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Full Length Research Paper

Detection of zearalenone, zearalenol and deoxynivalenol from medicinally important dried rhizomes and root tubers

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An investigation was carried to determine the natural contamination of three important fusarial mycotoxins; zearalenone (ZEN), zearalenol (ZOL) and deoxynivalenol (DON) in medicinally important dried rhizomes of *Acorus calamus, Bergenia ciliata, Curcuma longa, Zingiber officinale* and root tubers of *Pueraria tuberosa*. A total of 130 market samples were investigated for these mycotoxins. Contamination of ZEN was detected in 13.07 percent samples and that of DON was detected in 6.92 percent samples. However, only one sample of *P. tuberosa* was found to be contaminated with ZOL. Qualitative analysis with HPLC revealed very high concentrations of the detected fusarial toxins in the positive samples.

Key words: Medicinal plants, rhizomes, root tubers, zearalenone, zearalenol, deoxynivalenol.

INTRODUCTION

India has a rich wealth of more than 3000 medicinal plants (Shankar and Balasubramanian, 2001). Among these, a large number of plants have medicinally important rhizomes and root tubers. These medicinal plants are usually collected from wild sources, occasionally cultivated, sun-dried, and stored in traditional gunny bags or heaped as such on the ground till they are marketed or processed. Due to unscientific methods of harvesting, storage and cultivation, these dried medicinal plants are prone to deteriorating effects of storage microorganisms, especially the xerophilic fungal species, which flourish very well on them and may deteriorate their quality to such an extent that their therapeutic potential is considerably lost. The presence of rhizomes and root tubers below the soil line makes them more vulnerable to the huge diversity of soil borne opportunistic microbes especially the fusarial species. Some of these fusarial species may be toxigenic and produce mycotoxins during harvesting and storage. Further, it is anticipated that fleshy, underground plant parts may be more vulnerable to mycotoxin contamination, as they are rich in mineral salts and carbohydrates, including simple sugars like glucose, sucrose and fructose, which are known to stimulate mycotoxin production (Jarvis, 1971).

In view of this, an investigation was undertaken to verify contamination of fusarial toxins; zearalenone (ZEN), zearalenol (ZOL) and deoxynivalenol (DON) from the stored undergrounds of *Acorus calamus*, *Bergenia ciliata*, *Curcuma longa*, *Zingiber officinale* and *Pueraria tuberosa*. All these plants have undergrounds that are popularly used as ingredients in a large number of herbal cosmetics and 'Ayurvedic' formulations.

MATERIALS AND METHODS

Extraction of mycotoxins from dried underground medicinal plant parts

Market samples of medicinal plants were analyzed for zearalenone (ZEN), zearalenol (ZOL) and deoxynivalenol (DON) contamination by using modified multimycotoxin screening method developed by Roberts and Patterson (1975). In this method, 25 g of finely ground sample was taken in an Erlenmeyer flask (250 ml capacity) and 100 ml mixture of acetonitrile and 4% potassium chloride (90:10, v/v) was added to it. Extraction was done by horizontal shaking for 30 min. Thereafter, extract was filtered through Whatman no. 41 filter paper and the filtrate was defatted with 50 ml iso-octane in a sepa-

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rating funnel (250 ml capacity). When the layers separated clearly, upper iso-octane layer was discarded and the lower acetonitrile layer was re-extracted with 50 ml iso-octane. The upper lipid containing layer was discared and 12.5 ml distilled water was added to the lower acetonitrile layer. Mycotoxins were extracted thrice by using 20 ml chloroform each time. Lower chloroform acetonitrile layer was collected in a conical flask and passed through anhydrous sodium sulphate bed and evaporated to dryness on a water bath. In case of *A. calamus, B. ciliata, Z. officinale* and *P. tuberosa* samples, the residue was dissolved in 1 ml of chloroform and stored in small screw cap vials for qualitative and quantitative estimation of fusarial toxins.

In case of *Curcuma longa* samples, pigment separation is required, for which dried residue was re-dissolved in 1.25 ml of acetonitrile and transferred into a dialysis sac made from dialysis tubing, which was thoroughly washed with distilled water. The dialysis sac was equilibrated against 25 ml of acetone water mixture (30:70, v/v) in a stoppered conical flask for 16 h by gently shaking on a wrist action shaker. To improve recovery of fusarial toxins, dialysis sac was again equilibrated for 6 h against 25 ml of acetone water mixture (30:70, v/v). Aqueous acetone dilysates were combined and extracted with 15 ml of chloroform three times in a separating funnel. Methanol (3 ml) was added to it for the clear separation of layers. Chloroform extracts were combined, passed through anhydrous sodium sulphate bed and dried on a water bath. Dried residue was dissolved in 1 ml of chloroform and stored in a small screw cap vial for qualitative and quantitative analysis of ZEN, ZOL and DON.

