Chemistry International 8(4) (2022) 153-158

International Scientific Organization
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Chemistry International
www.bosaljournals.com/chemint/

# Phytochemical remediation and detoxification of aflatoxins in cattle feed

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**ARTICLE INFO**

**Article type:**
Short communication
**Article history:**
Received January 2022
Accepted August 2022
October 2022 Issue
**Keywords:**
Aflatoxins
Phytochemical remediation
Plant leaves
Feed additives
Detoxification

**ABSTRACT**

Contamination during the preparation and storage of feed are known to be environmental concerns. This study focusses on the use of *Mangifera indica, Citrus sinensis* and *Syzygium cumini* leaves that showed best anti-aflatoxigenic actions against *A. paraciticus* during the process of feed storage. *A. paraciticus* vaccinated feed of cattle had been cured through carefully chosen plant leaves powder in various concentrations and stored for a duration of 180 days with 15% humidity and at 25°C. Aflatoxins (AFTs) quantities had been measured at the end of each month. Selected plant leaves repressed AFTs production noticeably; yet, *Citrus sinensis* and *Syzygium cumini* blocked AFTs nearly 100% at higher percentage of leaves for the duration of feed storage for 180 days. Results exhibited that AFTs formation was blocked totally by plant leaves powder. These plant leaves could possibly be potent anti-aflatoxigenic materials to protect feedstuffs during storage and also recommended as a best solution for this environmental concern.



**Capsule Summary:** *M. indica, C. sinensis* and *S. cumini* leaves were employed for the detoxification of aflatoxin in cattle feed and selected plants showed promising anti-aflatoxigenic actions against *A. paraciticus* during feed storage.

**Cite This Article As:** A. Nazir, S. Hussain, M. Iqbal and N. Ahmad. Phytochemical remediation and detoxification of aflatoxins in cattle feed. Chemistry International 8(4) (2022) 153-158.
https://doi.org/10.5281/zenodo.7192548

## INTRODUCTION

Food has always been one of the most important materials both for animals and humans. Besides weather and nature severity, aflatoxins have been a serious threat to agricultural and livestock from the very first day of its presence. It causes loss of both economy and life of available sources humans, animals and feed. Aflatoxins have a cluster of nearly 20 linked fungal metabolic reagents formed mainly through fungi *Aspergillus flavus* and *A. paraciticus*. Simple carbohydrate and carbohydrates-based food ingredients are supposed to be the most significant foods affected by AFTs (Abbas et al., 2022; Hassan et al., 2021; Nazir et al., 2021; Yaqoob et al., 2021). The development of fungus takes place because of inappropriate dehydration of rice modicums having developed proportion of the humidity content requiring (>14%). Beans and groundnut, contrary to this, have been repeatedly recycled with a number of African foodstuffs to satisfy the cornflakes foods (Soro-Yao et al., 2014). Every year a huge amount of food and feed stuff is lost because of the aflatoxins.

The capability of progression of mushroom and construction of AFs in carbohydrates in small or more influenced by some factors humidity, soil type and temperature. Furthermore, seasonings are said to be exposed

to AFs infection and are significantly disposed in the treating and storing circumstances (Bokhari et al., 2021; Din et al., 2021; Iqbal et al., 2019a; Iqbal et al., 2019b). It has been concluded that AFs infection in huge assortment of spices self-possessed with different ingredients like cardamom, clove, pepper, cumin, ginger cinnamon and coriander in the county of Oman (Achaglinkame et al., 2017).

Different types of foodstuffs which consist of rice, copra, milo, peanuts, corn, sorghum, and grains had been diseased by aflatoxins. Even though infection due to the patterns can be well-known, stages for ultimate considerations in aflatoxins in the granule creation may or may not change within the range from 1 µg/kg (1 ppb) to 12,000 µg/kg (12 ppm). American Food and Drug Administration has laid down limits for aflatoxin in numerous food and feedstuff materials. In order to feed the mature non-lactating animals, the action level is ranging 100–300 ppb provisional upon feedstuff category. European Union has implemented considerably stricter principles and parameters for the presence of aflatoxins level in foods. Supervisory principles designed for aflatoxins initially spread in 1960s had been driven on the basis of analytical techniques which led them to work to the now a day (Henry et al., 1999).

