



Mycotoxin Contamination and Induced Biochemical Changes Associated with Selected Medicinal Plants

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Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

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ABSTRACT

Mycotoxin contamination and induced biochemical changes in medicinal plants namely *Azadirachta indica*, *Emblia officinalis*, *Plantago ovata* and *Vitex negundo* collected from different localities of Uttarakhand (India) were investigated. Mycotoxin producing fungi like *A.flavus*, *A.ochraceus*, *F.verticillioides* and *Penicillium citrinum* were recorded. In comparison to other mycotoxigenic fungi, percentage toxigenicity was higher in *Aspergillus flavus*. In case of *E. officinalis*, 32.69% isolates of *A.flavus* were toxigenic and produced aflatoxins up to 21 µg/ml in the liquid media followed by *A. indica* where 22.2% isolates produced aflatoxin in the range of 0.4-13.8 µg/ml. In case of *V. negundo* and *P. ovata* 14.28% and 8% isolates of *A. flavus* elaborated aflatoxin B₁B₂ and aflatoxin B₁ respectively at low concentration. Mycotoxin contamination in stored samples of *E. officinalis* and *V. negundo* showed aflatoxin B₁ and aflatoxin B₁B₂ as natural contaminant. Twenty eight percent (28%) samples of *E. officinalis* exhibited higher concentration of aflatoxins up to 0.98 µg/g whereas in case of *V. negundo* 6% samples were found naturally contaminated with aflatoxin B₁. Alkaloid content of medicinal plant produce understudy was estimated in healthy as well as *A.flavus* infested samples. There was an indication of inhibition in the total alkaloid content due to the toxigenic strain of *A. flavus*. Statistical analysis of the results show a decline in the level of total alkaloid content due to fungal contamination significant at 5% level of significance.

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1. INTRODUCTION

Indian forests have been a rich source of medicinal plant produce and these produce are widely used in the prevention, treatment and cure of disorders and diseases since ancient times. Literature on traditional herbal medicine preparation and survey of products of leading ayurvedic medicine manufacturer indicate use of plant produce in pharmacopoeial preparations. The concern with the quality of these natural products is due to the risk of potential fungal contamination and presence of mycotoxins. Practices used in harvesting, handling, storage, production and distribution make medicinal plants subject to contamination by various fungi, which may be responsible for spoilage and production of mycotoxins.

In spite of the extensive research on the occurrence of mycotoxins in agriculture, horticulture, Tree Borne Oil Seeds (TBOS) and other edible commodities [1-8], very few reports are available on the incidence of toxigenic mycobiota and mycotoxin contamination in medicinal plants and phytotherapeutic compounds [9-14].

In this background, present study was carried out on medicinal plant produce of *Azadirachta indica* (Neem), *Emblica officinalis* (Aonla), *Plantago ovata* (Isabgol) and *Vitex negundo* (Nirgundi) to assess natural contamination of mycotoxins, incidence of mycotoxin producing fungi, and induced biochemical changes.

2. MATERIAL AND METHODS

Fresh samples of fruits/seeds of *Azadirachta indica*, *Emblica officinalis*, *Plantago ovata*, *Vitex negundo* (60 samples) were collected from the forests of Uttarakhand during their harvesting season whereas stored samples (166 samples) were collected from different storage centres and pharmaceutical industries.

2.1 Isolation and Identification of Mycobiota

In order to record the fungal flora associated with fresh and stored samples, blotter test as well as agar plate methods as recommended by the International Seed Testing Association [15] were followed. Isolation of mycobiota was done from the surface sterilized samples to isolate the

internal fungi and unsterilized samples to isolate the surface fungi. For surface sterilization, the samples were soaked in 2% sodium hypochlorite (NaOCl) solution for 10 minutes. Subsequently the seeds, fruits were thoroughly rinsed in sterile distilled water and aseptically placed in Petri dishes containing three layered moistened blotter pads and Potato Dextrose Agar medium. The Petri dishes were then incubated for seven days at $25 \pm 2^\circ\text{C}$ and regularly examined under stereoscopic binocular microscope from third day and developing fungal colonies were recorded, isolated, identified and maintained on PDA. The isolated fungi were identified using research microscope with the help of standard literature and keys and matching the isolates with National Type Culture Collection at Forest Research Institute, Dehradun.

