

Effects of *Andrographis paniculata* (Burm.f.) Nees, *Azadirachta indica* A. Juss. and *Adhatoda vasica* Nees plant extracts on germination, seedling growth and food ingredients in Aflatoxin B₁ treated seeds of maize (*Zea mays* L.)

K. Ragni, K. Salahuddin*, L. Rani and G. Prasad

Seed Pathology and Mycotoxin Laboratory, Department of Botany,
L.N. Mithila University, Darbhanga- 846004, India
E. Mail: salahuddin212@gmail.com, drgpd261263@gmail.com

ABSTRACT

Maize (*Zea mays* L.) is a food ingredient in various forms (cornmeal, corn flour, cornstarch, and corn oil). It is also used for processing animal feeds and ethanol). Its seeds were contaminated with aflatoxin B₁ by inhibiting seed germination and seedling growth. The inhibitory properties of aflatoxin B₁ were determined in the extracts of different bitter plants. A fluctuation in the content of starch, total sugar (TS), reducing sugar (RS) and non-reducing Sugar (NRS) was observed in maize seeds due to treatments of toxin and bitter plants. The maximum starch (62.46), total sugar (0.08) reducing sugar (0.02), and non-reducing sugar (0.028) were found in maize at a 2 ppm concentration of aflatoxin B₁. The high levels of starch (14.12), total sugar (1.25), reducing sugar (0.36), and non-reducing sugar (2.46) were recorded in maize seeds due to aflatoxin B₁. The α -amylase activity was inhibited by aflatoxin B₁ treatment. The maximum O.D. values i.e., 0.68, 0.82, and 0.95 were recorded in maize seeds by treatment of aflatoxin B₁ with the extracts of *Andrographis paniculata*, *Azadirachta indica*, and *Adhatoda vasica*.

Keywords: *Adhatoda vasica*, Aflatoxin B₁, *Andrographis paniculata*, *Azadirachta indica*, Bitter plant, Food ingredient, Maize, Mycotoxin.

1. INTRODUCTION

Maize (*Zea mays* L.) is a major crop used as corn meal, corn flour, corn starch, and corn oil. It is directly consumed as food, indirectly as food products industrial raw materials and used as animal fodder (11). Food grains contain toxins called "Mycotoxins". These are toxic secondary metabolites and byproducts of mould infestations affecting one-quarter of food crops. Aflatoxin B₁ is the most potent, natural, highly toxic, and carcinogenic mycotoxin, its contamination of food grains causes fatal acute illness and increases cancer (31). Several mycotoxins are produced by *Aspergillus flavus*, *Penicillium* spp., and *Fusarium* spp. Fungi that grow on as maize, sorghum, barley, wheat, peanuts, and oilseeds due to environmental, post-harvest, poor storage and pH (29). Aflatoxins are produced by certain fungi like *Aspergillus flavus* and *Aspergillus parasiticus* (13,24,25). The inhibitory effects of Aflatoxin B₁ occurred on seed germination and seedling growth of *Lepidium sativum*, *Zea mays*, *Vigna radiata* (4,6,7,16,19,20-22).

*Correspondence author

Many plants contain extremely bitter-tasting phytochemicals (1) for their self-protection. The inhibitory properties of a few bitter plant extracts against aflatoxin B₁ production by *Aspergillus flavus* have been studied (5,17,28,). However, medicinal bitter plants played an important role in breaking seed dormancy, growth of seedlings, growth of *A. flavus*, and aflatoxin production in SMKY liquid media but not tested for this purpose (23,30).

2. MATERIAL AND METHODS

Study area: The culture study was conducted from March 2023 to December 2023 in Seed Pathology and Mycotoxin Laboratory, Department of Botany, L.N. Mithila University, Darbhanga- 846004, India (Latitude: 85.9000° E, Longitude: 26.1700 ° N, Altitude: 50 m above sea level, Rainfall: 1142.3 mm, Max. temp.:44 ° C, Min. temp.: 17 °C).

