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Exploitation of Aqueous Plant Extracts for Reduction of Fungal Growth and Detoxification of Aflatoxins

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Abstract

Aflatoxins are secondary metabolites of the molds *Aspergillus flavus* and *Aspergillus parasiticus* which can grow on a wide variety of agricultural commodities, known to be potent toxic and carcinogenic to test animals. Recent evidences indicate that these toxins may also be involved in the etiology of human liver cancer in certain parts of the world. To remove these aflatoxins from food and feed, the use of plant extracts for their anti-microbial and antifungal properties have been a subject of wide interest. Regarding the low phytotoxicity, more systemically and easily biodegradable nature, the antifungal products from higher plants may afford as the most suitable source for the development of effective fungicide. The selected plant extracts include *Terminalia chebula* (Karakkayalu), *Solanum xanthocarpum (Mullavanga), Syzygium cumini (Neredu), Capsicum annuum (Mirapakayalu), Ipomoea mauritiana (Nelagummadi).*Results indicate that only *S. xanthocarpum* fruit extract showed inhibitory property against aflatoxin production. *Terminalia chebula* and *Capsicum annuum* extracts also inhibited toxin production of *A. parasiticus* by 54%, 60% and of *A. flavus* by 45%, 52%, respectively. Plants having capacity for inhibiting aflatoxin did not exert identical effects on the growth of *A. flavus* and *A. parasiticus*. This is evident that whole plant extract of *Ipomoea mauritiana* only inhibited the growth by 73% and toxin inhibition by 30%. Fruit extracts inhibited the growth by 50% or more and there was no correlation between the inhibition of growth and aflatoxin production. The results show that some plants possess antifungal, antitoxic properties and that aflatoxin production is significantly reduced in some cases.

Keywords: Aflatoxin, Detoxification, Aqueous plant extracts, *A. flavus*, *A. parasiticus*.

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1. Introduction

Aflatoxins are poisonous, carcinogenic secondary metabolites of *Aspergillus flavus* and *Aspergillus parasiticus* and are the most studied mycotoxins. There are four major groups of aflatoxins: B_1, B_2, G_1 and G_2 (Figure 1). Aflatoxin M_1, a metabolite of aflatoxin B_1 in mammals, may be found in the milk of animals consuming feed contaminated with aflatoxin B_1 [1-3]. Aflatoxins B_1, B_2, G_1 and G_2 are classified as Group 1 human carcinogens whereas M_1 is classified as Group 2B probable human carcinogen [4]. Aflatoxin production has been reported from *A. flavus, A. nomius, A. parasiticus, A. zhaoqingensis, A. pseudotamarii* and *A. bombycis* [5-8] and the two varieties of *A. flavus (*var. *parvisclerotigenus* and var.*columnaris)*[9-11]. *Aspergillus flavus* produces aflatoxins in starchy cereal grains (for example, corn, wheat, sorghum, barley, millet and rice etc.) starting at a moisture content of about 18% that is, in equilibrium with 85% relative humidity (0.85 available water) and at temperatures of 12ºC to 42ºC with an optimum at 27ºC to 30ºC [12]. Under optimum growth conditions, *A. flavus* can produce some aflatoxin within 24 hours and a biologically significant amount in a few days. Contamination can occur in crops in the field, at harvest, during post-harvest operations and in storage. The rate and degree of aflatoxin contamination are dependent on temperature, humidity, soil and storage conditions [13]. Aflatoxicosis is a disease caused by the consumption of aflatoxins; the effects are dependent on the species, age, and condition of the animals involved, the amount of aflatoxin consumed and the length of exposure which leads to cancer and other diseases in humans [14]. In order to control these aflatoxins, either regulatory measures or detoxification strategies are to be followed. The object of detoxification was to find a detoxificant system capable of removing aflatoxin from contaminated food and feed and one that would be potentially acceptable under food and drug regulations [15]. Because aflatoxin contamination is unavoidable, numerous strategies for their detoxification have been proposed. These include physical methods of separation, thermal inactivation, irradiation, and solvent extraction, adsorption from solution, microbial inactivation and fermentation [16-20]. To have a practical, cost effective and non-toxic method, detoxification by plant extracts is one of the important methods. The present investigation focused on the inhibitory effects of aqueous plant extracts on aflatoxin production by *A. flavus* and *A. parasiticus*.