Qualitative estimation of fusarial toxins

For detection of ZEN, ZOL and DON, aliquots of sample extract (50 µl) were spotted on TLC plates along with the standards and developed in a solvent system consisting of toluene : ethyl acetate : formic acid (6:3:1, v/v/v). After drying, plates were observed under long wave UV light. ZEN spots were located as blue green fluorescent spots; ZOL spots were located as light blue fluorescent spots and that of DON as sky blue spots. Confirmation was done by spraying the plates with freshly prepared saturated solution of aluminium chloride in 95% ethanol and then heating it at 120°C for 10 min. The spots of ZEN, ZOL and DON became brighter in appearance.

Quantitative estimation of fusarial toxins

Quantitative analysis of ZEN, ZOL and DON was done through high performance liquid chromatography. The analytical equipment for HPLC (CLASS-LC10 SHIMADZU) consisted of a liquid chromatographic pump LC-10AT, an auto-injection system SIL – 10A with a 50 µl sample loop, and a variable wavelength absorbance UV – VIS detector SPD – 10 set at 365 nm. The analytical column was CLC – ODS (4.6 x 250 mm), filled with ODS (M), RP-18 material, 5 µm particle size (Merck).

Analysis of ZEN was done by using modified method of Scudamore and Patel (2000). For this, a variable wavelength absorbance fluorescent detector set at 274 nm excitation and 440 nm emission was used. The mobile phase consisted of acetonitrile : water (55:45, v/v) and was used at a flow rate of 0.5 ml/min. Injection volume for extract solution was 1, 2 or 5 µl for different samples. Analysis was performed at room temperature (25 - 30°C) and quantification of ZEN was done by comparison of the retention time (7.2 min) and peak area observed in the ZEN standard with those observed for samples.

For analysis of ZOL, a previously published method of James et al. (1982) was modified and used. By using a variable wavelength absorbance UV detector set at 236 nm estimation of ZOL was done. The mobile phase consisted of methanol : water (75:25, v/v) and was used at a flow rate of 1 ml/min. Injection volume for extract solution was 2 µl. Analysis was performed at room temperature (25 - 30°C) and quantification was done by comparison of the retention time (5.0 min) and peak area observed in the ZOL standard with that observed in samples.

Quantitative analysis of deoxynivalenol (DON) was done by using a modified method of Golinski et al. (1996), and for this a variable wavelength absorbance UV detector set at 229 nm was used. The mobile phase consisted of methanol : water (85:15) at a flow rate of 1 ml/min and the retention time was 2.95 min. Injection volume for extract solution was 2, 5 and 10 µl for different samples. Analysis was performed at room temperature (25 - 30°C) and quantification of DON was done by comparing the retention time and peak area observed in the DON standard with that observed in samples.

RESULTS

Detection of zearalenone (ZEN)

Zearalenone was detected as an important contaminant in all the test plants. A total of 130 dried samples of undergrounds of the selected plants were screened for this mycotoxin and 13.07 percent were found to be positive. Quantitative analysis by HPLC showed that the range of contamination in the ZEN positive samples varied between 0.52 and 14.51 µg/g (Table 1). There is a single report of ZEN contamination from Indian undergrounds by Roy and Chourasia (1990). Recently ZEN contamination and its estrogenic activity were reported from root extracts of American and Asian ginseng (Gray et al., 2004).

Detection of zearalenol (ZOL)

Among the investigated market samples of undergrounds, only one sample of *P. tuberosa* was found to be positive for ZOL (Table 1). Quantitative estimation by HPLC showed that the level of contamination in this sample was very high (1.28 µg/g). ZOL contamination has been reported from some medicinally important dried leaf and fruit samples collected from Jammu and Kashmir State of India (Sharma, 2005).

Detection of deoxynivalenol (DON)

All the investigated plants were positive for this mycotoxin and a total of 6.92 percent samples were contaminated (Table 1). In case of *P. tuberosa,* 7.69 percent samples were positive with contamination level upto 6.18 µg/g. In case of *A. calamus, B. ciliata* and *C. longa,* only one sample from each plant was contaminated and the levels of contamination detected were 7.39, 1.19 and 14.45 µg/g, respectively (Tables 1). So far, deoxynivalenol contamination has been reported only from medicinally important dried fruits of *Phyllanthus emblica* (Sharma, 2005).

