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ORIGINAL ARTICLE/ARTICLE ORIGINAL

Aflatoxin, proximate composition and mineral profile of stored broiler feed treated with medicinal plant leaves

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Summary
Objectives. — In the present investigation, the *Morus alba* (*M. alba*), *Vitis vinifera* (*V. vinifera*), *Ficus religiosa* (*F. religiosa*) and *Citrus paradisi* (*C. paradisi*) leaves anti-aflatoxigenic activities were evaluated in Aspergillus flavus (*A. flavus)* inoculated feed.
Methods. — The broiler feed inoculated with *A. flavus* was treated with selected medicinal plant leaf powder (5%, 10% and 15% w/w) and stored for the period of six months at 28 °C and 16% moisture. The aflatoxins (AFTs) were estimated at the end of each month by Reversed Phase High Performance Liquid Chromatography (RP-HPLC) method along with proximate composition and mineral contents.
Results. — Plant leaves controlled AFTs efficiently without affecting the feed proximate composition and mineral contents. The *M. alba* leaves completely inhibition (100%) the AFTs (B_1 and B_2) in feed at very low concentration (5%). Other plants also showed significant ($P < 0.05$) inhibition of AFTs production without affecting the feed quality over the storage period of six months.
Conclusion. — Based on promising efficiency of selected medicinal plant leaves, *A. flavus* produced AFTs could possibly be controlled in stored poultry feed.
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Introduction

The poultry feeds have been associated with toxins due to microorganism infestation during storage. Peanut, cotton seed, cereal grains, soya been, corn gluten, copra and sunflower are used in poultry feed preparation [1,2] and feed is stored for longer time to avoid feed interruption to poultry birds. Factors such as feed composition, temperature variation, prolonged storage and storage conditions play an important role in fungal growth and the synthesis of AFTs [3,4]. Among the toxins, AFTs not only deteriorate food and feed, but also adversely affect the health of organisms who consume contaminated feed [5–8]. In Pakistan, the poultry industry is well established and to date, $>$ 140 feed mills are operating with the capacity of $\sim$4 million tons of feed annually to meet the feed demand of poultry farms [9].

A. flavus is a common mold, responsible for feed and other agricultural commodities contamination. Among toxins, AFTs have received greater attention due to their negative effects on living organisms [3,4]. In tropical regions, it is difficult to control AFTs contamination and in spite of precautionary measures, absolute safety has never been achieved. Certain fungicides have been known to inhibit the *A. flavus* growth during storage; however, these may induce negative effects. In view of negative effects of chemical agents [10] and physical treatment methods [11], the biological approaches are more effective and safer for AFTs control [5]. The medicinal plants are the rich source of bioactive compounds, which contains phytochemicals and their fungicidal activities are well known [12]. In view of drawbacks of conventional treatment to save feed and food from toxin contamination, the biocontrol strategies to inhibit AFTs produced by fungai have been reported to be effective but unfortunately, this method is complex and associated with certain risks [13]. Therefore, the use of medicinal plants as anti-aflatoxigenic agents will be interesting since these are safer, eco-friendly and cost-effective. In view of current scenario of environmental pollution [14–31], there is a need to adopt safe and eco-friendly methods [10,11].

Medicinal plants fungicidal properties are well known; for this reason, they are investigated to control AFTs produced by *A. flavus* in stored broiler feed. Therefore, the principal objectives were to evaluate the AFTs inhibition in stored broiler feed using *M. alba*, *V. vinifera*, *F. religiosa* and *C. paradisi* leaves along with feed quality evaluation.

Materials and methods

Chemical and reagents

The media culture and standard discs were purchased from Oxoid (Hampshire, UK). Immunoaffinity column (AflaTest® WB Vicam, USA) and aflatoxin standards were purchased from Supelco (Bellefonte, PA, USA). Methanols, acetonitrile, *n*-hexane used were purchase from Merck, Darmstadt, Germany. Trifluoroacetic acid (TFA) of Riedel-de Haen (Seelze, Germany) was used as derivatizing agent.

Plant leaves collection

The leaves of *M. alba*, *V. vinifera*, *F. religiosa* and *C. paradisi* leaves were collected from Botanical Garden, University of Agriculture, Faisalabad, Pakistan. The specimens were further identified by Dr. M. Hamid, Department of Botany, University of Agriculture, Faisalabad, Pakistan (voucher specimen no. 831-15-1). The selected plant leaves were washed, dried under ambient conditions followed by oven drying at 70 °C to constant weight and then, grinded to fine powder.

Preparation of plant extracts

Antifungal extracts were prepared by dissolving 10 g of leaf powder (leaves) in 100 mL methanol (80%) and shaken (Gallenkamp, UK) for 24 h at room temperature, filtered and extracts were concentrated under reduced pressure at 45 °C (EYELA, N-N Series, Rikakikai Co. Ltd. Tokyo, Japan) and stored in refrigerator at 4 °C until further experimentation.

Preparation of inoculums

A. flavus (ATCC 9643) was obtained from the Department of Clinical Medicine and Surgery and identified by the Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan. The fungal strains were cultured on potato dextrose agar (Oxoid, UK) and incubated at 28 °C until sporulation. The spores were harvested in sterilized distilled water containing 0.1% Tween 80 (Sigma-Aldrich) and were counted in Nebauer chamber with microscopy. The suspension was further diluted to adjust spore density at 1×10^7 spores/mL and stored at −4 °C.

Antifungal activity screening and MIC determination

Disc diffusion assay was used to screen plants' leaf extracts for antifungal activity. PDA solution (20 mL) containing 1×10^7 spores/mL was spread on sterilized Petri plate. Sterilized discs (6 mm) were impregnated with plant extract (50 μL) and incubated at 28 °C for 48 h [32]. Fluconazole (Oxoid Company, Hampshire, UK) was used as positive control. The zone of inhibition diameter was measured by zone reader.

For the determination of MIC, initial concentration (100 mg/mL) of extract was transferred to 96 well plates and a series of dilution was performed (each time two folds) (1st row of 96 well plates). In a next row of 96 well plates, 50 μL of Sabouraud dextrose broth was added along with 10 μL of *A. flavus* suspension (1×10^7 spores/mL) (2nd row of 96 well plates). Finally, 50 μL dilutions from 1st row were added into 2nd row plates. The well plates were incubated at 28 °C for 48 h. The MIC was calculated from the preceding well where fungal growth was started and MIC was expressed in term of μg/mL [33].

Anti-aflatoxigenic

Broiler feed (4 kg) was obtained from Punjab Feed Corporation, Lahore Road, Sheikhupura, Pakistan. The broiler feed was dried in an oven at 60 °C and divided into 16 parts (200 g). Each lot was autoclaved and moisture was set at 16% with sterilized distilled water. The feed samples were inoculated with 4 mL of *A. flavus* suspension (1×10^7 spores/mL) under laminar airflow (Dalton, Japan). Powdered plant

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