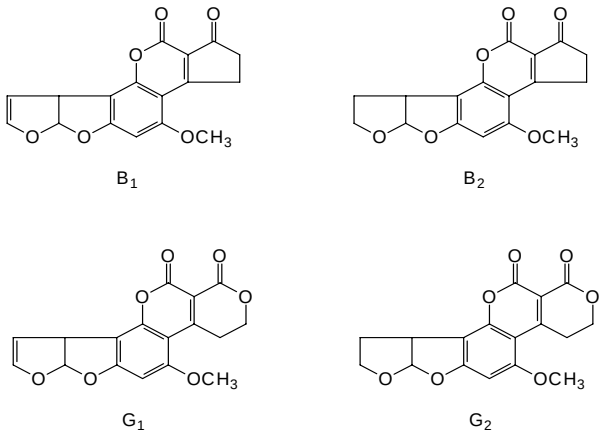


Figure 1 Structure of aflatoxins B_1, B_2, G_1 G_2

2. Materials and Methods

2.1 *Aspergillus* strains

The two fungal strains *Aspergillus flavus* (MTCC 2798) and *Aspergillus parasiticus* (MTCC 2797) were selected from MTCC, Chandigarh, INDIA which are known to have a special feature of aflatoxin production. The strains were subcultured on Czapek Yeast Extract Agar at 25ºC for 5 days under aerobic conditions.

2.2 Chemical confirmation of Aflatoxins

Chemical confirmation of aflatoxins was performed directly on the developed TLC plate. Two spraying reagents were used to visualize and increase the fluorescence intensity of the mycotoxins [21]. The plates were then sprayed with either 20% $AlCl_3$ solution or 20% sulfuric acid, heated to 110ºC and examined under UV light (365 nm)[22].

2.3 Preparation of aqueous plant extracts

For the detoxification studies initially a total of 24 plants were selected. On the basis of preliminary screening and their phyto active nature, five plants were selected viz., fruits of *Terminalia chebula* (Karakkayalu), *Solanum xanthocarpum* (Mullavanga) *and Capsicum annuum* (Mirapakayalu), leaves of *Syzygium cumini* (Neredu), *Ipomoea mauritiana* (Nelagummadi). Parts of the plants were thoroughly washed with sterile distilled water and dried in hot air oven at 60ºC for 4-7 days. Fifty grams of each plant were weighed and the aqueous extracts were prepared by blending in a mortar. Finally the blend was adjusted to one liter. The extract was filtered through several layers of cheese cloth and the filtrate was centrifuged for 30 min at 3500 rpm. The supernatant was used as test extract.

2.4 Testing of aqueous plant extracts on aflatoxin producing fungi

The plant extracts were prepared in different concentrations i.e. 5%, 10%, 15%, 20%, 25% along with a control. These different concentrations of extracts were enriched to the Czapek Yeast Extract medium separately for each set of experiment. After sterilization, the fungal cultures were inoculated with 1ml of spore suspension at 25 ± 2ºC for 8 days in the specified media along with different concentrations of aqueous plant extracts. After incubation the growth of the organism and toxin producing potential were analysed separately. The experiments were done in three replicates.

2.5 Extraction and Detection of *Aspergillus* toxins

After incubation, the contents of each flask along with medium and mycelium were homogenized for 5 min in a high-speed blender with 100 ml chloroform. The extraction procedure was repeated three times. The chloroform extracts were combined, filtered, and concentrated near to dryness and aflatoxin detected by TLC method [23]. Thin layer chromatographic technique of the clean extract was done on percolated silica gel plates (Merck, Silica Gel 60, 25 mm, 20x20). Detection of the different aflatoxins was carried out according to standard procedures. A mixture of toluene-ethyl acetate-90% formic acid (50:40:10) was used as a mobile phase. Aflatoxins were visualized under ultraviolet light at 365 nm in a chromatovisor. Aflatoxin had a retention time and fluorescent spot similar to the standard aflatoxins being tested. For quantitative estimation of the toxins in the sample extract, match the intensity of spots of the sample with that of standard toxin spots, by diluting both to extinction. The amount of toxin presented was calculated