Table 1. Zearalenone (ZEN), zearalenol (ZOL) and deoxynivalenol (DON) contamination in market samples of medicinally important dried rhizomes and root tubers ((µg/ g).

Sample	*Acorus calamus*			*Bergenia ciliata*			*Curcuma longa*			*Zingiber officinale*			*Pueraria tuberosa*		
	ZEN	**ZOL**	**DON**	**ZEN**	**ZOL**	**DON**	**ZEN**	**ZOL**	**DON**	**ZEN**	**ZOL**	**DON**	**ZEN**	**ZOL**	**DON**
1	-	-	-	-	-	-	-	-	-	-	-	-	1.14	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	1.75	-	-	-	-	-	-	-	-	-	-	-
4	0.93	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	4.85	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	8.41	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	2.32	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	3.67	-	-	-	-	-	-	-	-
11	1.49	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	1.55	-	-	-	-	-	-	-	-	-	-	-	-	-	5.60
14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	2.04	-	7.39	-	-	-	1.17	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17	0.52	-	-	-	-	-	0.64	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	13.44	-	10.35	-	-	6.18
19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21	5.42	-	-	-	-	-	-	-	-	14.51	-	-	-	-	-
22	-	-	-	-	-	1.19	5.39	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-	-	7.39	-	-	-
24	-	-	-	-	-	-	0.64	-	14.45	-	-	5.00	-	-	-
25	-	-	-				-	-	-	-	-	-	-	-	-
26	-	-	-				-	-	-	-	-	-	-	-	-
27	-	-	-				-	-	-	-	-	-	5.80	1.28	-
Total samples	26	26	26	24	24	24	27	27	27	27	27	27	26	26	26
Positive samples (%)	26.92	0.00	3.84	4.16	0.00	4.16	18.51	0.00	3.70	7.40	0.00	14.81	7.69	3.84	7.69

- = Not detected.

DISCUSSION

During the present investigation, very high levels of ZEN were detected from dried rhizomes of *C. longa* (0.64-5.39 µg/g), which are popularly used as tonic and ant-periodic after childbirth in all parts of India. In view of the oestrogenic activities of ZEN, its detection in very high concentrations from the *C. longa* may prove very dangerous. Instead of curing, it can produce adverse effects in the female reproductive system. Earlier, ZEN produced by some *Fusarium* species isolated from medicinal plants has been found to be cytotoxic to mammalian cells (Abeywickrama and Bean, 1992). Recently, oestrogenic effects caused by use of ginseng in some postmenopausal woman are suggested to be due to zearalenone contamination of these products (Gray et al., 2004). Oestrogenic activities of ZEN have been reported from other animal species also and may result in severe reproductive and infertility problems (Gaumy et al., 2001; Herrman and Trigo-Stockli, 2002). In children, it has been implicated in several incidents of precocious pubertal changes (Falkay et al., 1993). International Agency for Research on Cancer (IARC, 1993) evaluated the carcinogenicity of zearalenone and found it to be a possible human carcinogen. For this oestrogenic compound, limits between 60 and 200 µg/g in raw and finished products have been set in several countries (FAO, 1997). In view of the reported toxicity, detection of very high amounts of ZEN in the underground parts of the selected medicinal plants is of great concern.

Detection of very high amount of ZOL in a sample of *P. tuberosa* suggests that dried root tubers are good substrates for ZOL production. Absence of this mycotoxin from underground parts of other investigated plants suggests that probably they are not favourable substrates for ZOL production. Zearalenol is also known to show three to four times more severe oestrogenic properties than zearalenone (Bottalico et al., 1985).

Detection of DON in the dried rhizomes and root tubers of selected plants is very dangerous as instead of curing diseases it can affect the immune system of consumers and make them susceptible to microbial diseases. DON is known to have a variety of immunological effects in laboratory animals that lead to increased susceptibility to microbial diseases (Pestka and Bondy, 1994). Earlier, DON was also implicated in large-scale human toxicoses in Kashmir valley of North India (Bhat et al., 1989) although it was due to wheat consumption. The tolerance level of DON has been fixed by the U. S. Food and Drug Administration at 2 µg/Kg in finished wheat products intended for human consumption (Ueno, 1987).

Detection of three most important fusarial toxins from the investigated underground plant samples suggests that dried undergrounds are quite susceptible to fusarial toxin formation. It is possible that these toxins may interfere with the therapeutic potential of the healing underground plant parts and their ingestion may lead to deleterious health effects. Therefore, modern storage practices that improve the quality and decrease the probability of fusarial toxin contamination from the healing underground medicinal plant parts needs to be implemented.

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