Efficacy of Bitter plant extracts (*Adhatoda vasica)* on prevention of aflatoxin B_1 production and reverse the physiology of maize seeds (*Zea mays L.*).

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Abstract: Maize (*Zea mays L.*) is an important staple food grain crop throughout the world, especially in developing countries like India. It is used in foods in the form of flour, oil syrups, etc, and also used in the preparation of paints, cosmetics adhesives, starch, and oil are major products. However agronomic practices and climatic conditions favorable to mold growth severally affected the quality and quantity of maize seeds. The main toxigenic fungi associated with grains in tropical regions are species of *Aspergillus.* Aflatoxin is a secondary metabolite produced by *A. flavus.* Bitter plants like *Adhatoda vasica* extracts have played a significant role in the inhibition of mycotoxins especially Aflatoxin B_1 and the improvement of seedling growth, seed quality, viability, and field emergence of plant seeds. *Adhatoda vasica* exhibited maximum antifungal activity against *Aspergillus flavus.* However Bitter plants like *Adhatoda vasica* extracts are one of the approaches to control the inhibitory properties of mycotoxin to enhance food production to meet the growing population demand in developing countries like India. Our Research reveals that the maximum inhibitions in seed germination were 78.57% maize at a 2-ppm concentration of AFT B_1. Also report shows that, the minimum inhibitions recorded in root and shoot lengths were 2.0% and 8.66% in maize seedlings due to treatment of AFT B_1 with *Adhatoda vasica* at 8 mg/ml concentration. Ultimately, Bitter plant extracts reduce the inhibitory properties of AFT B_1 produced by *A. flavus.* Furthermore, the bitter plant like *Adhatoda vasica* investigated in the present study may provide potential leads for novel bioactive. This will ultimately lead to substantial, financial returns to the growers as these bitter plants like *Adhatoda vasica* are low priced and the potential return to the farmers is enormous.

Keywords: Adhatoda vasica, Maize seeds, Aspergillus flavus, aflatoxin B_1 and Mycoflora

Introduction:

Maize (*Zea mays L.*) is an important staple food grain crop throughout the world, especially in developing countries like India. Approximately 702.2 thousand hectares of land come under maize cultivation in India and produce about 8079 thousand tones every year. It is the third most important cereal crop after rice and wheat. It is used in foods in the form of flour, oil syrups, etc, and also used in the preparation of paints, cosmetics adhesives, etc. The maize starch and oil are major products. In Bihar, maize is cultivated in all three seasons summer, monsoon, and winter, and the production varies depending upon the climatic and agronomic conditions. Maize crop under field condition has zero tolerance for flooding (FAO, 2009; Duke, 1983).

However agronomic practices and climatic conditions favorable to mold growth severally affected the quality and quantity of maize seeds. Main toxigenic fungi associated with grains in tropical regions are species of *Aspergillus*, *Penicillium*, and *Fusarium* (Khosravi *et. al.*, 2007; Chulza, 2010; Dudoin *et. al.*, 2016; Diksha *et. al.*, 2022). Many mycotoxins are produced by these fungi of which aflatoxin is a secondary metabolite produced by *A. flavus* and *A. parasitics.*

Several reports have been reported that, Impact of AFB_1 on physiological and biochemical responses in maize seeds (Prasad G; Ph.D. Thesis, 1992; Prasad *et. al.*, 1993; Prasad, 1995; Prasad *et. al.*, 1996; K.K. Sinha and Prasad G. 1997).

Bitter plants like *Adhatoda vasica* extracts are one of the approaches to control the inhibitory properties of mycotoxin to enhance food production to meet the growing population demand in developing countries like India. *Adhatoda vasica sanctum* and *Holoptelea integrifolia* exhibited maximum antifungal activity against *Aspergillus flavus* (KB Ishnva, KH Chauhan, and CA Bhatt, 2012).

It attracts worldwide attention to the control of aflatoxins because of the significant economic losses associated with their impact on human health, animal productivity, and trade (Khlangwiset *et. al.*, 2011; Fernandez- Cruz *et. al.*, 2010).

Bitter plants like *Adhatoda vasica* extracts have played a significant role in the inhibition of mycotoxins especially Aflatoxin B_1 and the improvement of seedling growth, seed quality, viability, and field emergence of plant seeds.

Since, therefore, an attempt has been, made in the present investigation to record the comprehensive studies of bitter plants like *Adhatoda vasica* extracts on the physiological and biochemical processes of aflatoxin-treated maize seeds.

Material and Methods:

Collection of Materials:

Maize Seeds (*Zea mays L.*) of Syngenta Sugar 75 Sweet corn obtained from Pooja seeds, Agricultural seed store, Bazar Samiti Road, Shivdhara, Darbhanga, Bihar, 846004, were used throughout the experiments.

