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The mycobiota of herbal drug plants in Oman and possible decontamination by gamma radiation

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Summary. The mycobiota of seven herbal plant species were surveyed: *Nigella sativa, Zataria multiflora, Trigonella foenum-graecum, Rhazya stricta* (seeds and leaves), *Haplophyllum tuberculatum, Aristolochia bracteolata* and *Teucrium muscatense*. A total of 24 species of fungi were isolated from the plants (seeds, leaves, flowers and/or stems). No significant differences were found between the mycobiota of the herbal plant species or between the six samples of each plant. *Aspergillus niger* and *Penicillium* sp. were the most common species, followed by *A. flavus* and *Rhizopus* spp. *A. flavus* was found in all herbal plants except *R. stricta* (leaves) and *Z. multiflora*. Aflatoxins were extracted from a number of herbal plants. Some strains of *A. flavus* isolated from the plants were aflatoxigenic. Gamma radiation at 905.4 Gy showed an average percent inhibition of fungi on some herbal plants between 88.6 and 99.1%. Complete inhibition was obtained at 1836 Gy.

Key words: herbal plants, fungal mycoflora, aflatoxins.

Introduction

Herbal drug plants, sometimes referred to as 'phytopharmaceuticals' or 'phytomedicinals', are becoming popular worldwide with increasing demand. They are marketed in herbal shops all over the world and on the Internet as food supplements and hence they are not subject to drug-quality control regulations. In the USA, the Food and Drug Administration (FDA) does not class herbal preparations as drugs. In the Sultanate of Oman and elsewhere, herbal plants are utilized for the treatment of various human illnesses. Popular herbal plants used in Oman include *Haplophyllum tuberculatum*, *Nigella sativa* (black cumin), *Rhazya stricta*, *Teucrium muscatense*, *Trigonella foenum-graecum* (fenugreek), *Zataria multiflora* and *Aristolochia bracteolata*.

Herbal shops in Oman import dry plant parts from neighboring countries or purchase them locally from plant collectors who sun-dry them. Most herbal shops store their herbal plants in plastic containers, jute sacs, plastic barrels or paper bags under warm and humid tropical conditions that are conducive to microbial growth and subsequent

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mycotoxin accumulation (Hitokoto *et al*., 1978; El Maraghy, 1988; El Bazza *et al*., 1990; Abeywickrama and Bean, 1991; Al Jassir, 1992; Mahmoud *et al*., 1992; Chourasia, 1995; Efuntoye, 1996; Selim *et al*., 1996).

Herbal plants may acquire fungal pathogens during growth in the field (Bisht and Singh, 1993) or during storage (Efuntoye, 1996). Under favorable conditions, mycotoxigenic fungi produce different mycotoxins. Different concentrations of aflatoxins have been reported in herbal plants (El Maraghy, 1988; El Bazza *et al*., 1990; Chourasia, 1995; Selim *et al*., 1996) and also in plants intended to cure liver disorders (Kumar and Roy, 1993). Aflatoxins are reported to be carcinogens (Chen *et al*., 1996; Jackson and Groopman, 1999; Wogan, 1999).

Traditional methods of decontamination of spices, herbal plants and other foods by fumigation with ethylene, propylene oxide or heat treatment have been replaced by gamma radiation (Selim *et al*., 1996). Gamma radiation at the proper dose inhibits fungal growth on many herbal plants (El Bazza *et al*., 1990; Mahmoud *et al*., 1992) but does not inhibit mycotoxins already formed.

Gamma rays contain sufficient energy to break chemical bonds and ionize molecules. The two most common sources of high-energy radiation used in the food industry are ^{60}Co and ^{137}Cs. Radappertization (in the range of 20–30 kGy) is used for sterilization of foods, radurization (1–10 kGy) for targeting specific pathogens, and radicidation (less than 1 kGy) for extending shelf life. The maximum energy emitted by ^{60}Co and ^{137}Cs (< 1–33 MeV) is too low to induce radioactivity in food.

To our knowledge, this is the first report on the mycobiota and aflatoxins of herbal plants in the Sultanate of Oman. The effect of gamma radiation on the mycobiota of herbal plants is studied.