2.2 Screening of Fungal Isolates for Mycotoxin Producing Potential

The aflatoxin producing potentials of *Aspergillus flavus* isolates were tested in SMKY liquid medium [16]. The constituents of the medium were, Yeast extract-7g; Sucrose-200 g; Magnesium Sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)- 0.5 g; Potassium Nitrate (KNO_3)-3 g; Distilled water-1lt. Methods of Schwenk et al. [17] and Davis et al. [18] were followed for testing citrinin and ochratoxin producing abilities of *Penicillium citrinum* and *Aspergillus ochraceus* isolates, respectively. The composition of liquid medium used was, Sucrose-40 g; Yeast extract-20 g; Distilled water-1lt. Zearalenone producing ability of the different isolates of *Fusarium* was tested on moist - rice medium as suggested by Scott et al. [19]. In all the cases mycotoxins were finally extracted with chloroform and the chloroform extract used for qualitative and quantitative detection of mycotoxins.

2.3 Natural Occurrence of Mycotoxins

The fresh and stored samples were extracted chemically for the presence of aflatoxins [20]. Few samples, in which fungi producing other mycotoxins were associated, were extracted by the method of Roberts and Patterson [21]. Samples were powdered in a grinder and 50 g flour was blended with 250 ml methanol: water (60:40,v/v) for 2 minutes at high speed. The extract was filtered through Whatman No. 1 filter paper. 125 ml of this filtrate was then extracted with 30 ml saturated sodium chloride (NaCl)

solution and 50 ml n- hexane in a 250 ml separating funnel for 2 minutes. The lower methanol layer was transferred to another separating funnel. Finally, the lower methanol layer was extracted with 40 ml chloroform. The chloroform layer was drained into 125 ml flask containing 5 g cupric carbonate was allowed to settle and chloroform was decanted through Whatman No. 1 filter paper containing anhydrous sodium sulphate, extract transferred with careful washing to screw capped borosilicate vial and evaporated to dryness at 40°C or under gentle stream of nitrogen, dissolved in 200 µl benzene – acetonitrile (98 + 2), and spotted on TLC.

2.3.1 Qualitative and quantitative estimation of mycotoxin

Qualitative and quantitative estimation of the mycotoxins were carried out using Thin Layer Chromatography (TLC). Silica Gel - G (with 13 % CaSO₄ as binder) was used as stationary phase for the TLC. 50 µl of chloroform extract obtained for mycotoxin screening was spotted on TLC plates. The spotted chromatoplate was developed in the solvent system comprising Toluene: isoamyl alcohol: methanol (90: 32:2, v/v/v) . After developing, the plates were air dried and were observed under long (360 nm) and short (260nm) wavelengths UV-light for the detection of mycotoxins. Chemical confirmation of aflatoxin was done by Trifluoroacetic acid (TFA) as suggested by Stack and Pohland [22]. Presence of Ochratoxin on TLC plates was confirmed with ammonia fumes which changed blue green spot to a deep blue colour [18]. Confirmation of citrinin was done by spraying TLC plates with a freshly prepared mixture of 0.5 ml p- anisaldehyde in 85 ml of methanol containing 10 ml of glacial acetic acid and 5 ml of conc. H₂SO₄ and then by heating the plate at 130°C for 10 minutes. This changed yellow streak of citrinin to yellowish green under long wave UV- light [19]. Zearalenone was also confirmed by spraying TLC plates with acidic p- anisaldehyde solution [19] by which greenish blue fluorescence turned faint brown (in visible light) and faint yellow in long wave UV-light.

Aflatoxin being most potent mycotoxin, the quantitative estimation for the same was carried out. Quantity of aflatoxin was estimated spectrophotometrically [23] with the help of UV-Spectrophotometer.

2.4 Estimation of Total Alkaloid

Total alkaloid was estimated following the methodology of Waldi et al. [24] gm of powdered sample was soaked with 28% ammonium hydroxide solution and little dried up. Subsequently, the sample was soxhleted with a mixture of chloroform: ethanol (3:1, v/v) for 8 hrs. The extract was acidified with N/2 H₂SO₄. The acid extract was collected. The process was repeated thrice for complete extraction of alkaloids. The combined acid extract was made alkaline with dilute NH₄OH. The alkaloids were extracted from alkaline extract with 20 and 15 ml of chloroform. Chloroform was distilled on water bath, solvent was completely dried up and the residue was weighed on monopan balance to calculate the crude alkaloids. The crude alkaloids of each sample were dissolved in 0.5 ml of methanol. 50 µl of the solution was spotted on a TLC plate with the help of a micropipette. A mixture of cyclohexane, chloroform and diethyl amine (5:4:1, v/v/v) were used as solvent system. The air dried chromatoplates were then sprayed with Dragendroff reagent.