spectrophotometrically [24-25]. Trifluroacetic Acid (TFA) was directly super-imposed on to the aflatoxin extract spot before development. After reaction, the plate was developed and examined under UV light for the presence of the blue fluorescent spot of B_2, which can be recognized with the comparison to the B_1 standard, spotted on the same plate. As on additional conformation, sulphuric acid (50%) is sprayed on another part of the plate where unreacted aliquots of extracts and B_1 standard were developed. The sulphuric acid spray changes the fluorescence of aflatoxin from blue to yellow. This test only confirmed the absence of aflatoxin i.e. the spots which do not turn yellow are definitely not aflatoxin, because many materials other than aflatoxin may give a yellow spot with sulphuric acid. The same above process was adopted for the confirmation of aflatoxin G_1 [26].

3. Results and Discussion

The effects of aqueous plant extracts on mycelial growth as well as aflatoxin production are recorded. The aqueous extracts of *Terminalia chebula* were more effective on inhibiting the growth of *Aspergillus parasiticus* when compared to *Aspergillus flavus*. The maximum *A. parasiticus* mycelial inhibition was 74% whereas *A. flavus* inhibition was 63% at concentration of 25%. The aqueous extracts of *Terminalia chebula* were more effective on inhibiting aflatoxin production by *A. parasiticus* when compared to *A. flavus* (Figure 2). The maximum inhibition of aflatoxin production was 45% by *A. flavus* and 54% by *A. parasiticus* at 25% concentration of plant extracts.

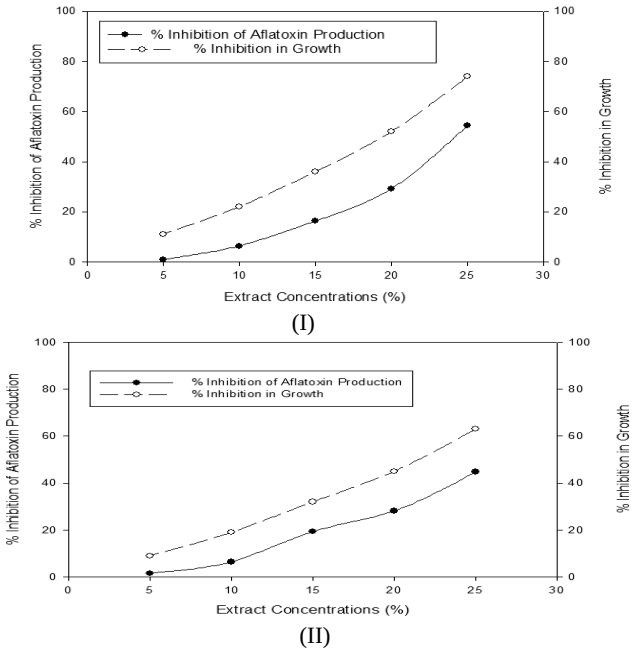
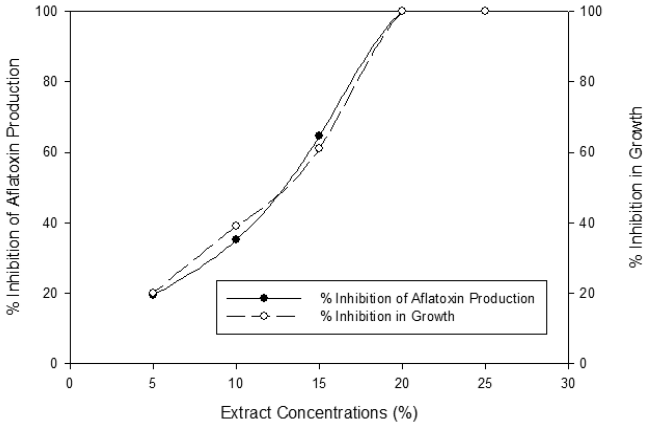
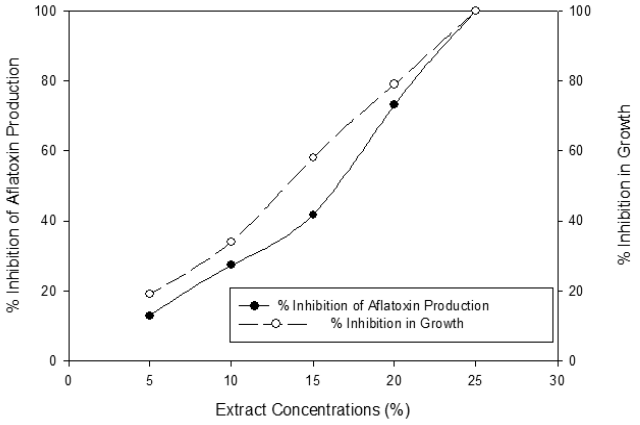