Substrate: Maize seeds (*Zea mays L.*) were purchased from Pooja Seeds.

Aflatoxin: The standards of aflatoxin B₁ were available in the Department. It was isolated as a result of infection of *Aspergillus flavus*. It contains aromatic ether and an aromatic ketone.

Bitter Plants: These were obtained from our university campus. The use of bitter plant extracts controls the mycotoxin inhibitory properties to enhance food production. Therefore, we studied the effects of some bitter plants (*Andrographis paniculata*, *Azadirachta indica*, and *Adhatoda vasica*) extracts on the physiology of aflatoxin-treated maize seeds.

A. *Andrographis paniculata* (Burm. f.) Nees: It belongs to the family, Acanthaceae. It is the most bitter plant in Bihar (27). It is widely used to treat high temperature in fevers and to remove toxins from the body. The plant is recommended to treat alleviation of leprosy, gonorrhoea, scabies and skin eruptions (Figure 1).



Figure 1. Whole plant and leaf of *Andrographis paniculata* Nees

B. *Azadirachta indica* A. Juss (Family, Meliaceae): It has a wide range of medicinal properties. It has biologically active compounds that are chemically diverse and structurally complex. It is a rich source of antioxidants and treat infections, acne and dandruff. It has antimalarial, antifungal and detoxifying properties (Figure 2).



Figure 2. Whole plant and leaf of *Azadirachta indica* A. Juss

C. *Adhatoda vasica* Nees (Family, Acanthaceae): It has wide applications in traditional Ayurveda as it has anti-inflammatory, anti-allergic and antioxidant properties. Its roots and bark have expectorant properties. It contains alkaloids such as vasicinone and vasicine that have bronchodilator properties. (Figure 3).



Figure 3. Whole plant and leaf of *Adhatoda vasica* Nees

Extract preparation: 10 g. leaves of each plant were separately, washed and surface sterilized. The extract was collected by the maceration method using 95 % ethanol and then filtered. The filtrate was collected, concentrated with a rotary evaporator and dried in a freeze-dryer. The different concentrations of each plant extract (2 ppm) were prepared.

2.1 BIOASSAY

2.1.1 Total Sugar and Starch: The total sugar and starch of the maize grains were estimated (8). For this, a 200 mg freshly ground sample was shaken thoroughly in 80 % warm ethanol. After 5 minutes, the supernatant was drained and centrifuged. Extractions were done thrice and the extracts were collected. Ethyl alcohol was dispersed in a Buchner funnel (evaporating disc) and the remainder left in the bottom was dissolved in 50 ml distilled water to form a stock solution of total sugar. 2 ml of this undiluted solution (4-20 mg of sugar) was assorted with 0.14 ml of 80 % saturated phenol followed by the rapid addition of 5 ml of concentration H_2SO_4 . After thorough vibration, the solution was permitted to resolve for 30 min. The optical density of yellow-orange mixture was measured at 490 nm with spectrophotometer. The standard curve of the glucose was prepared via a similar course of action and the outcome, thus obtained, was differentiated. From residue (procured after centrifugation and draining of the alcoholic extracts of the specimen) starch was estimated by adding 5 ml distilled water and mixing it with 6.5 ml of 55 % perchloric acid (adding 270 ml of 70 % perchloric acid into 100 ml water). This was then subsequently and constantly stirred for 10 min. After a 15 min, one more time 20 ml of distilled water was added, centrifuged and then the supernatant was drained. Extraction was done thrice and the supernatant was mixed. The volume was standardized to 100 ml by adding additional distilled water (stock solution). Now 1-2 ml of this stock solution was mixed with 10 ml at 0.1 %. Anthrone catalytic agent (760 ml conc. H_2SO_4 was diluted to 1 L and 1 g anthrone liquefy) and heated at 100 °C for 12 min. The bluish-green solution was cooled at room temperature and its optical density was recorded at 630 nm using a spectrophotometer. Readings were compared with the standard curve of starch prepared through a similar procedure.