Statistical analysis of the results was carried out using SPSS package. Results of control and contaminated samples were tested for their differences by paired t- test using SPSS package.

3. RESULTS

Mycobiota associated with fresh and stored fruits/seed samples of *Azadirachta indica*, *Emblica officinalis*, *Plantago ovata* and *Vitex negundo* was recorded. In case of fresh samples, nine fungi namely *Alternaria alternata*, *Aspergillus flavus*, *A.niger*, *A. ochraceus*, *Cladosporium cladosporioides*, *Curvularia lunata*, *Fusarium avenaceum*, *F. solani* and *Penicillium citrinum* were recorded. Among them, *A. niger* was the most common fungus appearing on all substrates except *E. officinalis*. *Aspergillus flavus* and *A. ochraceus* was recorded *A. indica* and *E. officinalis* whereas *P. citrinum* was detected only on *V. negundo* (Table 1).

All hues of known mycotoxin producing fungi i.e. *A. flavus*, *A. ochraceus*, *F. verticillioides* and *Penicillium citrinum* were recorded on the stored samples. Eight fungi namely *A. flavus*, *A. niger*, *A. ochraceus*, *A. spinulosus*, *F.semitectum*, *F. solani*, *P. citrinum* and *Penicillium* sp. were

Table 1. Percentage (%) incidence of mycobiota associated with fresh samples

| S.N. | Mycobiota | Medicinal plants | | | |
|------|---------------------------|---------------------------|-----------------------------|-----------------------|----------------------|
| | | <i>Azadirachta indica</i> | <i>Embllica officinalis</i> | <i>Plantago ovata</i> | <i>Vitex negundo</i> |
| 1 | <i>A. alternata</i> | - | - | - | 18.5 ± 1.25 |
| 2 | <i>A. flavus</i> | 37.0 ± 1.67 | 9.4 ± 1.4 | - | - |
| 3 | <i>A. niger</i> | 45.2 ± 1.28 | - | 13.5 ± 0.95 | 20.0 ± 0.89 |
| 4 | <i>A. ochraceus</i> | 14.2 ± 1.39 | 47.4 ± 1.98 | - | - |
| 5 | <i>C. cladosporioides</i> | 11.0 ± 1.09 | - | - | - |
| 6 | <i>C. lunata</i> | 12.4 ± 1.07 | - | - | - |
| 7 | <i>F. avenaceum</i> | 15.4 ± 0.92 | 9.75 ± 1.03 | - | - |
| 8 | <i>F. solani</i> | - | 40.5 ± 2.21 | - | 14.8 ± 1.01 |
| 9 | <i>P. citrinum</i> | - | - | - | 14.0 ± 0.95 |

* Value are mean ± deviation of three replicates

recorded on fruit of *E. officinalis*. In case of *P. ovata*, six fungi i.e. *A. flavus*, *A. niger*, *F. verticillioides*, *P. citrinum*, *Penicillium* sp. and *R. stolonifer* were found associated with the samples. On the other hand, seed mycobiota of *A. indica* comprised of *A. flavus*, *A. niger*, *Cladosporium cladosporioides*, *C. lunata*, *F. verticillioides*, *F. solani*, *Penicillium citrinum* and *Mucor hiemalis*, and only four fungi *A. flavus*, *A. niger*, *A. ochraceus* and *R. stolonifer* were recorded on *V. negundo*.

3.1 Mycotoxin Producing Potential of Fungi

Aflatoxins were the most common mycotoxins elaborated by *A. flavus* isolated from samples of *Azadirachta indica*, *Embllica officinalis*, *Plantago ovata*, *Vitex negundo*. In case of *Azadirachta indica* 14 isolates of *A. flavus* out of 63 screened produced aflatoxin. Aflatoxin B₁ was produced by 9 isolates whereas aflatoxin B₁B₂ were produced by 5 isolates. In *E. officinalis* 17 out of 52 isolates produced aflatoxin. Out of which 9 produced aflatoxin B₁ and 8 isolates produced aflatoxin B₁B₂. In case of *P. ovata*, only 4 out of 25 isolates produced aflatoxin B₁ in traces. In case of *V. negundo*, 3 isolates produced aflatoxinB₁ out of 32 and 21 isolates screened respectively. The amount of aflatoxins produced by the toxigenic isolates of *A. flavus* was in the range of 0.4 - 13.8 µg/ml in *Azadirachta indica*, 0.4-21.0 µg/ml in *E. officinalis* and 0.23 - 4.7 µg/ml in *V. negundo*, however, in case of *P. ovata*, aflatoxins were present in traces only.

