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Periodic Research

Detection of Mycoflora and Nephrotoxic Mycotoxin OTA from Kidney Curative Rhizomes of *Bergenia ciliata* (How.) Sternb

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Abstract

Bergenia ciliata (How.) Sternb. is a medicinally important perennial herb used as astringent, diuretic and tonic. It is also reported to cure fevers, diarrhoea, boils ophthalmia and cough. It is also used to dissolve kidney and bladder stones and forms an important ingredient in various kidney curative herbal drugs.

However, due to unscientific methods of collection, drying and storage these dried medicinal rhizomes 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 .Keeping in view this, a study was conducted to determine the mycobial load of these medicinal rhizomes and to determine the presence of nephrotoxic mycotoxin in this kidney curative medicinal plant. A total of 40 fungal species belonging to14 genera were recovered from the surface of underground parts of the selected medicinal plants. Analysis of rhizome samples for nephrotoxic mycotoxin OTA revealed that only one sample of *Bergenia ciliata* was positive out of 24 samples. The concentration of this toxin in the contaminated sample was 2.37 µg/g , which is very high, suggesting that these rhizomes are good substrates for OTA production. Further presence of this nephrotoxin mycotoxin in kidney curative drugs is alarming as instead of curing, it can have adverse effect on the health of patients.

Keywords: Medicinal Plants, Rhizomes, Ochratoxin A, Nephrotoxins

Introduction

Plants are medicinally important due to the presence of a variety of chemical substances such as essential oils, fatty oils, glycosides, resins, gums, mucilages, tannins, steroids and alkaloids, known as 'active principles'. The active principles of the medicinal plants are usually present in the seeds and underground storage organs (rhizomes, tubers, bulbs, corms, etc.) and to a lesser extent in the leaves, bark, wood and other plant parts. It is estimated that approximately 24-45 percent of the medicinal plants have the active principle in the underground storage organs.

Our state of Jammu and Kashmir, which is situated in the subtropical latitudes and surrounded by North-Western Himalayas, is very rich in plant wealth. Since early times, this plant wealth has been observed and scrutinized by different ethnic tribes of the state like Gujjars, Bakerwals, Paharis, Dards, Gaddies, Hanjis, Bishtis, Baltis, Bots, Purikpas, Drokpas, etc., for developing their own healing systems. More than 350 plant species from Jammu and Kashmir are found to possess medicinal value (Kaul, 1997). Among these, approximately 34 percent of the plants have medicinally important underground parts, which are commonly used in the preparation of varied types of medicinal formulations (Vidyarthi, 2003).

Bergenia ciliata (How.) Sternb. is a rhizomatic perennial herb growing on rocky and shady places. Dried rhizomes of this plant are used as astringent, diuretic and tonic. It is also reported to cure fevers, diarrhoea and cough. Paste of rhizomes is used to cure wounds, boils and ophthalmia. Most importantly it is used to dissolve kidney and bladder stones.

Aim of Study

This plant grows wild in our state of Jammu and Kashmir at higher altitudes. Medicinal rhizomes are collected from wild sources,

sun dried and stored in traditional warehouses till further use. However, due to unscientific methods of harvesting, drying and storage these medicinal rhizomes get infested with xerophytic fungi species. It is anticipated that because of their presence below the soil line, these plant parts are even vulnerable to the huge diversity of soil borne opportunistic microbes. Moreover, being rich in carbohydrates and other nutrients, they may form an excellent substrate for further proliferation of fungal species during storage. These carbohydrate rich, dried underground plant parts also have the ability to readily absorb moisture during rainy seasons and may thus attract some more groups of spoilage fungi. Some of the fungal species belonging to Aspergillus, Penicillium and Fusarium are even known to produce toxic secondary metabolites referred to as 'mycotoxins' Some of the mycotoxins of greater concern are aflatoxins, ochratoxins, citrinin, fumonisins, zearalenone, trichothecenes and sterigmatocystin (Pohland and Wood, 1987; Wood, 1992). Among these mycotoxins Ochratoxin A has been associated with nephropathy in livestock animals (Dalvi and Salunkhe, 1990; Kuiper-Goodman and Grant, 1993). In addition, it has been associated with Balkan endemic nephropathy and urogenital tract tumors in animals and possibly in humans (Plestina, 1992; Ceovic *et al*., 1992; Ferrufino *et al*., 2000).pre All these issues prompted the need to investigate the mycobial load and mycotoxin contamination associated with these kidney curative rhizomes.

Materials and Methods

Collection of Samples

Dried market samples of selected plant parts were collected in pre-sterilized polythene bags from wholesale and retail shops of nine districts of Jammu and Kashmir viz., Jammu, Kathua, Udhampur, Poonch, Rajouri, Doda, Anantnag, Srinagar, and Leh. The sample bags were brought to the laboratory, sealed over flame to avoid external contamination and kept in the refrigerator at 5-7°C to prevent undesirable changes till further studies were conducted.