Figure 2 Effect of *Terminalia chebula* extracts on percent inhibition of aflatoxin production and growth of *Aspergillus parasiticus* (I) and *Aspergillus flavus* (II)

In case of *Solanum xanthocarpum* inhibition of growth and aflatoxin production inhibitory effects were very significant when compared to both *A. flavus* and *A. parasiticus*. The maximum inhibition of growth was at 20% for *A. flavus* and at 25% for *A. parasiticus* .The maximum toxin inhibition was also recorded at the same concentrations. The maximum inhibition means a complete 100% inhibition of growth and toxin in this experiment (Figure 3). This may be due to the effect of solamargine and solasonine present in the fruit extract of the *Solanum xanthocarpum*. This is evident from the reports of Singh *et al*. [27] and Fewell *et al.* [28].



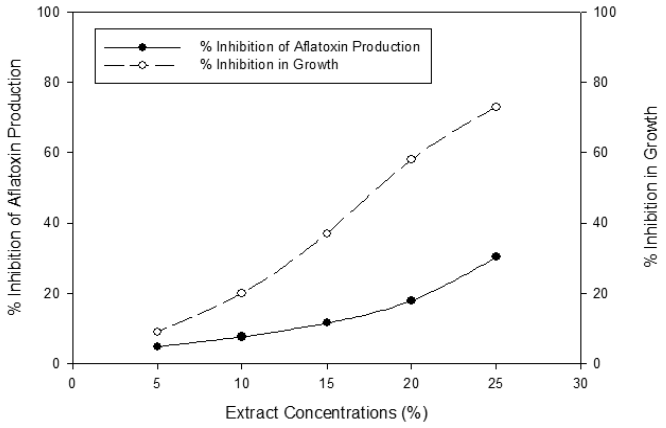
(I)



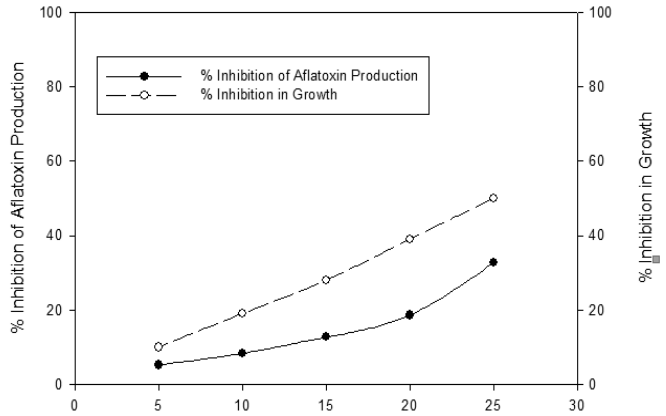
(II)

Figure 3 Effect of *Solanum xanthocarpum* extracts on percent inhibition of aflatoxin production and growth of *Aspergillus flavus* (I) and *Aspergillus parasiticus* (II)

The whole plant extract for *Ipomoea mauritiana* showed the growth inhibition of *A. flavus* (73%) when compared to the growth inhibition of *A. parasiticus* (50%). However, the inhibition of toxin production was moderate (30%, 33%) in both organisms (Figure 4).