Toxins

Standards of Aflatoxin B_1 were obtained from Sigma Chemical Co. St. Louis, U.S.A.

Bitter Plants

Bitter plants like *Adhatoda vasica* were obtained from the Janta nursery, Karamganj, west of Naka No-6, Darbhanga.

Preparation of plant extracts:
Adhatoda vasica was obtained from a Janta nursery, Darbhanga, washed initially with 2% aqueous NaOCl solution and subsequently with sterile distilled water. 20 g of plant parts (leaves) were blended with 100 ml of sterile distilled water for 5 min, the extracts were centrifuged at 4000 rpm (3000 g) for 10 min in a Sorvall Instruments RC-3B Refrigerated Centrifuge and filtered through Whatman no. 1 filter paper. The extracts were dried on a rotary evaporator at a temperature of between 55-60˚C. All the extracts were stored in desiccated at 4˚C before use.

SMKY Media:
An aqueous extract of *Adhatoda vasica* was prepared with different concentration viz; 4 mg/ml, 6mg/ml, 8mg/ml. The 1 ml extract of each concentration was added separately to 24 ml SMKY medium (Sucrose; magnesium sulfate; potassium nitrate; yeast extract, 200: 0.5:3: 7g/100ml) (Diener and Davis 1966), contained in a 100 ml, Erlenmeyer flask. In a control set, 1 ml of sterile distilled water was mixed with 24 ml SMKY medium. The flask was inoculated with 0.5 ml spore suspension (ca 10^6 spores/ml), prepared from 6-d-old culture incubated for 10 d under the maintained conditions of 28± 2˚C temperature and alternate cycles of 12 hrs light and 12 hrs dark. Triplicate replication was prepared for each treatment. After incubation for 7- 10 days, cultures were filtered and the dry mycelium mat was weighted after drying at 60˚c for 24 hrs.

Isolation of mycoflora and estimation of aflatoxin B_1:
Isolation of mycoflora associated with fifty sterilized (2% NaOCl) and fifty unsterilized kerels from each sample (Total-12 samples) was done by standard technique (ISTA; 1966). The plates were examined and fungal colonies were counted following the 5th day of incubation. The percent frequency of occurrence of each fungus was calculated on the following formula:

$$= \frac{Number\ of\ colonies\ of\ a\ particular\ species}{total\ number\ of\ colonies\ of\ all\ the\ species} \times 100$$

Seed germination and seedling growth:
The concentration of aflatoxin B_1 was used at 2ppm only. Since the effect of 5 different concentrations of aflatoxin B_1 Viz, 0.1, 0.25, 0.50, 1.0, and 2.00 ppm on seed germination, seedling growth and other biochemical contents of maize seeds were studied during Ph.D. work (Prasad, G, 1992). The maximum inhibition in the above parameters was recorded at a 2.0 ppm concentration of aflatoxin B_1 and hence, an already established concentration of aflatoxin B_1 (2.0 ppm) was used in the present study.

A stock solution was initially proposed in 1.00 ml ethanol from which the dilution (2.0 gm^{-3} change to ml) was made in sterile double distilled water solutions of aflatoxins B_1. Toxin and *Adhatoda vasica* were mixed separately and in combination with 4mg/ml, 6mg/ml, and 8mg/ml at 2 ppm of AFT B_1.

Maize Seeds were soaked in double distilled water for 1 hr and subsequently in different combinations of these toxins and *Adhatoda vasica* for 20 hr. One hundred healthy maize seeds were selected for each treatment in the triplicate set. The soaked seeds were subsequently placed on moist blotting paper and incubated in a seed germinator at 28 ± 2˚C. Seed germination index (GI) was calculated after 5- days of incubation, according to the formula given below:

$$GI = \frac{\text{Total number of seeds germinated}}{\text{Total number of seeds observed}} \times 100$$

Seedling growth was recorded on the 7th day by measuring the lengths of the radicle and plumule.

This data was analyzed statistically i.e., ‘t-test for seed germination and ‘F'- test for seedling growth. Statical calculations were carried out using the ANOVA test (Dospekhov; 1984).

Result:

Association of Mycoflora :
A total of 5 fungal species were found to be associated with maize with varying incidence (Table- 1) *Aspergillus flavus* and *A. niger* were recorded in all the samples. Whereas some other fungi were however isolated from both sterilized and non-sterilized kernels. As is evident from Table-1, the percent incidence of *A. flavus* was maximum (36.48) followed by *A. niger* and *Fusarium spp.*

The total number of colonies recorded were 340 and 515 in surface sterilized and non-sterilized seeds, respectively. The range of kernel infection in different samples varied between 4-43%.

