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ACUTE ORAL TOXICITY OF A PHYTOGENIC TOXIN BINDER IN FEMALE WISTAR RATS (*RATTUS NORVEGICUS*)

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ABSTRACT
Phytogenic toxin binders are being developed as alternatives to clay adsorbents for controlling aflatoxicosis in poultry, but each new preparation requires basic safety assessment before use. This study evaluated the acute oral toxicity of a polyherbal toxin binder prepared from fenugreek (Trigonella foenum-graecum), neem (Azadirachta indica), keelanelli (Phyllanthus niruri), turmeric (Curcuma longa), tulsi (Ocimum tenuiflorum) and ginger (Zingiber officinale). The limit test described in Organisation for Economic Co-operation and Development Test Guideline 423 was used. Female Wistar rats (Rattus norvegicus) received a single oral dose of 2000 mg/kg body weight in two sequential steps of three animals each and were observed for 14 days. Body weight was recorded on Days 0, 7 and 14, and gross necropsy was performed at the end of the observation period. No mortality, clinical signs of toxicity or behavioural changes were observed. All animals gained weight, with mean increases of 4.33% and 4.50% in the first and second steps, respectively. No gross lesions were observed in the liver, kidneys, heart, lungs, spleen or gastrointestinal tract. The acute oral LD50 cut-off was greater than 2000 mg/kg body weight; the preparation was not classified in GHS acute toxicity Categories 1–4 at the tested dose, with optional Category 5 applicability depending on the classification system adopted. The findings indicate low acute oral toxicity in rats. Repeated-dose toxicity testing and tolerance trials in chickens are required before efficacy evaluation against aflatoxicosis.

KEYWORDS: Acute oral toxicity; Aflatoxicosis; Phytogenic toxin binder; Polyherbal formulation; Wistar rat

INTRODUCTION
Aflatoxins are toxic secondary metabolites produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus* and they are among the most important contaminants of maize, groundnut cake and other ingredients of poultry feed (Bryden, 2012). Aflatoxin B_1 (AFB_1) is the most potent member of the group. Poultry are highly sensitive to it and continued intake of contaminated feed reduces growth and feed efficiency, damages the liver and suppresses immune function (Rawal et al., 2010). Losses in productivity have been recorded in commercial broiler flocks even when aflatoxin concentrations in the feed were low (Jones et al., 1982).

The usual way of limiting aflatoxin exposure in poultry is to add mineral adsorbents such as bentonite, zeolite or hydrated sodium calcium aluminosilicate to the diet. These clays bind AFB_1 in the gut and reduce its absorption but their binding is not selective and they may also take up micronutrients and veterinary drugs present in the same feed (Piva et al., 1995; Ramos and Hernández, 1997). These drawbacks have drawn attention to feed additives of plant origin that might protect the bird against the toxin as well as limit its uptake.

Plant-derived feed additives are used to promote growth and health in livestock and poultry, and many have antioxidant, antimicrobial or immunomodulatory activity (Hashemi and Davoodi, 2011). Several have reduced aflatoxin-associated injury in experimental studies. Turmeric powder improved weight gain and reduced liver lesions in broiler chicks fed AFB1 (Gowda et al., 2008). Resveratrol lessened aflatoxin-induced changes in broilers (Sridhar et al., 2015), and Urtica dioica seed extract had a protective effect in aflatoxicosis (Uyar et al., 2016). In rodents, extracts of Phyllanthus amarus and Adhatoda vasica limited AFB1-induced liver damage (Naaz et al., 2007; Brinda et al., 2013). A preparation that combines several such plants could potentially combine toxin-binding activity with protection of the liver and gut.

The binder examined in this study combines six plants used in Indian traditional practice: fenugreek (Trigonella foenum-graecum L.), neem (Azadirachta indica A. Juss.), keelanelli (Phyllanthus niruri L.), turmeric (Curcuma longa L.), tulsi (Ocimum tenuiflorum L., syn. O. sanctum L.) and ginger (Zingiber officinale Roscoe). Each has a history of use as food, medicine or feed additive; however, the safety of the combined preparation has not been established. Acute oral toxicity testing is an initial step in its safety assessment. Therefore, this study aimed to determine the acute oral toxicity of the polyherbal phytogenic toxin binder in female Wistar rats using the limit test described in Organisation for Economic Co-operation and Development (OECD) Test Guideline 423 (OECD, 2001), before evaluating the binder against aflatoxicosis in native chickens.

MATERIAL AND METHODS

Ethics statement

The study was approved by the Institutional Animal Ethics Committee, Veterinary College and Research Institute, Tirunelveli, under approval No. 24/SA/IAEC/VPT/VCRI/TNI/2026, and no procedure began before approval was received. All procedures followed OECD TG 423 (OECD, 2001).

