of arachidonic acid metabolism (e.g., leukotriene B₄) derived from the lipo-oxygenase pathway (Kanaoka and Boyce 2004). These mediators normally stimulate the production of pro-inflammatory cytokines, including IL-1 β and TNF- α . Further histological lesions observed in this study included development of sub-epithelial space and some evidence of villi degeneration.

The modulation of cytokine production is among new approaches to the treatment of various disease conditions and herbal extracts are currently being investigated as potent immunomodulators to regulate cytokine expression (Spelman et al. 2006). The pathology of many inflammatory conditions involves excessive generation of reactive oxygen species (ROS) by polymorphonuclear leucocytes (Babior 2000). Naturally occurring compounds or those isolated from natural sources can be useful to modulate the oxidative and/or inflammatory damage associated with toxic conditions. Oral administration of flavonoids from *C. odorata* prevented the AFB1-induced elevation of IL-1 β and histologic changes in the duodenum and ileum, while only the higher dose was effective in

the liver. Immuno-modulatory effects of methoxylated flavonoids from *C. odorata* have been associated with inhibition of oxidative stress, and this was found to be related to the number and positions of methoxy and hydroxyl groups on the flavonoid rings (Kanashiro et al. 2004; Taleb-Contini et al. 2006).

Although melatonin is naturally secreted in the pineal gland and many other tissues including the bone marrow, testes, retina, lymphocytes, and the gastrointestinal tract (Tijmes et al. 1996; Conti et al. 2000; Bubenik 2002), exogenous administration of this anti-oxidant and anti-inflammatory compound has also been found to be beneficial in many models of organ damage (Mathes 2010). Its role in the suppression of inflammatory response related to increased formation of pro-inflammatory cytokines in models of carbon-tetrachloride and dimethyl nitrosamine toxicities have been documented (Carrillo-Vico et al. 2005; Wang et al. 2005; Jung et al. 2009). In the present study, melatonin clearly ameliorated cytokine-related changes. This effect was positively correlated with its protective effects in the liver tissues, but not in the intestines. It appears, therefore, that while extracts of *C. odorata* produced amelioration of tissue injury in both liver and intestines, melatonin was effective predominantly in the liver. The differences observed in the effectiveness of these compounds could be related to their availability in the tissues examined, due to the differences in their routes of administration.

Taken together, aflatoxin B1 produced alterations in pro-inflammatory cytokines as part of an inflammatory and/or immuno-modulatory process that also included associated histologic changes in the liver and small intestines. Modulation of cytokine activity may partially play a role in protection of tissue integrity observed with treatment with melatonin and/or flavonoids from *C. odorata*, and this may, in part, justify their potential incorporation in diet against aflatoxicosis.

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Compliance with ethical standards

Conflict of interest The authors report no conflicts of interest whatsoever.

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