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EVALUATION OF AFLATOXIN B_1 CONTAMINATION LEVELS IN THE OIL SEED SAMPLES

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ABSTRACT Aflatoxins are the toxic secondary metabolites produced by *Aspergillus* species in food and feed commodities, when stored under warm and damp conditions. Aflatoxin B_1 (AFB_1), a prototype of the aflatoxins is widely recognized as the most hepato-carcinogenic compound and classified as group I human carcinogen by the international agency for research on cancer(IARC). Contamination of oil seeds with the AFB_1 pose serious threat to the food and feed industry. The present investigation efforts on the percentage incidence of seed borne mycoflora and assessment of AFB_1 content in selected nine eight seed samples by the qualitative and quantitative methods, which involves the principle of chromatography methods like thin layer chromatography(TLC) and high-performance liquid chromatography(HPLC). The percentage incidence of infection was observed to be higher for the groundnut seed sample showing the mean percentage of infection to be about 48.57% than with all the seed samples. The genus *Aspergillus* was most predominant, with A, niger topping the list followed by *Rhizopus spp*. and the least was *Trichoderma*. Among 55 isolates of *Aspergillus flavus*,42 were found to be aflatoxigenic strains by determinative method of TLC and amount of aflatoxin B_1 in oil seeds is estimated to be in the range of 29-35µg/ml by quantitative analysis of HPLC. The results indicate that the selected oil seeds are prone to fungal contamination with AFB_1 production when stored under ambient conditions. Therefore, proper storage and regular inspection of its quality should be practiced ensuring the safety of animals including human beings.

KEYWORDS : Oil seeds; *Aspergillus flavus*; Aflatoxin B_1; TLC; HPLC.

INTRODUCTION:

Aflatoxins are a group of closely related heterocyclic compounds produced predominantly by filamentous fungi viz., *Aspergillus flavus* and *A. parasiticus* (Yu et al. 2002*)*. Aflatoxin contaminates a vast array of food and agricultural commodities such as cereals, nuts, dried fruits, coffee, cocoa, spices oil seeds, dried peas and bean and fruit (Turner et al., 2009: Reddy et al. 2011). *Aspergillus* species can grow on a variety of substrates under different environmental conditions (Reddy et al. 2011). Oil seeds are the most widely distributed food crops in the world and its contamination by these fungi can lead to mycotoxin accumulation during the stages of growing, harvesting, storage, transporting and processing. Aflatoxin contamination of foods and feeds has gained global importance because of its deleterious effects on human as well as animal health (Okoli et al. 2006). Mycotoxins contamination intensity in food crops vary geographically and groundnut is main source of mycotoxins. Groundnut seed is predominantly infected with *Aspergillus flavus* and *Aspergillus niger* (Gebreselassie, Dereje, & Solomon,2014). The reported outbreaks of aflatoxicosis in man were due to the consumption of contaminated food and feed (Reddy and Raghavender, 2007). The economic consequences of mycotoxin contamination are profound, as the crops contaminated with high levels of mycotoxin are often destroyed (Fakruddin et al. 2015). This study was undertaken to isolate, identify the seed borne mycoflora and estimation of aflatoxin B_1 content produced by them, qualitatively and quantitively by TLC and HPLC respectively.

MATERIALS AND METHODS

CHEMICALS & REAGENTS

The standard AFB_1 was procured from Sigma-Aldrich (Steinheim, Germany) and thin layer chromatography (TLC, Silica gel 60) plates from Merck (Demhadt, Germany). All analytical HPLC grade solvents and culture media were purchased from Hi-Media Mumbai (India).

COLLECTION OF SAMPLES

To obtain a representative sample, all the oil seed samples viz., Groundnut, Castor, Mustard, Sesame (Husked and Dehusked), Sunflower, Guizotia, Soya bean and Cotton seeds were collected by random sampling method. The samples were collected from regulated market yards which are situated in major oil seed growing and selling regions of Karnataka and Andhra Pradesh(India). A total of eight different types were collected and subjected to estimate the incidence of seed borne fungi

SCREENING FOR SEED BORNE MYCOFLORA BY STANDARD BLOTTER METHOD

The Standard blotter method was employed to assess the incidence of fungal infection in the oil seeds on contamination. Seeds were surface sterilized with 0.1% mercuric chloride solution for 3-4 minutes rinsing thoroughly in sterile distilled water. About twenty-five seeds of each sample were placed in a petri dish containing three circular discs of blotting paper. The seeds were placed equidistant from one another and incubated at temperature of 26 ± 2^0 C for 7 days. The pure fungal cultures were examined under microscope ((Fakhrunnisa *et al*,.2006). The *Aspergillus flavus* isolates were maintained on PDA slants as a pure culture for the analysis of AFB_1.