The percentage of toxigenic isolates was comparatively low in other mycotoxin producing fungi isolated from all the samples. In case of *A. ochraceus*, 7 out of 37 from *E. officinalis*, and 3 out of 15 from *V. negundo* elaborated Ochratoxin A in liquid medium. Zearalenone was produced

by 3 isolates of *F. verticillioides* isolated from *Azadirachta indica* (out of 26), 3 from *P. ovata* (out of 22) whereas Citrinin was elaborated by 21.8% isolates in *A. indica*, 14.81% in case of *E. officinalis*, 22.22% in *P. ovata* (Table 2).

3.2 Mycotoxin Contamination of Medicinal Plant Produce

Natural occurrence of mycotoxin contamination in fresh and stored samples was analyzed by following the standard procedures. Mycotoxin contamination in stored samples of *E. officinalis* and *V. negundo* showed aflatoxin B₁ as natural contaminant. Twenty eight percent (28%) samples of *E. officinalis* exhibited higher concentration of aflatoxins, up to 0.98 µg/g whereas in case of *V. negundo* 6% samples were found naturally contaminated with aflatoxin B₁ and mycotoxins were not detected in case of *A. indica* and *P. ovata* (Table 3).

3.3 Changes in the Alkaloid Content of Medicinal Plant Produce

Changes in the level of alkaloid content in medicinal seeds/ fruits due to infestation of toxigenic strain of *A. flavus* have been carried out. *A. flavus* caused considerable changes total alkaloid content of the medicinal seeds/ fruits under study during their infestation (Table 4). There was an indication of inhibition in the total alkaloid content due to infestation with toxigenic strain of *A. flavus*. The percent amount of alkaloid in healthy medicinal seeds/fruits was 0.065%, 0.020%, 0.004%, 0.042% in, *A. indica*, *P. ovata*, *E. officinalis*, and *V. negundo* respectively. On the other hand, in infested fruits/seeds of *A. indica*, *P. ovata*, *E. officinalis*, and *V. negundo*, the percent (g/100 g) amount of total alkaloid recorded was 0.062%, 0.020%, 0.003%, and 0.039% respectively. Statistical

Table 2. Screening of mycobiota for mycotoxin producing potential

| Substrate | Mycobiota | No. of isolates screened | No. of toxigenic isolates | % toxigenicity | Mycotoxin produced | Range of toxin production ($\mu\text{g/ml}$) |
|-----------------------|---------------------------|--------------------------|---------------------------|----------------|--|--|
| <i>A. indica</i> | <i>A. flavus</i> | 63 | 14 | 22.22 | Aflatoxins | 0.4-13.8 |
| | | | 9 | 14.28 | AflatoxinB ₁ | |
| | | | 5 | 7.93 | AflatoxinB ₁ B ₂ | |
| | <i>F. verticillioides</i> | 26 | 3 | 11.53 | Zearalenone | - |
| | <i>P. citrinum</i> | 32 | 7 | 21.87 | Citrinin | - |
| <i>E. officinalis</i> | <i>A. flavus</i> | 52 | 17 | 32.69 | Aflatoxins | 0.4-21 |
| | | | 9 | 17.30 | AflatoxinB ₁ | |
| | | | 8 | 15.38 | AflatoxinB ₁ B ₂ | |
| | <i>A. ochraceus</i> | 37 | 7 | 18.91 | Ochratoxin | - |
| | <i>P. citrinum</i> | 27 | 4 | 14.81 | Citrinin | - |
| <i>P. ovata</i> | <i>A. flavus</i> | 25 | 2 | 8.00 | AflatoxinB ₁ | Traces |
| | <i>F. verticillioides</i> | 22 | 3 | 13.63 | Zearalenone | - |
| | <i>P. citrinum</i> | 18 | 4 | 22.22 | Citrinin | - |
| <i>V. negundo</i> | <i>A. flavus</i> | 21 | 3 | 14.28 | AflatoxinB ₁ B ₂ | Traces |
| | <i>A. ochraceus</i> | 15 | 3 | 20.00 | Ochratoxin | - |

Table 3. Natural occurrence of aflatoxins in medicinal plant produce

| S.N. | Substrate | % of contaminated sample | Mycotoxin produced | | | Range of mycotoxin ($\mu\text{g/g}$) |
|------|-----------------------------|--------------------------|-------------------------|--|--|--|
| | | | AflatoxinB ₁ | AflatoxinB ₁ B ₂ | Aflatoxin B ₁ B ₂ G ₁ | |
| 1 | <i>Azadirachta indica</i> | Nil | - | - | - | - |
| 2 | <i>Embllica officinalis</i> | 28 | 15 | 6 | 2 | 0.14-0.98 |
| 3 | <i>Plantago ovata</i> | Nil | - | - | - | - |
| 4 | <i>Vitex negundo</i> | 6 | 1 | - | - | traces |

