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Fungal contamination of raw materials of selected herbal drugs available in retail shops in Matara area

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Abstract

As a result of fungal contamination of herbal raw materials, the risk of mycotoxin production, especially aflatoxin, should be taken into consideration in the manufacturing process of herbal drugs. Vortex and ground methods were employed to isolate fungal species contaminating selected herbal raw materials of four different drug plants, *Terminalia chebula* (Sin. Aralu), *Piper longum* (Sinh. Thippili), *Glycyrriza glabra* (Sinh. Valmi) and *Terminalia belerica* (Sinh. Bulu). These materials stored for 6 to 8 months by local suppliers and imported from India were sampled for selective isolation of fungal species. Fifty microlitre aliquots from 10-fold dilutions were aseptically plated and distributed on the culture medium. Identification of fungal species was done using culture and morphological characteristics.

A total of 131 fungal isolates resulted from the raw materials. The highest number of fungal isolates was isolated from *T. belerica* whilst the least number of fungal isolates was isolated from *P. longum.*

According to frequency of occurrence, vortex method resulted in higher number of colonies than ground method. *T. belerica* was contaminated with higher number of fungal species yielded from both ground and vortexed samples.

Aspergillus appeared at the highest relative density than other genera. *Mucor* sp., *Curvularia* sp., *Rhizoctonia* sp., *Rhizopus* sp. and *Penicillium* sp. were the other dominant and frequently isolated species.

Keywords: herbal drugs, fungal isolates, frequency of occurrence, relative density

Introduction

Herbal drug is a dried medicinal plant or any part thereof, such as leaf, stem, root, flower, or seed (Hiroshi *et al.*, 1978). In spite of their origin, natural drugs should not be viewed as simple tools of folk medicine since they are a class of pharmaceutical products and should meet the requirements of quality, safety and efficacy (Bugno *et al.*, 2006).

Herbal medicine has a long history, probably extending over 2000 years, and is quite popular with many people, particularly Asians and Northern Europeans (Hiroshi *et al.*, 1978). Even though the pharmaceutical effects of all of these drugs have not been thoroughly investigated in recent years, the low level of side effects has increased the demand for the drugs (Hiroshi *et al.*, 1978).

The unscientific methods of collection, storage, transportation and congenial climatic conditions make the raw materials of herbal drugs prone to fungal infestations (Dubey *et al.*, 2008). Since they are natural products, the drugs are quite often deteriorated by microorganisms before harvesting and during handling and storage. Quality control to prevent growth of fungi and bacteria is essential for processors (Hiroshi *et al.*, 1978).

As a result of fungal contamination, the risk of mycotoxin production, especially aflatoxin, should be taken into consideration in the manufacturing process. At present, some investigators have reported fungal population of the herbal drugs and several kinds of spices (Bugno *et al.*, 2006).

The objective of the present investigation was to survey the most common raw materials of medicinal plants found in retail shops around Matara, Sri Lanka, such as *Terminalia chebula* (Aralu), *Piper longum* (Thippili), *Glycyrriza glabra* (Valmi) and *Terminalia belerica* (Bulu) in order to isolate the fungal species that colonize the stored products in storehouses.

Materials and Methods

Collection of Samples - Samples of four different drug plants *Terminalia chebula, Piper longum, Glycyrriza glabra* and *Terminalia belerica* stored by local suppliers for six to eight months after harvesting were collected from four storage centers located in and around Matara, Sri Lanka. Four samples from each herbal raw material were collected separately in sterilized polythene bags and sealed to avoid further contamination.

Mycological Analysis – Composite samples were prepared for each herbal raw material by mixing four collected samples. Vortex and ground methods were used to obtain fungal species. One gram of each composite sample was separately weighed and tenfold dilution series were prepared.

Three types of PDA media were prepared including normal PDA, acidic PDA and PDA supplemented with tetracycline for selective isolation of fungal species. pH of the PDA medium was adjusted to pH 4 for preparation of acidic PDA. All three PDA media were sterilized by autoclaving. Two milliliters of 0.6 g/L tetracycline was added to 1 L of PDA for preparation of PDA with antibiotics.

From appropriate 10 fold dilution series 50 µl aliquot of each dilution was aseptically plated and distributed on the culture media with the help of sterilized glass spreader. Colonies which have different morphologies were isolated into separate plates containing the same PDA medium.

Identification of fungal species was done by culture and morphological characteristics using three replicates from each isolate. The percentage relative density of different fungi on the samples was calculated using the following formula (Singh *et al.*, 2008).

$$\text{Relative density (\%)} = \frac{\text{Number of colonies of the fungus}}{\text{Total number of colonies of all fungal species}} \times 100$$

Percentage frequency of occurrence of mycobiota on individual raw materials of herbal drug samples was determined using the following formula (Singh *et al.*, 2008).

$$\text{Frequency of occurrence (\%)} = \frac{\text{Number of fungal isolates from a drug sample}}{\text{Total number of fungal isolates on all drug samples}} \times 100$$

Results and Discussion

A total of 131 fungal isolates were obtained from the herbal raw materials. The highest number of isolates were recorded from *Terminalia belerica* (49), followed by *Glycyrriza glabra* (34) and *Terminalia chebula* (32), whereas the lowest number was isolated (16) from *Piper longum* (Table 01).

Out of 131 isolates, 29 were identified up to the genus level using their morphological and reproductive characters under light microscope according to Singh *et al.*, (2008). Rest of the 102 isolates were observed but was unable to identify up to the genus level (Table 01).