2.1.2 Reducing and non-reducing sugars: These were evaluated by the method of Somogyi- Nelson (14). 100 mg of samples were added in 3 ml distilled water and centrifuged. The supernatant was mixed rigorously with 0.2 ml 5 % ZnSO_4 solution. The mixture was then centrifuged with 0.5- 1 ml alkaline copper reagent (4 g dissolved in water) and was added in 1 ml of supernatant. The resulting solution was heated in a boiling water bath. After 15 min it was cooled and 1 ml arsenomolybdate catalytic agent was added and left for a few minutes till effervescence was ceased (stock solution). The blue-stained solution was diluted to 10 ml and optical density was recorded at 510 nm as opposed to blank with spectrophotometer. The optical density was differentiated from that of the standard loop of glucose mixture. The non-reducing sugar was determined by abstracting the value of bringing down sugar from total sugars.

2.1.3. α -amylase activity: The activity of α -amylase was assayed as per the method of Bernfeld (2). One ml of starch solution was taken in a test tube. Now 0.5 ml enzyme homogenate (100 mg powder/ml acetate buffer; pH 4.8) was added to the control tube. One and the other monitoring tubes were incubated for 30 min at 37 °C. After incubation, both were dipped in a boiling water bath for 5 min and subsequently washed in effluence tap water. Now 3.0 ml of IKI solution was added in both the tubes and mixed with a cyclomixer. The colorimetric reading was taken at 620 nm and the difference between the control and experimental reading gave the activity of the α - amylase. The data recorded at each stage were the mean of the three replicates.

Starch solution: Potato starch=150 mg, KH_2PO_4 =600 mg, Anhydrous CaCl_2 =20 mg, Distilled water=100 ml

IKI solution: I_2 =25.4 mg, KI=400 mg, Distt. Water=100 ml

Data analysis: The data was subjected to standard error and it was incorporated in the figures (4 and 5).

3. RESULTS AND DISCUSSION

A fluctuation in the content of starch, total sugar (TS), reducing sugar (RS), and non-reducing sugar (NRS) was observed in maize seeds due to treatments of toxins and subsequently with the extracts of bitter plants (Table 1). The maximum amount of starch i.e., 62.46 was exhibited in maize at a 2-ppm concentration of aflatoxin B_1 . The low levels of starch i.e., 12.25 were recorded in maize seeds due to the treatment of aflatoxin B_1 with the extract of *Andrographis paniculata*. However, more or less similar results (13.16 % and 14.12%) were recorded due to aflatoxin B_1 with the extracts of *Azadirachta indica* and *Adhatoda vasica* treatment, respectively.

Table 1. Influence of *Andrographis paniculata* (A), *Azadirachta indica* (B), and *Adhatoda vasica* (C) on Aflatoxin B_1 treated maize seeds on starch and sugar contents.

Treatment	Starch $\bar{X} \pm \text{S. E}$	T. Sugar $\bar{X} \pm \text{S. E}$	R. Sugar $\bar{X} \pm \text{S. E}$	N.R Sugar $\bar{X} \pm \text{S. E}$
Control	4.78 \pm 0.025	4.58 \pm 0.017	1.47 \pm 0.025	3.16 \pm 0.030
Plant A (2ppm)	0.93 \pm 0.027	4.05 \pm 0.056	1.17 \pm 0.030	2.78 \pm 0.039
Plant B (2ppm)	1.2 \pm 0.027	4.06 \pm 0.035	0.97 \pm 0.015	2.96 \pm 0.032
Plant C (2ppm)	1.03 \pm 0.028	2.84 \pm 0.029	0.58 \pm 0.019	3.09 \pm 0.023
Aflatoxin B_1 (2 ppm)	62.46 \pm 0.041	0.08 \pm 0.034	0.02 \pm 0.005	0.028 \pm 0.0035
Aflatoxin B_1 + Plant A (2 ppm)	12.25 \pm 0.032	1.25 \pm 0.056	0.28 \pm 0.021	2.46 \pm 0.027
Aflatoxin B_1 + Plant B (2 ppm)	13.16 \pm 0.032	0.74 \pm 0.032	0.36 \pm 0.053	0.53 \pm 0.038
Aflatoxin B_1 + Plant C (2 ppm)	14.12 \pm 0.065	0.61 \pm 0.046	0.18 \pm 0.030	0.53 \pm 0.030