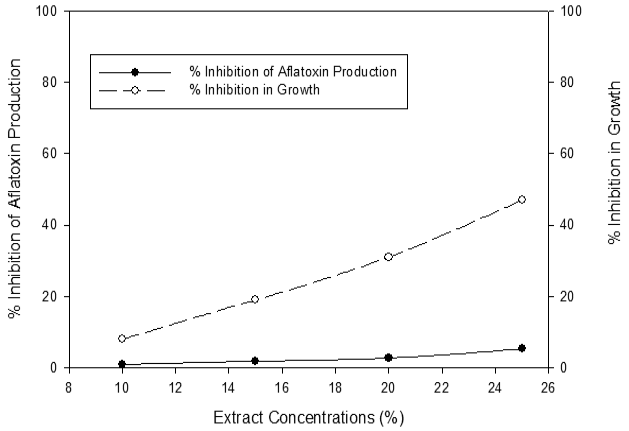
(I)



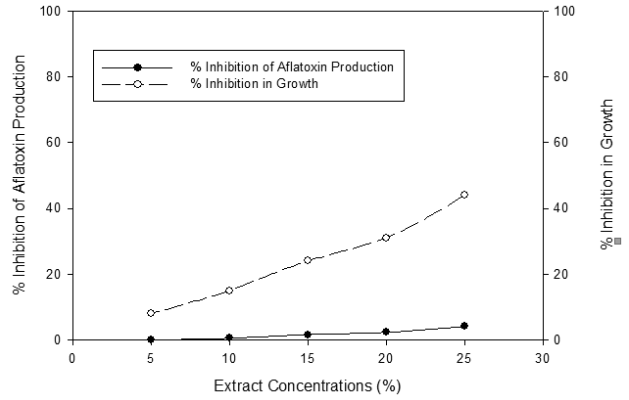
(II)

Figure 4 Effect of *Ipomoea mauritiana* extracts on percent inhibition of aflatoxin production and growth of *Aspergillus flavus*(I) and *Aspergillus parasiticus*(II)

The growth inhibition of both organisms was moderate at 25% concentration of the extract. The growth of *A. flavus* was slightly stimulated when 5% extract was applied. The inhibitiory effect was insignificant on both *A. flavus* and *A. parasiticus* (Figure 5) when leaves extract of *Syzygium cumini* (Neredu) was applied.



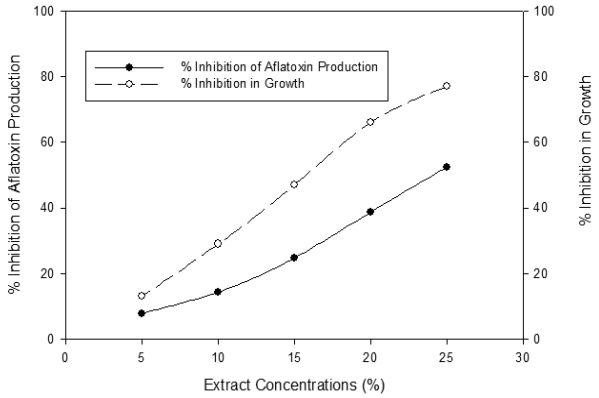
(I)



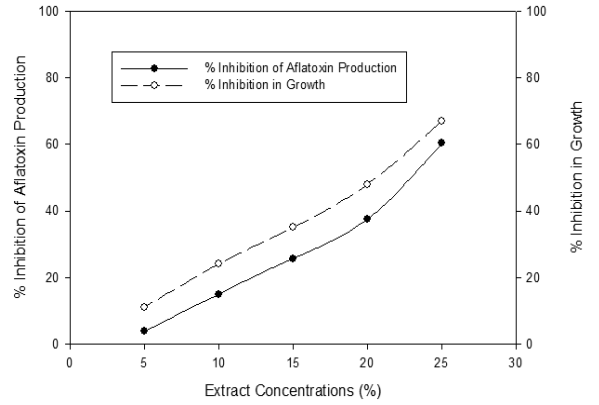
(II)

Figure 5 Effect of *Syzygium cumini* extracts on percent inhibition of aflatoxin production and growth of A. *flavus*(I) and A. *parasiticus* (II).