Table 4. Changes in total alkaloid due to fungal contamination

| S.N. | Substrate | | Total alkaloid (g/100g) | T- value | Significance CD (0.05) |
|------|-----------------------------|---|-------------------------|----------|------------------------|
| | | | Mean \pm S.E. | | |
| 1 | <i>Azadirachta indica</i> | C | 0.065 \pm 0.001 | 4.630 | 0.044 |
| | | I | 0.062 \pm 0.000 | | |
| 2 | <i>Plantago ovata</i> | C | 0.021 \pm 0.000 | 2.60 | 0.122 |
| | | I | 0.020 \pm 0.000 | | |
| 3 | <i>Embllica officinalis</i> | C | 0.004 \pm 0.000 | 15.24 | 0.000 |
| | | I | 0.003 \pm 0.000 | | |
| 4 | <i>Vitex negundo</i> | C | 0.042 \pm 0.000 | 29.00 | 0.001 |
| | | i | 0.039 \pm 0.000 | | |

Note: C-control, I- infested

analysis of the results showed that a decline in the level of total alkaloid content due to fungal infestation in the substrates is significant at 5% level of significance.

4. DISCUSSION

The results of the present investigation indicate that a large number of fungi were associated with the medicinal plant produce of *Azadirachta indica*, *Embllica officinalis*, *Plantago ovata* and *Vitex negundo*. Association of fungi as well as their incidence are governed by the nature of the substrates, methods of storage and prevailing environmental conditions. Earlier reports also indicate varied pattern of fungal incidence with *E. officinalis* [25,13] and *Azadirachta indica* [26].

Four mycotoxigenic fungi viz. *Aspergillus flavus*, *A. ochraceus*, *Fusarium verticillioides* and *Penicillium citrinum* were commonly associated with the stored samples, however their incidence in fresh samples was comparatively low. The range of toxin production by these fungi in liquid medium varied with the type of the substrate. It was also noted that aflatoxin B₁ was produced by majority of the toxigenic isolates of *A. flavus*, however, the frequency of aflatoxins other than B₁ was comparatively low.

The results of the natural occurrence of mycotoxin contamination of the stored samples under study indicate that aflatoxin B₁ was detected in both fresh and stored samples of *E. officinalis* and the concentration of aflatoxin B₁ in

the stored samples was quite high i.e. up 0.98 µg/g in stored samples. *V. negundo* also exhibited presence of aflatoxins as natural contaminant. In case of *P. ovata* and *A. indica* mycotoxins were not detected as natural contaminant although 20% *A. flavus* isolates from *A. indica* were toxigenic and produced aflatoxins up to 13.8 µg/ml in the liquid medium. It is well established that aflatoxins can be produced only under particular environmental conditions. The actual growth of aflatoxigenic fungi on any substrate does not necessarily mean that aflatoxins are also present. Moisture, temperature and insect or other injury as well as the *A. flavus* isolate, the substrate and the environmental conditions are particularly important factors in determining whether aflatoxins are actually produced as the fungus grows within the fruits/ seeds or grains [27,28].

The variation in natural occurrence of mycotoxins may be due to differences in their moisture contents and constitutional make up. Earlier reports also indicate association of large number of mycobiota and mycotoxins in edible and medicinal fruits/seeds of forest origin [3-8,13]. There is an indication of decline in the level of total alkaloid in infested substrates. Inhibition in the level of total alkaloid content might be due to their utilization or degradation into simpler forms. Dutta and Roy [29] worked on deterioration in total alkaloid content of *Strychnos nux-vomica* by some fungi and reported that *A. flavus* and *P. citrinum* significantly inhibited the level of alkaloid content. Bilgrami et al. [30] also reported significant biochemical changes in dry fruits during aflatoxin elaboration by *A. flavus*.

5. CONCLUSION

Presence of mycotoxin producing fungi and high concentration of aflatoxins in the medicinal plant samples is a matter of great concern as these raw materials are commonly used for the preparation of herbal drugs. Due to fungal infection, quality of the product is deteriorated and use of aflatoxin contaminated herbal drugs may cause severe health hazard. Therefore, it is necessary for the Indian herbal industries to set up appropriate standards for screening the crude herbal drugs and medicinal plant produce to be used as raw material in the pharmacopoeial industry.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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