Table 1 Showing association of Mycoflora with maize seeds

Sl. No.	Fungi	No. of the positive sample (12)	Total No. of colonies		% Relative infection		Range of kernel infection (%)
			Surface Sterilized	Non sterilized	Surface Sterilized	Non sterilized	
1.	***Aspergillus flavus***	12	133	208	36.48	26.14	7-43
2.	***A. niger***	10	115	145	26.78	19.82	6-31
3.	***Penicillium spp.***	8	18	40	3.88	5.16	5-16
4.	***Rhizopus nigricans***	7	29	42	7.59	6.07	4.19
5.	***Fusarium spp.***	6	45	80	10.47	10.62	8-22
Total no. of colonies		—	**340**	**515**	—	—	—

100 seeds per sample (1:1; S: NS); NS= Non sterilised, S= Sterilized.

Estimation of Aflatoxin B_1:

Altogether 50 isolates of *A. flavus* representing 5- isolates from each sample were screened for their aflatoxin-producing potentialities. Out of which 20 isolates were found to be toxigenic (Table-2).

Among the toxigenic strains, 12 isolates elaborated aflatoxin B_1 only, whereas 7- isolates were capable of producing aflatoxin B_1 and B_2. One isolate could be produced B_1, B_2, and G_1. *A. flavus* isolates produced aflatoxin B_1 in the range of 2.2- 10.8 µg/ml.

Table 2 also shows that out of 12 samples of maize analyzed for the natural contamination of aflatoxin; 4 were found to be positive for aflatoxin B_1. The amount of aflatoxin B_1 was, however very low i.e., only 1.2 and 25 µg/kg in those contaminated samples.

Table-2 Aflatoxin producing potentiality of *A. flavus* isolated and aflatoxin contamination in maize seeds sample.

No. of *A. flavus* isolates/maize samples screened	No. of toxigenic isolates/contaminated sample	Types of aflatoxin produced			Range of aflatoxin B_1 Concentration
		B_1	B_1, B_2	B_1, B_2, G_1	
50 Isolates	20	12	7	01	2.2 – 10.8 µg/ml
12 Sample	4	3	—	—	1.2 & 25 µg/kg

Table- 2 also shows that out of 12 samples of maize analyzed for the natural contamination of aflatoxin, 3- were found to be positive for aflatoxin B_1. The amount of aflatoxin B_1 was, however very low, only 1.2 and 25 µg/kg in those contaminated samples.

Seed germination:

The effect of AFT B_1 individually and in combinations with *Adhatoda vasica* was also depicted on maize seed germination (Table-3). A significant fall and stimulation in the seed germination were noticed at that concentration/combination of aflatoxin B_1 and *Adhatoda vasica* (at 2ppm; t-test), respectively. The maximum inhibitions in seed germination were 78.57% maize at a 2ppm concentration of AFT B_1, whereas minimum inhibitions in seed germination were 26.53 and 13.2% in maize seeds due to a combination of AFT B_1 + *Adhatoda vasica* (6 mg/ml) and AFT B_1 + *Adhatoda vasica* (8 mg/ml), respectively (Table-3 & Fig 1).

Table-3 Impact of the combined effect of AFT B_1 and *Adhatoda vasica* of different concentrations on maize seed germination

Observation	**Control**	**Aflatoxin B_1**	***Adhatoda vasica* (4mg/ml) + AFT B_1**	***Adhatoda vasica* (6mg/ml) + AFT B_1**	***Adhatoda vasica* (8mg/ml) + AFT B_1**
Germination Index **X ± S.E**	98 ± 0.94	21 ± 0.57	65 ± 0.81	72 ± 0.83	85 ± 0.54
't' Difference with control	—	77	33	26	13
% Inhibition	—	78.57%	33.67%	26.53%	13.2%

Figure 1 Impact of the combined effect of AFT B1 and Adhatoda vasica of different concentrations on maize seed germination

Seedling growth:

Seedling growth (Shoot and root lengths) was drastically reduced due to the inhibitory or stimulatory effects of AFT B_1. *Adhatoda vasica* on the other hand increased the root and shoot lengths (Table- 4). The minimum inhibitions recorded in root and shoot lengths were

2.0% and 8.66% in maize seedlings due to treatment of AFT B_1 with *Adhatoda vasica* at 8 mg/ml concentration. However, the treatment of AFT B_1 with *Adhatoda vasica* exhibited somewhat greater inhibitions such as 24.6 and 38.8 % than the above treatment at 4 mg/ml concentration respectively (Table- 4 & Fig- 2).

Table-4 Impact of *Adhatoda vasica* extracts on seedling growth of AFT B_1 treated maize seeds.