Experimental animals

Healthy adult female Wistar rats (*Rattus norvegicus*), 9–11 weeks old, were obtained from Cape Bio Lab and Research Centre, Marthandam, Kanyakumari, Tamil Nadu, India, a breeding facility registered with the Committee for Control and Supervision of Experiments on Animals. The rats weighed 320–348 g at the time of dosing (Table 3) and were acclimatized to laboratory conditions before the study. They were housed in polypropylene cages on sterilized rice husk bedding at 22–26 °C and 30–70% relative humidity under a 12-h light/12-h dark cycle with standard pelleted feed and drinking water available *ad libitum*.

Preparation of the phytogenic toxin binder

The binder was prepared from fenugreek, neem, keelanelli, turmeric, tulsi and ginger mixed in fixed proportions. Each plant material was shade-dried and ground separately, and the weighed powders were mixed thoroughly to obtain a uniform product. The binder was stored in airtight containers until use.

Acute oral toxicity test

The limit test of the acute toxic class method was conducted according to OECD TG 423 (OECD, 2001). A limit dose of 2000 mg/kg body weight was selected with reference to the reported low toxicity of the constituent plants in earlier rodent studies (Muralidhara et al., 1999; Rong et al., 2009; Asare et al., 2011; Gautam and Goel, 2014). Food, but not water, was withheld overnight before dosing. Immediately before administration, the binder was suspended in 0.25% carboxymethyl cellulose and administered once by oral gavage. In the first step, three rats received 2000 mg/kg body weight. As none died or showed signs of toxicity, a further three rats were dosed at the same level in the second step, following the sequential procedure specified in the guideline. All six rats were observed for 14 days after dosing.

Clinical observations and body weight

Each rat was observed individually during the first 30 min after dosing, periodically during the first 4 h, at 24 h, and once daily thereafter for 14 days. Observations included general behaviour, locomotor activity, skin and fur, eyes and mucous membranes, salivation, tremors, convulsions, diarrhoea and mortality. Individual body weights were recorded immediately before dosing (Day 0) and on Days 7 and 14.

Necropsy and classification

At the end of the observation period, all rats were euthanized under isoflurane anesthesia and subjected to gross necropsy. The liver, kidneys, heart, lungs, spleen and gastrointestinal tract were examined for visible lesions. The binder was classified according to the criteria of OECD TG 423 (OECD, 2001).

Data analysis

Body weights are presented for individual rats and as mean ± standard error (SE) for each step (N = 3). Body weight gain from Day 0 to Day 14 was expressed as a percentage of the Day 0 weight. No inferential statistics were applied because the guideline uses three animals per step.

RESULTS

Mortality

No rat died during the 14-day observation period in either step, and all six animals were euthanized as scheduled at the end of the study (Table 1).

Table 1. Mortality of female Wistar rats after a single oral dose of the phytogenic toxin binder.

Step	Dose (mg/kg body weight)	Number of animals	Animals sacrificed	Mortality
I	2000	3	3	0
II	2000	3	3	0

Deaths were recorded at any time during the 14-day observation period.

Clinical signs and behavior

All rats remained alert and active throughout the study and no change in behaviour or appearance was observed. The skin, fur, eyes and mucous membranes remained normal and no tremors, convulsions, salivation, diarrhea or impaired locomotion occurred at any time (Table 2).

Table 2. Clinical and behavioral observations in female Wistar rats after a single oral dose (2000 mg/kg body weight) of the phytogenic toxin binder.

Parameter	Day 0	Day 7	Day 14
General behaviour	Normal	Normal	Normal
Locomotor activity	Normal	Normal	Normal

Skin and fur	Normal	Normal	Normal
Eyes and mucous membranes	Normal	Normal	Normal
Salivation	Absent	Absent	Absent
Diarrhoea	Absent	Absent	Absent
Tremors/Convulsions	Absent	Absent	Absent
Mortality	Nil	Nil	Nil

Body weight

All rats gained weight between dosing and the end of the study (Table 3). In the first step, mean body weight increased from 334.33 ± 7.17 g on Day 0 to 341.00 ± 5.57 g on Day 7 and 348.67 ± 4.48 g on Day 14. In the second step, the corresponding values were 341.00 ± 5.57, 348.67 ± 4.91 and 356.33 ± 5.24 g. Individual gains over the 14 days ranged from 9 to 20 g, equal to a mean increase of 4.33 ± 1.05% in the first step and 4.50 ± 0.17% in the second step.

Table 3. Individual and mean body weights (g) of female Wistar rats after a single oral dose (2000 mg/kg body weight) of the phytogenic toxin binder.