Materials and methods

Mycological sampling and examination

Seven herbal plants were studied: *Nigella sativa* (seeds), *Zataria multiflora* (leaves), *Rhaza stricta* (seeds and leaves), *Trigonella foenum-graceum* (seeds), *Teucrium muscatense* (flowers, stems and leaves), *Haplophyllum tuberculatum* (flowers, stems and leaves) and *Aristolochia bracteolata* (flowers and leaves). They were purchased from local markets where they had been stored in plastic containers, jute sacs, or paper bags under warm humid conditions, sometimes for over a year. Six samples (500 g) of each plants were examined.

Samples were ground immediately after purchase. One gram of each ground sample was mixed aseptically in 9 ml of sterile distilled water and shaken vigorously. Appropriate serial dilutions were made and 0.1 ml of the dilution was transferred to Petri dishes containing potato dextrose agar (Oxoid, Basingstoke, UK) with 0.1 g l^{-1} chloramphenicol. Triplicate Petri dishes of each sample were incubated at room temperature (22±2°C) for 7 days. The mean number of fungal colony-forming units (cfus) was recorded. Fungi were identified using the reference literature.

Extraction of aflatoxins from broth culture and ground herbal plants

The ability of 22 *A. flavus* isolates to produce aflatoxins was studied by culturing the isolates in yeast extract broth (Oxoid). Stationary cultures were incubated at room temperature (22±2°C) for 10 days. Aflatoxins were extracted from the culture filtrate using the method of the Association of Official Analytical Chemists (Helrich, 1990). Extraction was also carried out from ground samples of medicinal plants contaminated with *A. flavus*.

Determination of aflatoxin with high pressure liquid chromatography (HPLC)

Aflatoxin standards B_1, B_2, G_1 and G_2 were purchased from the Sigma-Aldrich Company (Gillingham, Dorset, UK) and standard calibration chromatograms were made. Waters HPLC having the following features was used: Waters autoinjector, Waters quadretic pump, Millenium 32 Software data station, photodiodetector PDA, and C18 Reversal phase column. The mobile phase was water: methanol: acetonitrite (60:25:15) at a flow rate of 1 ml min^{-1}.

Irradiation of samples with gamma rays

Three grams of each of the six ground samples of three highly contaminated medicinal plants were placed in vials. Three replicas were made. The samples were irradiated with Cesium (^{137}Cs) using a 1000 Elite gamma cell (Nordion International Inc., Ottawa, Ontario, Canada) at 0.035 kGy min^{-1}. The dose used ranged from 343 to 1836 Gy.

Analysis of the data

The data were analyzed with one-way analysis of variance (Tukey Test P=0.05) using Minitab 1998 software.

Results

The mean cfu g^{-1} of six samples of each herbal plant were studied. No significant differences were found between samples (P=0.05). The mean number of cfu g^{-1} were: *T. foenum-graecum*, 19; *H. tuberculatum*, 2544; *T. muscatense*, 1447; *N. sativa*, 729; *Z. multiflora*, 42; *R. stricta* leaves, 495; *R. stricta* seeds, 422; and *A. bracteolata*, 275. The mean number of cfus g^{-1} of all samples ranged from 19 to 2544 (mean cfu g^{-1} 747; Table 1).

Table 1 shows a total of 24 fungal species belonging to sixteen genera. The maximum number of species (11) was found on *H. tuberculatum* leaves, the minimum (4) on *R. stricta* seeds. Species of the genus *Aspergillus* represented 33% of all fungal species and included potential mycotoxigenic species (*A. flavus* and *A. ochraceus*). Other potential mycotoxin producers among isolates were *Fusarium, Penicillium* and *A. alternata*. The most common species, occuring in all herbal plants, were *A. niger* and *Penicillium*. *A. flavus* was found in six out of the eight herbal plants.

Table 2 shows percent inhibition of microorganisms by gamma radiation at a dose of 905.4 Gy on some samples of *H. tuberculatum, A. bracteolata* and *T. muscatense*. Gamma radiation at 905.4 Gy showed percent inhibition of 88.6, 93.3 and 99.1% for *H. tuberculatum*, *A. bracteolata* and *T. muscatense* respectively. Radiation at 1836 Gy completely inhibited all microorganisms in the samples, while a dose of less than 905.4 Gy caused less than 70% inhibition.