The concentration of *Adhatoda vasica*	Root Length (c.m)			Shoot length (c.m)		
	X ± S.E	Difference with control	% Inhibition	X ± S.E	Difference with control	% Inhibition
Control	9.74 ± 0.06	—	—	6.12 ± 0.02	—	—
AFT B_1 (2ppm)	3.38 ± 0.01	6.36	65.29	2.27 ± 0.008	3.85	62.90
AFT B_1 + 4 mg/ml	12.14 ± 0.01	2.4	24.6	8.50 ± 0.43	2.38	38.8
AFT B_1 + 6 mg/ml	10.60 ± 0.30	0.8	8.21	7.20 ± 1.22	1.08	17.64
AFT B_1 + 8 mg/ml	9.49 ± 0.47	0.2	2.0	5.59 ± 12.01	0.53	8.66

Figure 2 seedling growth of AFT B1 treated maize seeds.

Growth and Aflatoxin B_1 analysis:
The growth of *A. flavus* and aflatoxin B_1 production were inhibited to a substantial level by treatment with different concentrations of *Adhatoda vasica* extract. The maximum percent aflatoxin B_1 inhibition was at an 8 mg/ml concentration of extract (74.90% with AFT B_1) and was minimal at 4 mg/ml (48.40 % with AFT B_1). The growth of *A. flavus* was also correspondingly decreased by increasing the concentration of extract (Table- 5). The reduction of growth of the *A. flavus* and of aflatoxin B_1 production by treatment with *Adhatoda vasica* was possibly due to interference by active principles of the extract.

Present findings suggest, however, that growth and aflatoxin B_1 production by *A. flavus* are proportionate processes. The active principles of *Adhatoda vasica* also affected the production of AFT B_1. It is widely accepted that aflatoxin B_1 is the precursor of the other related congeners (Badii and Moss 1988).

Table- 5 The effect of *Adhatoda vasica* on growth and aflatoxin B_1 production by *Aspergillus flavus* in SMKY liquid media.

Concentrations (mg/ml) of *Adhatoda vasica* extracts	Mycelium weight of *A. flavus* (mg/25 ml) X ± S.E	Aflatoxin B_1 production (µg/ml) X ± S.E	Differences with control		% Inhibition	
			B_1	Mycelium	B_1	Mycelium growth
Control	1367 ± 4.80	16.34 ± 0.003	—	—	—	—
4	1085 ± 1.50	8.43 ± 0.005	7.91	282	48.40	20.62
6	715 ± 12.29	6.22 ± 0.07	10.12	652	61.93	47.69
8	337 ± 12.29	4.10 ± 0.51	12.24	1030	74.90	75.34

Discussion:
The fungal isolates showed varying degrees of sensitivity and tolerance to the Bitter plant like *Adhatoda vasica* extracts. The results showed that there was no uniform response within or between fungal isolates of the same species in terms of susceptibility to antifungal activity in the *Adhatoda vasica* extracts. Variation in sensitivity of fungal isolates has been previously reported (Mazzola *et. al.*, 1995; Sandrock and Van Etten, 1998).

Conidia formation is known to occur when fungi are under stress because of fungicides, nitrogen fertilizers, and environmental factors such as temperature, pH, water availability, or co-inoculation (Leandro *et. al.*, 2003; Paterson, 2007; Xu *et. al.*, 2007).

The whole herb of *Adhatoda vasica* is bitter and is a source of several diterpenoids of which a bitter water-soluble lactone "andrographolide" is important.

Significance and Impact of the Research; Food and Feed are subject to infection by a variety of microorganisms that can induce spoilage and/or produce metabolites that are toxic to humans and animals. Extracts of *Adhatoda vasica* were most active and maybe developed into environmentally eco-friendly fungicides, which are affordable to rural farmers in developing countries.

Conclusion:
The result obtained from this study indicates that Bitter plants like *Adhatoda vasica* contain chemical constituents that can be developed as a potential antifungal agent for agricultural use. This bitter plant may be ecologically adapted to withstand fungal infection, i.e., they seem to have developed a huge armament of secondary metabolites to resist fungal attack because of their constant exposure to fungi because of co-existence with crop plants (Eloff *et. al.*, 2007).

Adhatoda vasica is a significant inhibitor of the growth of *A. flavus* and aflatoxin B_1. If inhibitory factors could be examined at a physiological and biochemical level, *Adhatoda vasica* might be used in controlling aflatoxin B_1 contamination in food and feed. It is argued that the *Adhatoda vasica* may be exploited for preventing aflatoxin contamination in agricultural commodities.

Furthermore, the bitter plant like *Adhatoda vasica* investigated in this study may provide potential leads for novel bioactive. This will ultimately lead to substantial, financial returns to the growers as these bitter plants like *Adhatoda vasica* are low priced and the potential return to the farmers is enormous.

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