The studies of sub-acute toxicity of aflatoxin B1 in many animals showed a restrained to brutal damage of liver. For example, in monkeys, sub-acute toxicity level studies clarified fatty change and portal inflammation. The studies of chronic toxicity for aflatoxin B1 displayed reduced liver micro somalcyto-chrome P-450 meditation in chicken, decline in feed utilization and decreased mass once again. The studies of sub-chronic toxicity of aflatoxins B1 in fish clarified from past to present with pre-neoplastic abnormal change in cells, at the same time with change in spleen, gill, intestine and pancreas. Cure of the human being liver cells by means of aflatoxin B1 with different doses which ranges commencing 3-5 µmol/l resulting in the configuration of the aflatoxin B1-DNA adducts 8-hydroxy-guanine anomalous change and triggered DNA damage. Carcinogenicity in the aflatoxin B1 that is signified by the development of liver cells cancer had been described in various studies conducted on rats. The death of embryo and improperly combined embryonic enlargement of bursa of Fabricious in poultry especially in chicken due to aflatoxin B1 has been described (Hassan et al., 2017; Iqbal et al., 2019a; Nazir et al., 2019).

Aflatoxins have been recorded with Teratogenic type in rabbits pooled a number of different types of symptoms micro-pthalmia, cardiac defects, micro-pthalmia, lenticular degeneration, reduced immature weights, enlarged eye socket, agenesis of caudal vertebrae and wrist drop among the many others. Various types of studies on fish have clarified aflatoxins B1 to have significant immunity diminishing effects self-possessed with lowered serum and total globulin and decreased actions of bacterial creatures (Sur and Celik, 2003).

Inclusive metaphors like this category of bio-measured interferences have been far away from the capability of following study and assessment; the successive section shall predominantly emphasis on the utilization of aflatoxins biological units' increase and authenticate extra prime and derived prevention approach. For a number of years, sodium calcium alumino-silicate has been promoted the same as Nova-Sil mud, a knowledgeable anti-coating characteristic mixed in animal feed, has assisted to fascinate on the surface of aflatoxins in large intestine area of the animals and shrink the biologically availability and bad properties of such pollutants (Phillips et al., 2002).

It has been witnessed that introduction of aflatoxins may have any consequence on child development and faintness against the contamination, as the HCC risk is disturbed, it assists in a number of ways to put emphasis on additional communal health is looked-for for the progression and achievement of interference (Iqbal et al., 2017; Qamar et al., 2017; Younas et al., 2017). The detoxifying aflatoxins from polluted food materials and animal feed is a worldwide present problem because aflatoxins are very poisonous and are capable to pass through metabolic processes and restore themselves in tissues which cause a severe effect on both of animals and human beings in life and economy both. Even though a number of detoxifying techniques have been reported after testing but none of them seems to be satisfy different factors such as safety, efficacy, costs requisites and safeguarding of nutritional elements.

Hence, in order to reduce food contamination of mycotoxins, a number of ways and techniques are being utilized to handle and tackle aflatoxins from the pre-harvest and post-harvest levels. The main objective of this study is detoxification of mycotoxins in an agricultural product (Mukhtar Wanda; cattle feed) using different plant leaves in powdered form.

## MATERIAL AND METHODS

### Samples, chemical and reagents

The leaves of various plants of Syzygium cumini, Mangifera indica and Citrus sinensis respectively had been gathered from botanical garden, University of Bulleh Shah Kasur, Pakistan. The plant leaves had been washed using distilled water, shade dried and crushed into fine particles for experimental purpose. All the required chemical reagents were of laboratory grade and were purchased from Merck (Germany). 20 g sample of crushed and powdered leaves in 150 mL of 80% methanol was placed in an orbital shaker (Gallenkamp, England) for 24 h at 25 °C (Sultana et al., 2007). The extracts were kept into refrigerator at 4 °C for further analysis.