As for sugar, there was a depletion in TS, RS, and NRS contents of maize seedlings due to the toxic effects of aflatoxin B_1 at 2 ppm concentration (Table 1). The levels of TS, RS, and NRS were 1.25, 0.28, and 2.46 % and 0.74, 0.36 and 0.53 % and 0.61, 0.18, and 0.53% in maize seeds due to the treatment of aflatoxin B_1 with *Andrographis paniculata*, aflatoxin B_1 with *Azadirachta indica* and aflatoxin B_1 with *Adhatoda vasica*, respectively. The lesser amount of TS, RS, and NRS was recorded due to aflatoxin B_1 with *Andrographis paniculata* treatment in comparison to aflatoxin B_1 with *Azadirachta indica* and *Adhatoda vasica* in maize seeds, respectively.

Both aflatoxin B₁ with *Andrographis paniculata* and aflatoxin B₁ with *Azadirachta indica* and *Adhatoda vasica* reduced α -amylase activity (increase in O.D. values) of germinating maize seeds (Table 2). The optical densities detected in control sets of maize were 0.77 at 0-time intervals. The α -amylase activity was inhibited maximum due to the treatment of aflatoxin B₁ (13).

Table 2. Influence of *Andrographis paniculata* (A), *Azadirachta indica* (B), and *Adhatoda vasica* (C) on α - amylase activity in Aflatoxin B₁ Treated maize seedlings

Treatment	α - amylase Activity			
	00 min.	30 min.	60 min.	90 min.
Control	0.77 \pm 0.026	0.44 \pm 0.028	0.18 \pm 0.035	-
Plant A	0.53 \pm 0.030	0.23 \pm 0.024	-	-
Plant B	0.63 \pm 0.026	0.34 \pm 0.021	-	-
Plant C	0.80 \pm 0.034	0.49 \pm 0.045	-	-
Aflatoxin B ₁ (2 ppm)	0.97 \pm 0.011	0.66 \pm 0.016	0.36 \pm 0.016	0.18 \pm 0.042
Aflatoxin B ₁ + Plant A	0.68 \pm 0.031	0.37 \pm 0.020	0.08 \pm 0.021	0.06 \pm 0.009
Aflatoxin B ₁ + Plant B	0.82 \pm 0.032	0.45 \pm 0.019	0.26 \pm 0.019	0.03 \pm 0.015
Aflatoxin B ₁ + Plant C	0.95 \pm 0.014	0.57 \pm 0.021	0.38 \pm 0.028	0.01 \pm 0.010

The maximum O.D. values i.e., 0.68, 0.82, and 0.95 were recorded in maize seeds due to the treatment of aflatoxin B₁ with *Andrographis paniculata*, aflatoxin B₁ with *Azadirachta indica* and aflatoxin B₁ with *Adhatoda vasica*, respectively. More or less similar results were observed due to the treatment of aflatoxin B₁ with *Azadirachta indica* and *Adhatoda vasica*. The enzyme activity was completely lost in the control as well as *Andrographis paniculata*, *Azadirachta indica*, and *Adhatoda vasica* treatments in maize seeds after 90-minute intervals. However, enzyme activity was also lost in maize seeds due to the treatment of *Andrographis paniculata*, *Azadirachta indica*, and *Adhatoda vasica* individually after 60-minute intervals.