Table 3 shows that eight out of twenty isolates (40%) produced aflatoxins. Some isolates produced all types of aflatoxins while others produced only one or two types. We analyzed 10 drug plant sam-

Table 1. Mean number of fungal cfu g^{-1} on 7 herbal plants.

	Fungus	*Nigella sativa*	*Haplophyllum tuberculatum*	*Teocrium muscatense*	*Aristolochia bracteolata*	*Rhazya stricta* (seeds)	*Trigonella foenum graecum*	*Rhazya stricta* (leaves)	*Zataria multiflora*
1	*Aspergillus flavus*	225.5	138.9	0.5	1.1	62.8	0.5	0	0
2	*A. fumigatus*	0	0	0	0	0	0	0	1.7
3	*A. nidulans*	0	0.5	26.7	117	0	1.1	0	4.4
4	*A. niger*	111.7	324.5	82.8	134	357	3.3	15	12.2
5	*A. ochraceus*	111.1	0	0	0	0	0	0	0
6	*A. terreus*	0	0	0	0	0.5	0.5	0	1.1
7	*A. versicolor*	0	0	0	0	0	2.2	12.5	0
8	*Aspergillus* spp.	0	0	0	0	0	0	0	2.8
9	*Alternaria alternata*	0	0.5	0.5	2.2	0	0	0.8	0
10	*Aureobasidium pullulans*	0	498.3	0	5.6	0	0	0	10.5
11	*Chaetomium* spp.	0	0	0	4.4	0	0	0	0
12	*Cladosporium* spp.	0	1377	0	0	0	0	0	0
13	*Coelomycetes* spp.	0	143.3	0	0	0	0	458	0
14	*Dreshslera spicifera*	0	0	0	1.12	0	0	0	0
15	*Emericellula nidulans*	0	0	1251	0	0	0	0	0
16	*Eurotium amystelodami*	1.7	0	0	4.4	0	2.2	0	0
17	*Fusarium roseum*	0	0	2.2	0	0	0	2.8	0
18	*Fusarium* spp.	0.5	53.9	0	3.3	0	0	0	0
19	*Mucor* spp.	0	5.0	4.4	09	0	0	0	0
20	*Myrothecium roseum*	0	0.5	0	0	0	0	0	0
21	*Penicillium* spp.	278.3	1.1	76.7	1.1	2.2	8.9	5.8	8.9
22	*Trichothecium roseum*	0	0	1.1	0	0	0	0	0
23	*Rhizopus* spp.	0	0	0.5	1.1	0	0.5	0.8	0.5
24	*Ulocladium botrytis*	0	0	0.5	0	0	0	0	0

Table 2. Survival (expressed as percentage of inhibition) of fungi irradiated with Gamma rays (905.4 Gy).

Sample No.	*H. tuberculatum*	*A. bracteolata*	*T. muscatense*
1	100	100	100
2	60	100	100
3	97.4	89.6	100
4	99.4	100	100
5	78.5	100	97.4
6	96	100	97.4
Average	88.6	98.3	99.1

Table 3. Aflatoxins produced by *A. flavus* isolates obtained from herbal plants.

Isolate No.	Amount of aflatoxins (ppm)			
	B_1	B_2	G_1	G_2
1	11	0	0	0
2	0	0	84	17
3	3	4	6	5
4	16	4	36	70
5	7	13	3	80
6	6	37	90	229
7	0	113	0	0
8	9	7	0	14

ples that showed high contamination with *A. flavus*; only two contained aflatoxin of the B group at a level of 28 ppm (data not shown).

Discussion

Fungal counts on most herbal plants were higher than maximum counts (350 cfu g^{-1}) reported on herbal drug plants in Nigeria (Efuntoy, 1996). The maximum count in this study was 2,544 cfu g^{-1} which is seven times greater than that in Nigeria. The cfus g^{-1} varied between 19 and 2,544 cfus g^{-1}. This variation could be due to differences in the plant sources, the chemistry of the plants, field contamination, and/or poor storage conditions.