### Preparation of inoculum

Fungal strain had more developed on potato dextrose agar (PDA) diagonally and incubation was processed at 28 °C for a week (7 days) in anticipation of reproductive structure of suitable size. The following fungal spores had been reaped

through pasteurized distilled water carrying 0.1% Tween 80 and fungal count had been made in Neuberger chamber by the means of microscope. It had been additionally diluted to make spore concentration in the range of 10-12 spores $mL^{-1}$. The spore suspension had been vacuum-packed at 4 °C in unconditional dry containers.

**Disc diffusion procedure**

The antifungal activity of selected plant leaf distillates had been checked against *A. paraciticus*. More or less 20 mL PDA solution which controlled 10-12 spores $mL^{-1}$ of the mold suspension which had been spread on disinfected petri plates. Pasteurized discs were nearly in the size range of 6mm with a plant abstract of 50µL had been kept on agar plates. Fluconazole (30 µg $disc^{-1}$) had been employed as a positive reference whereas disc showing no reaction as a negative control. The plates had been sterilized at the temperature of 28 °C for 48 h (two days). The thickness of control region had been calculated in mm with the help of zone reader.

**Treatment of the feed with plant leaves powder**

The plant leaves had been transferred in three various considerations (20% w/w) in every bag having 200 g of inoculated feed (Mukhtar Wanda). Unreacted samples of feed had been well thought-out as control. After the reliable intervals of time, feedstuff had been drawn and aflatoxins had been determined with the help of HPLC analysis.

**Analysis of aflatoxins**

Aflatoxins had been removed from the feed samples subsequently following in earlier times determined technique of (Beltran et al., 2011) with minor alterations and modifications. 5 g feed sample was placed in a pointed hipflask. Aflatoxins had been mined in 20 mL of acetonitrile-water (84:16) mixture by the means of shaking and mixing the mixture at 30 °C for 90 min. The extracts had been filtered. The filtrate was concentrated to a very small size of 2 to 5 mL. The LC-system had been used both for quantitative calculation as well as for the estimation of aflatoxins which had belonged to LC-10A Sequences (Shimadzu, Japan), operational through booklet vaccination arrangement containing circle dimensions (20 µL), Mediterranean Sea 18® column (5 µm; 25 × 0.46 cm) Serial No. N45074 (Teknokroma, Barcelona, Spain) had been formfitting through CTO-20A® (Shimadzu, Tokyo, Japan) column oven and LC-20AT® (Shimadzu, Kyoto, Japan) propel had been employed.

Isocratic mobile phase is composed of acetonitrile, water and methanol (1: 2: 1) has been employed at a current amount of 1 mL min−1. Spectro-fluorometer detector RF-10AXL ® (Shimadzu, Kyoto, Japan) had been set at both of hyper leveling and discharge wavelengths of 360 nm and 440 nm respectively. The following chromatographic settings have prearranged good and prominent resolutions of peaks. A variety of measurements made by some new and standard device and finally after associating curves had been drawn by using a sequence of various solutions of aflatoxins in acetonitrile with different percentages in ascending order 0.05, 0.1, 0.5, 1.0, 5.0, 10 µg L-1 correspondingly. The separation of (resolution) scheme of aflatoxins had been evaluated and examined by using converse phase HPLC column. Degree of aflatoxins and existence had been shown with expressions of ppb and aflatoxins had been calculated by means of the following Eq. 1.

$$\text{Inhibition } (\%) = \frac{Y-X}{Y} \times 100 \qquad (1)$$

Where, "X" and "Y" are composition expressions used for amount of aflatoxins in sample and amount of control in aflatoxins correspondingly.

**Statistical analysis**

All the readings had been made to count in repeatedly was nearly same three times the data had been described in the form of mean (n = 3 × 3) ± SD (n = 3 × 3). Examining the variance (ANOVA) had been carried out on all calculated values at a level of $p < 0.05$ with Minitab 2000 Version 13.2 arithmetic software (Minitab Inc. Pennsylvania, U.S.A).