The mycotoxins in combination with *Andrographis paniculata*, *Azadirachta indica*, and *Adhatoda vasica* have also been found to change the levels of starch, sugars as well as α - amylase activity in germinating maize seeds (Tables 1 and 2). High levels of starch as well as low levels of sugar and α - amylase activity have earlier been observed at 2-ppm concentration of Aflatoxin B₁ treatments in maize seeds. The degradation of starch was due to the activation of starch hydrolyzing enzymes like α - amylase, which convert the starch molecules into simpler sugars (10,15). Because of the reduction in α - amylase activities at a 2-ppm concentration of toxins, there were comparatively high levels of starch in the toxin-treated seeds. This might also be one of the reasons for the low level of sugars in the treated seeds (Figure 4).

The activity of α -amylase at the first two stages in the control sets indicates the development of the enzyme in a very early event. This is indicative of the initial burst of starch hydrolysis by active α -amylase which is synthesized in the scutellum of rice and sorghum seeds and the aleurone layer of barley seeds (9). Its activity was, however, slowed down at the subsequent stage of germination. This corresponded with the enzyme

activity in radicle and plumule tissue. For the inhibition of seed germination starch hydrolysis is primarily conducted by α - amylase. However, low level of starch in toxin and plant extracts treated seeds also suggests major participation of this enzyme in the process. There was no previous report on the toxic effects of mycotoxins with bitter plant extracts on α - amylase activity in the germinating seeds.

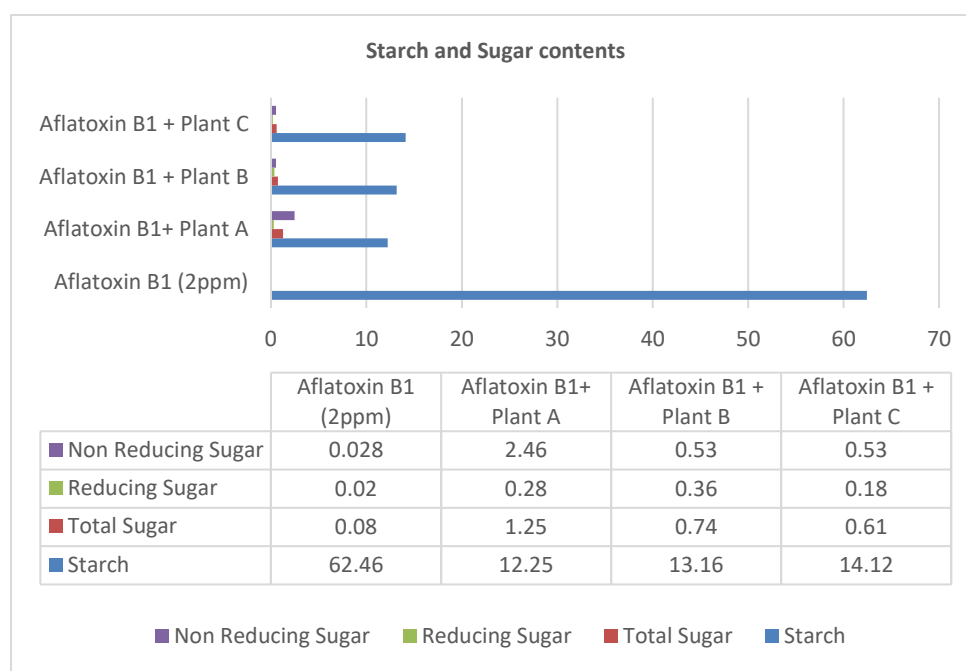


Figure 4. Influence of extracts of bitter plant A, B, and C on aflatoxin B₁ treated maize seeds on starch and sugar content.