The *Capsicum annuum* fruit extract was more effective on *A. flavus* when compared to *A. parasiticus*. The maximum growth inhibition of *A. flavus* and *A. parasiticus* were 77% and 67%, respectively at 25% concentrations. The maximum inhibition of toxin production being recorded as 60% in *A. parasiticus* and that of *A. flavus* was 52% only (Figure 6).



(I)



(II)

Figure 6 Effect of *Capsicum annuum* extracts on percent inhibition of aflatoxin production and growth of *Aspergillus flavus* (I) and *Aspergillus parasiticus* (II).

Aflatoxin contamination of edible commodities is one of the greatest problems of biological environmental pollution. Considering the existing state of aflatoxin problem and the need of developing suitable control measures, a current approach has been made in the present investigations by screening plant extracts which showed potent inhibitory action towards the inhibition of aflatoxin production. Aqueous extracts of five plants were tested against aflatoxin production and growth of *A. flavus* and *A. parasiticus*. The confinement of antifungal principles to specific plant parts i.e. root, stem, wood, bark, flower, and fruit has been reported [29-31]. In order to locate the inhibitory compounds in different parts of plants viz., root, stem, leaves, fruits, these parts were extracted separately. Extracts of different parts of the plants varied in inhibitory property. Fruit extracts of *Terminalia chebula*, *Solanum xanthocarpum*, *Capsicum annuum* were substantially potent in their inhibitory effect. Leaf extract of *Syzygium cumini* is not effective on

inhibiting the aflatoxin. It is thus evident that inhibitory property varies not only with the nature of the plant but also with the part of the plant. It is, therefore, essential to locate the proper plant component for the inhibitory factors. The selected parts exhibiting maximum efficacy on solid substance like maize, wheat, rice and groundnut, when treated with aqueous extracts of plants and the inhibition was 50% or more were reported [32, 33]. In the present investigation, although some of the aqueous extracts of plant fruits inhibited aflatoxin production by more than 50%. Only *Solanum xanthocarpum* fruit extract showed percent inhibitory property against aflatoxin production. *Terminalia chebula and Capsicum annuum* extracts also inhibited toxin production by 54% and 60% on *A. parasiticus* and 45% and 52% by *A. flavus*, respectively. Preliminary screening showed that some of the plant extracts possess enormous potential for inhibiting aflatoxin production and growth of *A. flavus* and *A. parasiticus*. Plants having capacity for the inhibition of aflatoxin did not exert identical effects on the growth of *A. flavus* and *A. parasiticus*. This is evident that *Ipomoea mauritiana* whole plant extract inhibited the growth by 73% and toxin inhibition by 30% only while fruit extracts inhibited the growth by 50% or more. There was no correlation between the inhibition of growth and aflatoxin production. From the results, it is clear that some plants possess antifungal and/ or anti toxic properties and that aflatoxin production is significantly reduced in some cases. This may be the effect of plant extracts on the metabolism of fungi. Variation in the antimicrobial property of plants belonging to different families and also in different genera of the same family has been reported by Misra *et al*. [34].

From the present investigations it is evident that the plants can be exploited for prevention of aflatoxins on liquid and solid substances [35-39]. The results also show that the direct application of plant extracts or their active ingredient could be substantially helpful in preventing aflatoxin contamination on commercial scale. Direct spray of aqueous plant extract is convenient for the farmers because these can be easily prepared and its application does not require any technical knowledge. Moreover, any residual effect is not expected because these can be easily and quickly degraded.

4. Conclusions

In the Government godowns the elaboration of aflatoxin can be avoided by spraying the seeds with tolerable doses of acids, but for the farmers it might not be convenient. The main strategy should be aimed at preventing the growth of molds on stored seeds and for that purpose the application of plant extracts were effective. There is also need to screen more plants, specially the wild ones for effective inhibition on aflatoxins and toxigenic fungal strains.

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