**RESULTS AND DISCUSSION**

Aflatoxins are known to be the prominent toxins than any of the other fungal mycotoxins because of their heavily

**Table 1:** Aflatoxin permissible limits for various food stupp worldwide

| S. No | Country | Food stuff | Limits (µg/kg) |
|---|---|---|---|
| 1 | Japan | All of the food stuffs | 10 |
| 2 | UK | Carbohydrates, Tree nuts, milk, cocoa, coffee | 0.5 - 20 |
| 3 | USA | Figs, nuts and related products | 2 - 12 |
| 4 | China | Barley, sorghum, rice and nuts etc. | 5 - 50 |
| 5 | EU | Peanuts, groundnut, spices, other foods | 2 - 12 |
| 6 | Australia | Except Peanuts all food stuff | 5 |
| 7 | India | Cereals, spices, nuts and other food stuff | 30 |

**Table 2:** Total aflatoxins (ng) in *A. paraciticus* inoculated feed and treatment with *Mangifera indica* (Mango) leaves for the period of 180 days of storage.

| Storage time (days) | *Mangifera indica* | | *Syzygium cumini* | | *Citrus sinensis* | |
|---|---|---|---|---|---|---|
| | Control (ng) | Treatment (ng) | Control (ng) | Treatment (ng) | Control (ng) | Treatment (ng) |
| 0 | 7.50 ± 0.50 | 0 | 7.50 ± 0.50 | 0 | 7.50 ± 0.50 | 0 |
| 30 | 12.20 ± 1 | 0 | 12.20 ± 1 | 0 | 12.20 ± 1 | 0 |
| 60 | 19.50 ± 1.20 | 0.60 ± 0.20 | 19.50 ± 1.20 | 0 | 19.50 ± 1.20 | 0 |
| 90 | 27.75 ± 0.90 | 1.25 ± 0.30 | 27.75 ± 0.90 | 0.40 ± 0.20 | 27.75 ± 0.90 | 0 |
| 120 | 38.10 ± 1.10 | 2.10 ± 0.15 | 38.10 ± 1.10 | 0.90 ± 0.25 | 38.10 ± 1.10 | 0.30 ± 0.20 |
| 150 | 44.80 ± 1.50 | 4.50 ± 0.25 | 44.80 ± 1.50 | 1.40 ± 0.35 | 44.80 ± 1.50 | 0.80 ± 0.15 |
| 180 | 52.10 ± 2.10 | 6.30 ± 0.30 | 52.10 ± 2.10 | 3.20 ± 0.20 | 52.10 ± 2.10 | 2.00 ± 0.30 |

cancer-causing effects and severe poisoning. Naturally four types of the aflatoxins are found with the names including B1, B2, G1 and G2 and two types of metabolic products named as M1 and M2 are found. These can affect badly on the food and feed of both animals and humans as cottonseed, wheat, corn, sorghum, soya, peanut and nuts.in order to control the aflatoxins a number of physical techniques bare being used named as fermentation, solvent extraction, radiography, deactivation by heat, microbes and physical separation (Mallakian et al., 2017).

Feed samples had been collected from Mukhtar Wanda as a well-known brand in market for the sake of research and analysis. Plant based material had been used in order to avoid the toxic chemicals. Some samples of feed had been collected to check the different levels of aflatoxins based on different time interval and production of aflatoxins as compared to a control. Remarkable achievements have been made through the use of HPLC tests in the comparison of control as the results are surprising based on plant leaf used. This process consisted of 180 days of examining cattle feed of layers during the storage. All the observations have been recorded with keen and careful ways and recorded for each plant separately in order to check and compare efficacy of results shown by plants.

A number of procedures for aflatoxins detection have been constructed on the basis of chromatography. HPLC-FLD is an inexpensive and applicable method used for the recognition of the aflatoxins. All the plants have shown a significant antifungal action against *A. paraciticus*. The antifungal activity revealed by *P. granatum* and *M. alba* are proportional by anti-mycological action of the fluconazole, which shows the occurrence of a considerable quantity of antifungal means in distillates which have been found to be accountable being capable of antifungal phenomenon. Antifungal behaviors of distillates of plants are because of derived metabolic reagents as biologically reactive compounds (Sharma and Kumar, 2008). The distillates of derived reagents from methanol have confirmed the powerful antifungal action and had been associated it with phenols, tannins and flavonoids in the extracts (Dahham et al., 2010).