Aspergillus niger, Penicillium spp., *A. flavus* and *A. nidulans* were the most common species. This could be linked to the fact that mitosporic fungi produce larger number of conidia than other fungi, which are usually found as mycelial fragments, and are therefore less easily detectable. Similar results were reported on herbal plants studied in Nigeria (Efuntoye, 1996) where the most common species isolated were: *A. niger, A. flavus, F. moniliforme, Trichoderma viride*, *Penicillium expansum,* and *Mucor fragilis*. Common species on drug plants in India were *Aspergillus, Fusarium, Alternaria, Emericella, Mucor, Penicillium* and *Chaetomium* species (Chourasia, 1995), in Sri Lanka *A. flavus*, *A. niger*, and *Fusarium* species (Abeywickrama and Bean 1991), and in Japan *A. flavus, A. glaucus, A. niger* and *Penicillium* (Hitokoto *et al*. 1978). Udagawa *et al*. (1976) found that *A. niger, A. glaucus, A. flavus* were the most common on crude drug plants. It appears that herbal plants, spices, grains, stored products, textiles and paper are usually contaminated with *Aspergillus* species (mainly *A. flavus, A. niger, A. ochraceus*), *Penicillium,* and *Fusarium* species. These genera are adapted to growing at low water activity levels.

Table 2 shows the effect of gamma radiation at 905.4 Gy on three heavily contaminated herbal plants. Average fungal inhibition at this dose ranged from 88.6 to 99.1% This dose is one fifth the safe dose for food (10 kGy) recommended by a joint committee of the Food and Agriculture Organization (FAO), the International Atomic Energy (IAEA), and the World Health Organization (WHO) (FAO/IAEA/WHO, 1981). Similar results were obtained by Mahmoud *et al*. (1992) who found that at 2 kGy fungi on tea and fenugreek were completely inhibited, while 3–4 kGy inhibited fungal growth on anise and liquorice. Gamma radiation has been extensively studied for sterilizing and reducing microbial contamination of spices (Vajdi and Pereira 1971; Tjayberg *et al*., 1972; Eiss 1984; Mossel, 1985; Farkas 1985; Alam *et al*., 1992).

On spices, 6.5–10 kGy ^{60}C was sufficient to reduce fungi to negligible levels, with no difference

between control and irradiated samples detected by chemical analysis (Eiss, 1984; Narvaiz *et al*. 1989). Andrews *et al*. (1995) reported that irradiation of ginger powder with 10 kGy from a ^{60}C source reduced the aerobic microflora from 10^8 to 10 cfu g^{-1} with no change in color or odor. Gamma radiation has also been shown to have an effect on growth and aflatoxin production of *A. flavus* (Srinivas *et al*., 1996).

In this study 40% of the *A. flavus* isolates were aflatoxigenic. Similar results were obtained by Kumar (1993), who reported that 48% of *A. flavus* strains isolated from herbal plants were aflatoxigenic, and by Roy *et al*. (1988) who found that of 158 isolates of *A. flavus* from drug plants, 49 (31%) were aflatoxigenic. Chourasia (1995) also reported that 42% of 95 strains of *A. flavus* screened for aflatoxins, produced aflatoxin. Although not all *A. flavus* strains are aflatoxigenic, a high percentage of aflatoxigenic strains (50–100%) is usual (Cannole *et al*., 1981; Lisker *et al*., 1993; Heperkan *et al*., 1994; Elshafie *et al*., 2002). In our study aflatoxins were detected in three samples of *N. sativa* and two of *H. tuberculatum* at average levels of 58 and 42 ppm respectively. We analyzed 10 drug plant samples that have showed high contamination by *A. flavus*, only two of which contained aflatoxin of the B group at a level of 28 ppm. Roy *et al*. (1988) reported that 14 out of 15 samples of drug plants analyzed for aflatoxins were aflatoxin-positive.

Fungal-contaminated herbal plants are a health hazard to people, particularly in the case of *A. flavus, A. ochraceus, Fusarium* species and *Penicillium* species. Since most of the fungi encountered in this study were storage or post-harvest contaminants, better storage conditions may reduce their incidence. Humans use medicinal plants to treat diseases. It would be ironic if the treatment of one disease with a herb was the unintended cause of another. Setting standards for microorganisms and mycotoxins in herbal plants will reduce the risk of mycotoxin contamination. Gamma radiation before storage is a useful tool for this purpose.

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