Step	Dose (mg/kg body weight)	Sex	Animal No.	Day 0	Day 7	Day 14
I	2000	F	1	320 g	330 g	340 g
			2	341 g	348 g	355 g
			3	342 g	345 g	351 g
			Mean±SE	334.33 ± 7.17	341.00 ± 5.57	348.67 ± 4.48
II	2000	F	1	345 g	352 g	360 g
			2	348 g	355 g	363 g
			3	330 g	339 g	346 g
			Mean±SE	341.00 ± 5.57	348.67 ± 4.91	356.33 ± 5.04

Group values are mean ± standard error (SE), N = 3 per step. Day 0, day of dosing (weights taken immediately before dosing).

Gross pathology

Gross necropsy revealed no treatment-related abnormality. The liver, kidneys, heart, lungs, spleen and gastrointestinal tract were normal in color, size and texture, and no hemorrhage, congestion or other lesion was found.

Acute toxicity classification

Because none of the six rats dosed at 2000 mg/kg body weight died, the acute oral LD50 cut-off of the binder is considered to be greater than 2000 mg/kg body weight. Under the OECD TG 423 decision scheme, the substance is not classified in GHS acute toxicity Categories 1–4 at this dose; it may be considered under the optional GHS Category 5, where that category is applied.

DISCUSSION

A single oral dose of 2000 mg/kg body weight of the polyherbal binder caused no deaths, clinical signs of toxicity or gross lesions in female Wistar rats during the 14-day observation period. The acute oral LD50 cut-off was therefore greater than 2000 mg/kg body weight. Under the OECD TG 423 decision scheme, the preparation was not classified in GHS acute toxicity Categories 1–4 at the tested dose; the optional Category 5 may be applicable where adopted (OECD, 2001).

This result agrees with the toxicity data available for the individual plants. Debitterized fenugreek powder caused no deaths or signs of toxicity in mice and rats at single doses of up to 2 and 5 g/kg body weight, respectively, and no adverse effects when fed to rats at up to 10% of the diet for 90 days (Muralidhara et al., 1999). An aqueous leaf extract of *P. niruri* produced no toxicity in female rats at single doses of 2000 and 5000 mg/kg body weight (Asare et al., 2011). An ethanolic leaf extract of *O. sanctum* caused no deaths or signs of toxicity in mice at 2000 mg/kg body weight and no adverse changes when given to rats for 28 days at up to 800 mg/kg body weight per day (Gautam and Goel, 2014). Ginger given by gavage to rats for 35 days at up to 2000 mg/kg body weight per day did not increase mortality, although testis weight fell slightly at the highest dose (Rong et al., 2009). For neem, toxicity depends on how the material is processed: non-aqueous extracts appear to be the most toxic neem products, whereas aqueous extracts and unprocessed seed oil are less toxic (Boeke et al., 2004). The part of the neem plant used and its processing should therefore be specified and kept constant in future batches of the binder.

Changes in body weight are a useful indicator of general condition in toxicity studies. All rats gained weight during the 14-day observation period, with mean increases of 4.33% in the first step and 4.50% in the second step. These findings, together with the absence of observed clinical signs, indicate that no overt acute toxicity was detected under the conditions of this study. Gross examination of the major organs also revealed no visible abnormalities.

Several limitations should be noted. TG 423 uses females only, because females are generally slightly more sensitive than males in acute oral tests (OECD, 2001), and the small number of animals per step yields a hazard class rather than a precise LD_{50}. The design has no concurrent vehicle control, and its endpoints are limited to

clinical observation, body weight and gross pathology. Hematology, serum biochemistry and histopathology were not examined, so subclinical effects on the liver or kidneys cannot be excluded. A single dose also does not reflect the continuous dietary exposure that a feed additive delivers. Finally, the binder was not chemically standardized, and medicinal plant materials can themselves be contaminated with mycotoxins (Ashiq et al., 2014). Each batch intended for feeding trials should therefore be screened for aflatoxins and characterized for its main active constituents.

The next step is a repeated-dose 28-day oral toxicity study in rodents according to OECD TG 407 (OECD, 2008), which adds clinical pathology and histopathology, followed by tolerance and efficacy trials in native chickens fed aflatoxin-contaminated diets. Poultry are particularly sensitive to AFB_1 because of the way they metabolize it (Rawal et al., 2010), so safety and efficacy shown in rats must be confirmed in the target species.

CONCLUSION

A single oral dose of 2000 mg/kg body weight of the phytogenic toxin binder caused no deaths, observed clinical signs of toxicity or gross organ abnormalities in female Wistar rats during the 14-day observation period. All animals gained weight. The acute oral LD50 cut-off was greater than 2000 mg/kg body weight, indicating low acute oral toxicity under the conditions of this study. Further repeated-dose safety testing and tolerance studies in the target poultry species are required before efficacy trials against aflatoxicosis are undertaken.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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