Recovery of Mycoflora Associated With Market Samples of Medicinal Plants

Mycobial load of market samples was recovered by using surface washing technique. For recovery of maximum number of fungal species, three different media –modified Czapek's Dox Agar (CDA), Dichloran 18% Glycerol Agar (DG-18) and Malt Salt Agar (MSA) were used, and for each medium five replicates were maintained.The medium was poured by making gentle rotational movement of Petri-plates so as to ensure uniform spreading of the sample. These Petri-plates were incubated upside down at 28$\pm$2ºC for 7 days. After incubation, the number of colony-forming units (CFU) was calculated. Relative abundance of each fungal species was calculated by the formulae given below:

Relative Abundance % =Total number of colonies of a particular fungal species in the all replicates / Total number of fungal colonies in all the replicates x100

Extraction of Ochratoxin A

Samples of dried underground parts of *Bergenia* 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 minutes.Thereafter, extract was filtered through Whatman no. 41 filter paper and the filtrate was defatted with 50 ml iso-octane in a separating 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. Discarded the upper lipid containing layer and added 12.5 ml distilled water 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 drained through Whatman no. 41 filter paper having a bed of anhydrous sodium sulphate. The extract was collected in a beaker and marked as extract I. For the complete recovery of this acidic mycotoxin the aqueous layer left in the separating funnel was acidified with 1 ml of 1.0 N HCl and extraction was done from it thrice by using 10 ml chloroform each time. Lower chloroform layers were combined, passed through anhydrous sodium sulphate bed, collected in a beaker and marked as extract II. Extracts I and II were combined and then evaporated to dryness on a water bath. The residue was dissolved in 1 ml of chloroform and stored in small screw cap vials for qualitative and quantitative estimation of OTA

Estimation of Ochratoxin A

For detection of ochratoxin A (OTA), known amount of sample extracts (50 µl) were spotted on the activated TLC plates along with the OTA standard. Plates were developed in a solvent system consisting of toluene: ethyl acetate: 90% formic acid (50: 40: 10 v/v). Spots of OTA were located under long wave UV light after comparing the fluorescence colour and Rf with the standard spots. Confirmation of OTA was done by spraying the plate with saturated solution of $AlCl_3$ in 90% ethanol, which changes blue-green fluorescent spot to deep blue-green colour.

Quantitative estimation of OTA was done by modifying the HPLC method of Vrabcheva *et al*. (2000). The mobile phase consisted of methanol: 0.05% aqueous phosphoric acid (65: 35 v/v), at flow rate of 1.25 ml/minute. Injection volume for extract solution was 5 µl and analysis was performed at room temperature (25-30°C).

Fluorescence detector RF – 10 AXL set at 333 nm excitation and 470 nm emission was used and quantification of OTA was done by comparison of the retention time (8.0 minutes) and peak area observed in the OTA standard with those observed in the samples.

Results and Discussion

Association of Mycoflora

Bergenia ciliata (Pashanbhed) is a rhizomatous herb growing on bare rocks at high altitudes. It is an important medicinal plant whose rhizomes are used for dissolving kidney and bladder stones.

Market samples of *Bergenia ciliata* were collected from nine districts of Jammu and Kashmir and screened for their mycobial load. A total of 40 fungal species belonging to 14 genera were recovered from their surface (Table 1).Deuteromycetes were represented by 30 species,species. Among the

recovered fungal species, 6 were dominant, 1 was fairly dominant, 9 were sub-dominant and 24 were of rare occurrence. Maximum number of fungal species were recovered from samples of Kathua and Poonch districts (17 each), followed in decreasing order by Doda (16), Jammu, Rajouri and Srinagar (15 each), Udhampur and Anantnag (9 each) and Leh (8).

Table1.Table Showing Detected Surface Mycoflora of *Bergenia Ciliata* Rhizomes

S.No.	Fungal Species
	ZYGOMYCETES
1.	Absidia corymbifera
2.	Rhizopus stolonifer
3.	R. oryzae
4.	Syncephalastrum racemosum
	ASCOMYCETES
5.	Chaetomium globosum
6.	Emericella nidulans
7.	E. quadrilineata
8.	Eurotium amstelodami
9.	E. chevalieri
10.	E. rubrum
	DEUTEROMYCETES
11.	Alternaria altertnata
12.	Aspergillus awamori
13.	A. candidus
14.	A. deflectus
15.	A. flavus
16.	A. fumigatus
17.	A. niger
18.	A. niveus
19.	A. ochraceus
20.	A. puniceus
21.	A. sydowii
22.	A. tamarii
23.	A. terreus
24.	A. ustus
25.	A versicolor
23.	A. viridi-nutans
27.	Cladosporium oxyporums
28.	C. sphaerospermum
29.	Curvularia pallescens
30.	Drechslera australiensis
31.	Paeliomyces herbarum
32.	P. variotii
33.	Penicillium brevicompactum
34.	P. citrinum
35.	P. corylophilum
36.	P. expansum
37.	P. funiculosum
38.	P. glabrum
39.	P. waksmanii
40.	Scopulariopsis candida