The content of starch was also influenced by a combination of plant extracts and toxins at 2 ppm concentration. It was maximum i.e., 62.46 and 0.93 % at 2 ppm concentration of aflatoxin B₁ and *Andrographis paniculata* in maize seeds, respectively. The least amount of starch was recorded in bitter plant-treated seeds whereas the low levels of starch i.e., 0.93 and 12.25 were recorded in maize seeds due to aflatoxin B₁ with *Andrographis paniculata* followed by the combination of *Azadirachta indica* and *Adhatoda vasica*, respectively.

The levels of TS, RS, and NRS were 1.25, 0.28, and 2.46 % in maize seeds due to the treatment of aflatoxin B₁ with *Andrographis paniculata*, respectively. The lesser amounts of TS, RS, and NRS 0.61, 0.18, and 0.53 % were recorded due to Aflatoxin B₁ with *Adhatoda vasica* treatment in comparison to aflatoxin B₁ with *Andrographis paniculata* in maize seeds, respectively (18).

Aflatoxin B₁ reduced the α -amylase activity (increases in O.D. values) of germinating maize seeds. The optical density detected in control sets of maize seeds were 0.77, 0.44, and 0.18 at 00, 30- and 60-time intervals, respectively. The maximum O.D. values i.e., seeds due to Aflatoxin B₁ were observed with *Adhatoda vasica*, *Azadirachta indica* and *Andrographis paniculata* treatments, respectively (12) (Figure 5).

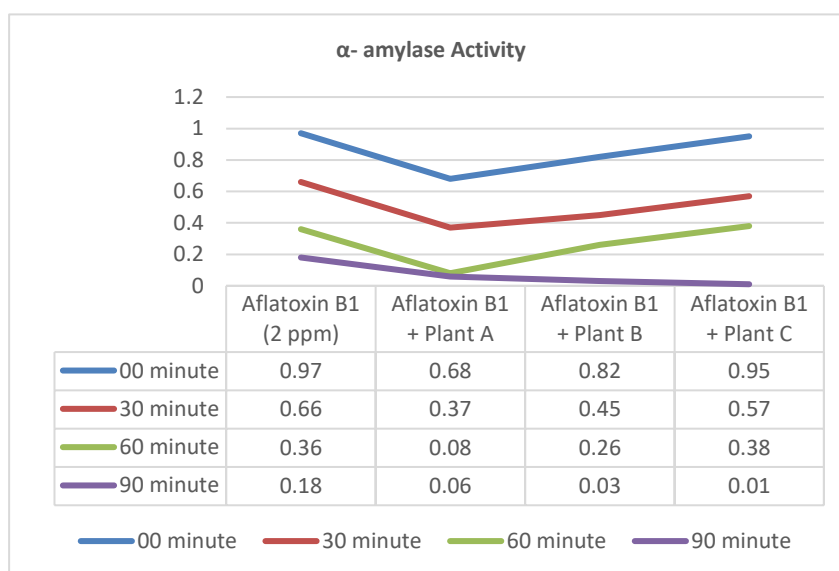


Figure 5. Influence of extracts of bitter plant A, B, and C on α -amylase activity in aflatoxin B₁ treated maize seedlings.

4. CONCLUSIONS

The extracts of bitter plants are indispensable for crop plants to protect from pests and animal grazing. There is sharp variation in the content of starch, total sugar, reducing sugar and non-reducing sugar. The contamination of maize seeds with aflatoxin B₁ caused a drastic decrease in crop productivity, especially the starch content. The activity of α -amylase was less due to inhibitory actions of mycotoxins in different concentrations. Still, once the extracts of bitter plants were applied in mycotoxin-treated seeds their increased.

DECLARATION

We declare that all authors of this Ms. have made significant contributions. We did not exclude any author who substantially contributed to this Ms. We have followed the ethical norms established by our institutions.

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration with all authors. All authors finally approved and drafted the manuscript.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICAL STATEMENT

This is to inform you that in this study, we have not been involved in any animal and human studies.

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