STUDIES ON EFFECT OF CULTURE FILTRATES AND ANALYSIS OF AFB_1:

To study the ability of the isolates of *Aspergillus flavus* to produce aflatoxins, they were grown by inoculating the 1 ml of spore suspension into the sterilized yeast extract sucrose (YES) media and incubated under room temperature for 7 days. After the completion of incubation period, the culture fluid was filtered through Buchner funnel using Whatman's No.1 filter paper. The mycelia growth was discarded. The culture filtrates were used for the bioassay test and for estimation of aflatoxin B_1.

EFFECT OF CULTURE FILTRATES ON SEED GERMINATI ON OF FENUGREEK SEEDS

The toxicity of the aflatoxin obtained from culture filtrates of *Aspergillus flavus* on plant growth was assayed using seed germination and seedling vigor tests. Twenty ml of culture filtrates were utilized for the bioassay test. Fenugreek seeds were surface sterilized with 0.1% mercuric chloride solution for 3-4 minutes rinsing thoroughly in sterile distilled water before soaking in culture filtrates of various isolates of

A. flavus. Seeds were given a 24-hour soaking in water and were plated equidistantly from one another on two moist blotters in horizontal and vertical rows. A third blotting paper moistened with water was placed on the seeds. Then all the three blotting papers along with the seeds were rolled in and kept in a tray containing water. Care was taken not to immerse the seeds completely in water. Seeds soaked in sterilized water and in liquid media were kept as controls. Observations on germination were recorded after 8 days. Vigour index was also calculated using below formula.

Vigour index = (Average root length +Average shoot length) x % of germination.

EXTRACTION AND QUANTIFICATION OF AFB_1:

THIN LAYER CHROMATOGRAPHY: Aflatoxins were determined by spotting 10 µl of chloroform extracts of 55 isolates of *A. flavus* of different oil seed samples on pre-coated TLC plates(Merck) along with Standard AFB_1. Plates were developed in a solvent system consisting of solvent mixture of Butanol: Acetic-acid: water in the ratio of **5:1:4** in an unlined chamber. After the development, the plates were viewed under long UV light at 365nm. Blue fluorescence was produced similar to that of standard AFB_1 indicating the presence of AFB_1(Aycicek, Hasan et al., 2005)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY: AFB_1 was separated isocratically on Perkins Elmer S- LC-10ATVP, Japan HPLC chromatograph, connected to a reverse phase column PHENOMENEX of particle size 5 µm C18(2) 100 Å, LC Column 250 x 4.6 mm with Shimazu SPD10A uv-vis, Japan fluorescence detector (Perkin-Elmer) and LCI-100 computing integrator. Measurements were made by peak area. The mobile phase was combination of Acetonitrile and orthophosphoric acid in the ratio of 70:30 (Filtered through 0.2 µm Millipore filter) at a flow rate of 1ml/min and detection was observed by fluorescence with excitation at 362nm (λ_{ex}). Quantification of aflatoxin was performed by measuring peak areas at their retention times, and comparing them with the relevant standard calibration curve. The identity of AFB_1 was confirmed in all the analyzed samples by injecting the sample extracts sequentially and comparing the peak area ratio with the corresponding standard AFB_1 (Younis M.H. *et al*., 2003).

RESULTS AND DISCUSSION:

MYCOFLORA ASSOCIATED WITH OIL SEEDS, AS OBSERVED BY THE STANDARD BLOTTER METHOD.

The storage fungi like *Aspergillus flavus, A. niger, A. oryzae, A. flaviceps* dominated the mycoflora of groundnut and were recorded in all the samples. Apart from the *Aspergillus* species, *Penicillium* species were also recorded in almost all the samples. *Rhizopus* species and *Mucor* were also commonly encountered. In addition to the storage fungi, field fungi like *Fusarium* species were also observed.

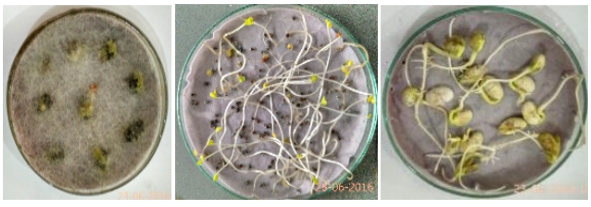
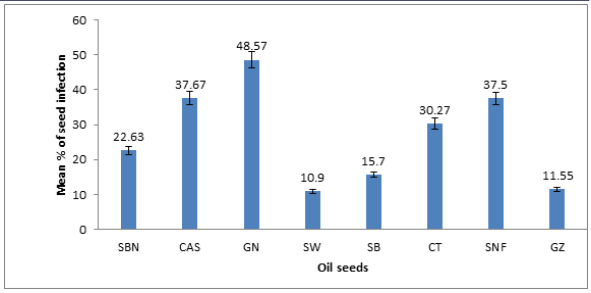


Fig 1.0 Seed borne mycoflora on ground nut, sesame and soya oil seeds

In soyabean the incidence of *A. niger and A. flavus* was more as compared with the other *Aspergillus* species. *Aspergillus oryzae* was always frequent but its frequency was less compared to *A. flavus* or even *A. niger., Penicillium and Rhizopus* species were also frequently recorded. *Fusarium* species was found to be prevalent in three samples. *Trichoderma and Neurospora* species were also found, in three and one samples respectively.