Among the recovered fungal species, percentage abundance of Aspergillus awamorii was maximum (2.61 74.42), followed by Penicillium glabrum (0.00 – 70.89), Aspergillus flavus (0.37 – 65.62), Aspergillus puniceus (3.77 – 61.36), Eurotium chevalieri (1.00 – 55.33), Aspergillus niger (1.72 – 50.40), Penicillum corylophilum (7.69 – 42.01), Aspergillus viridi-nutans (1.60 – 37.75), Emericella quadrilineata (0.74 – 36.00), Emericella nidulans (0.57 – 32.40) , Aspergillus fumigatus (1.92 – 32.07) , Penicillum citrinum (3.00-30.76), Aspergillus tamarii (1.13-27.83) and A. sydowii (0.51-20.80). Differences in mycobiota population of crude drug samples may be attributed to diverse factors operating in the storage systems.

During the present investigation, Emericella nidulans was recovered from Bergenia ciliata samples of all the nine districts, and Aspergillus and Penicillium species Prevalence and dominance of Aspergillus species, Penicillium species and their perfect states has also been reported by Misra and Bhargava (1976), Roy and Chourasia (1990a), Aziz et al. (1998), Sharma (2005) and Bugno et al. (2006) while investigating mycobial load of other medicinal plants.

Detection of Ochratoxin A

Ochratoxin A is an immunosuppressive, teratogenic, genotoxic, mutagenic and carcinogenic fungal metabolite produced by different species of Aspergillus and Penicillium.

Analysis of rhizome samples for nephrotoxic mycotoxin OTA revealed that only one sample of Bergenia ciliata was positive out of total 24 rhizome samples (Table 2). Absence of this toxin in rest of the samples may be because of absence of toxigenic strains of Aspergillus ochraceus.

Table 2
Analysis of Mycotoxin Contamination in Market Samples of *Bergenia Ciliata* (Dried Rhizomes)

Sample code	Place of collection	Samples found positive for OTA contamination (µg/g)
BER J1	Jammu	-
BER J2	Jammu	-
BER J3	Jammu	-
BER J4	Jammu	-
BER K1	Kathua	-
BER K2	Kathua	-
BER U1	Udhampur	-
BER U2	Udhampur	-
BER U3	Udhampur	-
BER P1	Poonch	-
BER P2	Poonch	-
BER P3	Poonch	-
BER R1	Rajouri	-
BER R2	Rajouri	-
BER B1	Doda	-
BER B2	Doda	-
BER B3	Doda	-
BER A1	Anantnag	-
BER A2	Anantnag	-
BER S1	Srinagar	-
BER S2	Srinagar	-
BER L1	Leh	-
BER L2	Leh	-
BER L3	Leh	2.37
Positive samples		1
%age of +ve samples		4.12

- (Not detected)

The concentration of this toxin in the contaminated sample was 2.37 µg/g , which is very high. The joint expert committee on food additives of the WHO and FAO have set a provisional maximum intake of 100 ng/kg body weight (WHO, 1996). Presence of high concentration of this toxin in contaminated sample suggests that these rhizomes are good substrates for OTA production.

Further presence of this nephrotoxin mycotoxin in kidney curative drugs is alarming as instead of curing, it can have adverse effect on the health of patients.

Few other mycotoxicologists have also reported OTA contamination from dried medicinal plants and their products(Roy and Chourasia,1990 a,b; Chourasia, 1995; Aziz *et al*., 1998; Halt, 1998; Sharma, 2005)

The International Agency for Research on Cancer (1993) has classified OTA in group 2B because of its toxicity to humans. Committee on toxicity (COT) of chemicals in food, consumer products and environment considers OTA as a genotoxic carcinogen and proposed that its level in food be reduced to the lowest (COT, 1997). The joint expert committee on food additives of the WHO and FAO have set a provisional maximum intake of 100 ng/kg body weight (WHO, 1996). However, few countries have legislative limits ranging from 5 to 50 μg/g (Webley *et al.*, 1997). Ochratoxin A is known to have a remarkably long resistance time in the animal body (Moss, 2002). Presence of any amount of this toxin in herbal products is dangerous because Ayurvedic practitioners usually recommend these formulations for very long durations and this may be sufficient to produce ill effects in the body of consumers.

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