Mango plant leaves-based material was proved to be very beneficial against the aflatoxins. In first two months, there were zero aflatoxins produced where leaves-based protection was used while control was showing a prominent production of aflatoxins up to 12.2 ng. Similarly, in 3$^{rd}$ and 4$^{th}$ month, production of the aflatoxins was 0.6 and 1.25 ng respectively while large quantity of aflatoxins had been produced in control 19.5 and 27.75 ng respectively were noticed. In last two months, treatment had been proven to be very valuable and a small quantity of aflatoxins had been produced compared to control. Hence, it can be concluded that detoxification process was enhanced with the help of plant-based material.

The plant leaves of Jamun (*Syzygium cumini*) were employed for the process of detoxification counter to aflatoxins. In earlier two months, zero aflatoxins were produced where Jamun (*Syzygium cumini*) leaves-based safety had been used while control had been viewing a noticeable construction of aflatoxins of 7.5 and 19.5 ng correspondingly. In the same way, in 3$^{rd}$ and 4$^{th}$ month, manufacture of the aflatoxins had been calculated 0.4 and 0.9 with little quantities in that order whereas comparatively larger magnitude of aflatoxins was formed. In 5$^{th}$ and 6$^{th}$ months, same behavior was recognized with slight increase in aflatoxins.

Malta (*Citrus sinensis*) is a well-known fruit being very useful in herbal, therapy sections and lot of other uses. It showed most prominent and effective results in feed where it was implemented. Its effectiveness is revealed when aflatoxins had not been produced for three months consecutively when treatment showed zero reading while control showed a significant amount of aflatoxins 12.20 ng, 19.50 ng and 27.75 ng which reflects the efficacy of plant based material. For last three months, aflatoxins were produced in very minute quantities 0.30 to 2 ng whereas aflatoxins in control were produced in 38.10 ng, 44.80 ng and 52.10 ng respectively. Control shows a continuous increase in the growth of aflatoxins in animal feed where chemical-based material had been used material but it was not effective as the plant-based materials were proven during the six months of testing. HPLC analysis demonstrates that AFB1 was the most prominent type of

aflatoxin present amongst the others followed by AFG1, AFG2 and AFB2 (Iqbal et al., 2020; Iqbal et al., 2019b).

Neeff et al. (Neeff et al., 2018) proposed aflatoxins are known as the second level metabolites produced by fungus family species aspergillus, mainly *A. flavus*, *A. paraciticus* and *A. nomius*. Aflatoxin B1 (AFB1) is known to be the most poisonous species, having human hepatotoxic and carcinogenic nature and effects in vertebrates especially mammals and human beings. The pathway of toxicity aflatoxin B1 shows oxidative stress, which finally leads to Deoxyribose Nucleic acid lipid and protein damage at cellular level. Antioxidants have been utilized at particular extent as food additives to protect against the degradation because of oxidation process and have been well known because of prevention of fungal growth. This study shows the updated information and knowledge on the use of natural antioxidants and show phenomenon of detoxification for animal and human feed contaminated with aflatoxins.

## CONCLUSIONS

Anti-aflatoxins producing events of Mangifera indica, Citrus sinensis, Syzygium cumini have been used for the treatment of aflatoxins produced in feed of animals. The leaves had been investigated against the *A. paraciticus* produced in Mukhtar Wanda for the duration of 180 days of storage. *Mangifera indica, Citrus sinensis, Syzygium cumini* showed a good efficacy at lower concentration but was recommendable at relatively higher concentrations. Results revealed that these plant leaves are potential applicants in order to govern AFTs in storing feedstuff because they have been found completely benign, effective and environmentally friendly against those based on chemical treatments. HPLC is Sensitive, selective, repeatable and reliable technique while other techniques including LCMS is very expensive, needs specialist operator and internal standards. Similarly, ELISA shows possible cross reactivity with related mycotoxins matrix interference and possible false positives/negatives. On the other hand, immunoaffinity assay destruct the sample and is limited to total aflatoxin assay.

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