The Mycoflora of castor includes the *Aspergillus* species*, A. flavus, A. versicolor and A. niger* were most frequently observed; but the frequency of *A. niger and A. versicolor* was less than *A.flavus. Phoma and Candida* species were also encountered. The incidence of *Penicillium* species was less while *Rhizopus* species were observed frequently. Among the field fungi *Fusarium and Curvularia* species were encountered. *Trichoderma* and *Neurospora* species were also recorded in many samples, but in lower frequencies.



GN=Groundnut
CA=Castor
SNF=Sunflower
SBN=Soyabean
GT=Guizotia
SB=Sesame black
SW=Sesame white
CT=Cotton

Fig 1.1 Graph showing the incidence of seed borne mycoflora

EXTRACTION AND QUANTIFICATION OF AFB_1

Among 55 isolates, 42 isolates of *Aspergillus flavus* strains are found to be Aflatoxigenic in their nature based on the ability of AFB_1 production by qualitative method of TLC and the results of seed germination and viguor index, indicating that culture filtrates of different isolates were showed non-phytotoxicity by enhancing the growth of seedlings (Table 1.1). From the method of HPLC, the amount of AFB_1 from selected isolates of seed samples was estimated and found to be in the range of 29-35 µg/ml in all the selected seed samples (Fig 1.4). The amount of $AFB_1$was quantified by using below formula.

$$\text{Content of Aflatoxin } B_1 = \frac{\text{sample area}}{\text{standard area}} \times \frac{\text{standard weight}}{\text{sample weight}} \times \frac{\text{sample dilution}}{\text{standard dilution}} \times \text{purity} \times 100$$

Table 1.1 Effect of culture filtrates of Aspergillus flavus isolates and their aflatoxin B1 producing ability:

Sl no.	Isolate no.	% Inhibition of germination	Viguor index	Presence/absence of AFB_1	Rf value
1	GN1	66.6	319.96	-	-
2	GN2*	66.6	-	+++	0.90
3	GN3	66.6	379.96	+	0.68
4	GN4	66.6	226.64	-	-
5	GN5	66.6	-	++	0.77
6	GN6	66.6	166.65	++	0.91
7	GN7	66.6	240.41	++	0.88
8	CA8	100.0	310.00	+++	0.83
9	CA9	100.0	390.00	+	0.87
10	CA10	100.0	360.00	++	0.75
11	CA11	100.0	410.00	+++	0.84
12	CA12*	100.0	340.00	+++	0.92
13	CA13	100.0	280.00	+	0.82
14	CA14	100.0	430.00	+++	0.73
15	SNF15	50.0	-	-	-
16	SNF16	50.0	155.00	+++	0.88
17	SNF17	50.0	160.00	+++	0.81
18	SNF18	50.0	-	-	-
19	SNF19*	50.0	95.00	+++	0.93
20	SNF20	50.0	-	-	-
21	H21	100.00	350.00	-	-
22	H22	100.00	350.00	+++	0.97
23	H23	100.00	320.00	+++	0.84
24	H24	100.00	490.00	-	-
25	D25	100.00	420.00	-	-
26	D26	100.00	460.00	++	0.80
27	D27	100.00	450.00	+	0.90
28	D28	100.00	550.00	+	0.43
29	SB29	100.00	262.00	+	0.81
30	SB30	100.00	181.50	+++	0.80
31	SB31	100.00	560.00	++	0.93
32	SB32	100.00	540.00	+	0.63
33	SB33	100.00	440.00	+	0.34

34	**Sb34**	100.00	300.00	++	0.30
35	**SB35***	100.00	520.00	+++	0.96
36	**SB36**	100.00	240.00	+	0.41
37	**GT37**	100.00	490.00	+++	0.83
38	**GT38**	100.00	390.00	++	0.88
39	**GT39**	100.00	420.00	-	-
40	**GT40**	100.00	380.00	++	0.32
41	**GT41**	100.00	430.00	+++	0.73
42	**GT42**	100.00	580.00	++	0.91
43	**GT43**	100.00	490.00	-	-
44	**M44**	50.00	80.00	++	0.82
45	**M45**	50.00	135.00	+	0.86
46	**M46**	50.00	-	-	-
47	**M47**	50.00	-	-	-
48	**CT48**	83.33	249.99	+	0.82
49	**CT49**	83.33	291.60	+	0.92
50	**CT50**	83.33	458.31	+++	0.85
51	**CT51**	83.33	141.66	+++	0.85
52	**CT52**	83.33	301.48	++	0.81
53	**CT53**	83.33	320.00	++	0.42
54	**CT54**	83.33	269.00	+++	0.96
55	**CT55**	83.33	-	-	-

Vigour index = (Average root length +Average shoot length) x % of germination

+++ = Bright fluorescence
++= Faint fluorescence
- = No fluorescence

GN=Groundnut
CA=Castor
SB=Soyabean
H=Husked sesame
SNF=Sunflower
D= Dehusked sesame
GT=Guizotia
M=Mustard
CT=Cotton

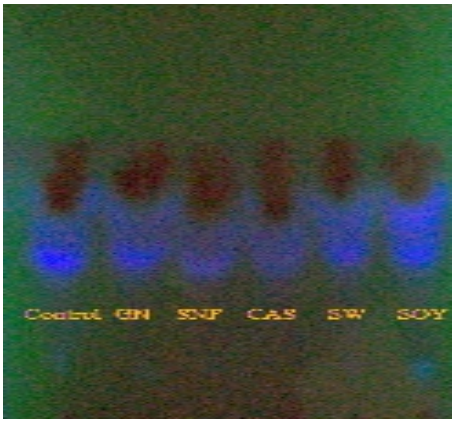


Fig 1.2 TLC plate showing presence of AFB_1 of selected isolates with standard AFB_1 (control) under UV light (365 nm).

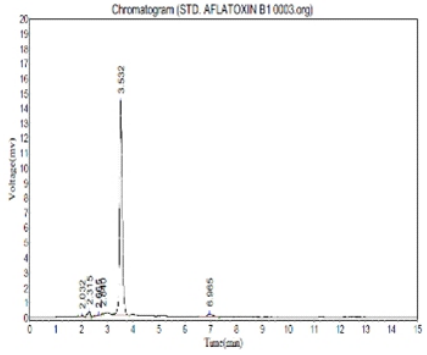


Fig1.3 Chromatogram of Standard AFB_1

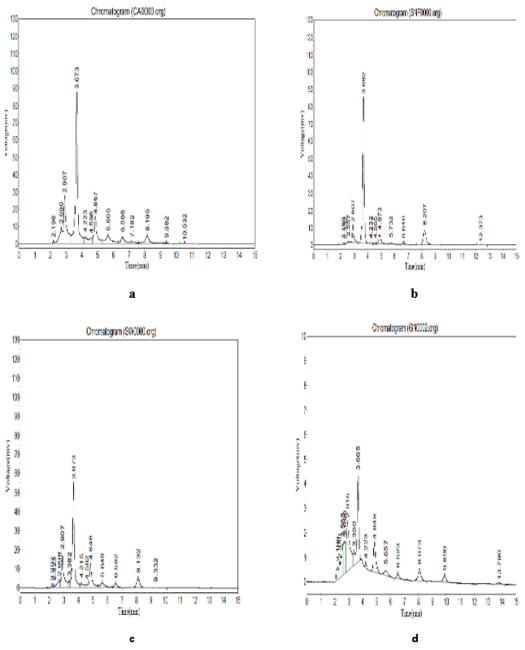


Fig1.4 Chromatogram of oil seed samples a) Castor b) Sunflower c) Sesame d) Groundnut.

CONCLUSION

The present study on **Evaluation of aflatoxin B_1 contamination levels in oil seed samples** is summarized as follows:

The present study involved the screening of seed borne mycoflora of various oil seeds by using standard blotter method, followed by identification of seed borne mycoflora using macroscopic and microscopic methods like morphological and lactophenol cotton blue staining method respectively. The percentage incidence of infection was observed to be higher for the groundnut seed sample showing the mean percentage of infection to be about 48.57% than with all the seed samples. The genus *Aspergillus* was most predominant, with A, niger topping the list followed by *Rhizopus spp*. and the least was *Trichoderma*. In addition to storage fungi, the field fungi *Fusarium* and *curvularia species* were recorded. The bioassay of culture filtrates of various *A. flavus* isolates showed non-phytotoxicity by enhancing the growth of seedlings. Among 55 isolates of *Aspergillus flavus*,42 were found to be aflatoxigenic strains by determinative method of TLC and amount of aflatoxin B_1 in oil seeds is estimated to be in the range of 29-35µg/ml by quantitative analysis of HPLC. This study suggests that proper storage and regular inspection of the quality of oil seeds should be practiced to ensure the safety of animals including human beings.

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