Twenty nine identified isolates belonged to six different fungal genera. Namely; *Mucor* sp. (4), *Aspergillus* spp. (17), *Curvularia* sp. (4), *Rhizopus* sp. (1), *Rhizoctonia* sp. (2), *Penicillium* sp. (1) (Table 01). Among them *Aspergillus* spp. was the most frequently occurring species (Table 02). Similar results were reported by Takatori *et al.*, (1977), Ayres *et al.*, (1980). Misra, (1981) and Roy *et al.*, (1988).

Different frequency of occurrence values for fungi for ground and vortexed samples of each herbal raw material are as follows: *Terminalia chebula* (Local) Ground (12.97%) and Vortexed (11.45%), *Terminalia belerica* (Local) Ground (16.03%) and Vortex (21.37%), *Piper longum* (Local) Ground (3.05%) and Vortex (5.34%), *Piper longum* (Imported) Ground (0%) and Vortex (3.82%), *Glycyrriza glabra.* (Imported) Ground (6.87%) and Vortex (19.08%) (Table 03).

Table 01: Number of individuals of each fungal species isolated from herbal raw materials using ground and vortexed methods

Herbal material / Fungal species	*Terminalia chebula* (Local)		*Terminalia belerica* (Local)		*Piper longum* (Local)		*Piper longum* (Imported)		*Glycyrriza glabra* (Imported)	
	G	V	G	V	G	V	G	V	G	V
Mucor sp.				2						2
Aspergillus sp.	5	5	1			1		1	1	3
Curvularia sp.	2	1							1	
Rhizopus sp.									1	
Rhizoctonia sp.	1		1							
Penicillium sp.				1						
Identified	8	6	2	3	0	1	0	1	3	5
Unidentified	9	9	19	25	4	6	0	4	6	20
Total	17	15	21	28	4	7	0	5	9	25

G: - Ground V: - Vortexed

Relative density of the *Aspergillus* spp. was 58.62%, where as those of *Mucor* sp. (13.79%), *Curvularia* sp. (13.79%), *Rhizoctonia* sp. (6.90%), *Penicillium* sp. (3.45%) and *Rhizopus* sp. (3.45%), ranged from 13.79%, to 3.45% (Table 02).

In Sri Lanka, both local and imported herbal raw materials are used for traditional medicine. Therefore, in this investigation, some of the samples contained both imported and local samples and others were either local or imported samples. Fungal attack can occur either on the outer surface or inside the raw materials. Therefore vortex method was used to isolate fungal species present on outer surfaces and ground method was used to isolate fungal species present inside the

raw materials. According to our observations countable numbers of different colonies were observed at 10^{-2} fold dilution.

In the initial isolations higher number of bacterial contaminants occurred on PDA medium. Therefore, antibiotic and acid PDA were used to reduce the bacterial contaminations.

Table 02: Relative density of each fungal species in herbal raw materials

Fungal species	Relative density (%)
Mucor sp.	13.79
Aspergillus sp.	58.62
Curvularia sp.	13.79
Rhizopus sp.	3.45
Rhizoctonia sp.	6.90
Penicillium sp.	3.45

Table 03: Frequency of occurrence of fungi in each herbal raw material

Herbal material No: of fungal species	*Terminalia chebula (Local)*		*Terminalia belerica (Local)*		*Piper longum (Local)*		*Piper longum (Imported)*		*Glycyrriza glabra (Imported)*	
	G	V	G	V	G	V	G	V	G	V
Identified	8	6	2	3	0	1	0	1	3	5
Unidentified	9	9	19	25	4	6	0	4	6	20
Total	17	15	21	28	4	7	0	5	9	25
Frequency of occurrence (%)	12.97	11.45	16.03	21.37	3.05	5.34	0	3.82	6.87	19.08

G: - Ground V: - Vortex

Most of the colonies during our survey were isolated from vortex solution. According to that isolation, most of the species are present on the surface of the deteriorated raw materials. While grinding of the raw materials accumulation of inhibiting chemical components of the raw materials can take place. Therefore, numbers of fungal colonies are less in ground solutions than the vortex solutions. According to Singh *et al.* (2008), the chemical profile of the substrate may also be a deciding factor for fungal association. The individual raw materials contain different types of secondary metabolites. According to Dubey *et al.* (2008) the specific secondary metabolites of a raw material may be suppressive to association by specific fungal species.

Most often the desired biological response is not due to one constituent but a mixture of bioactive constituents and the relative proportion of active constituents which can vary from plant to plant of the same species and also in different plant parts. Since herbal medicines are prepared from materials of plant origin they are prone to contamination, deterioration and variation in composition. This gives rise to inferior quality of herbal products with little or no therapeutic efficacy (Bugno *et al.*, 2006).

Relative density of the Genus *Aspergillus* was greater than other Genera. Similar studies conducted by Udagawa *et al.* (1976) and Bugno *et al.* (2006) indicated that *Aspergillus* was a main component of the mycoflora of herbal drugs and *Aspergillus niger*, *Aspergillus glaucus* and *Aspergillus flavus* were the most prevalent species among the *Aspergillus* Genus. Recently, Singh *et al.* (2008) have reported mould and aflatoxin contamination in stored raw materials of six medicinal plants including, *Glycyrrhiza glabra* and *Terminalia chebula.*

According to frequency of occurrence, vortexed samples were heavily contaminated than the grounded samples. When considering the ground samples, *Terminalia belerica* was contaminated with higher number of fungal species followed by *Terminalia chebula* (Local), *Glycyrriza glabra* (Imported), *Piper longum* (Local) *and Piper longum* (imported) in decreasing manner.

According to number of fungal isolates obtained from each herbal raw material, *Terminalia belerica* was the most contaminated raw material with fungi. Same results have been obtained by Singh *et al.* (2008) and Gautam *et al.* (2009). The least number of fungal colonies were obtained from *Piper longum.* This may be due to presence of inhibiting chemicals (Sunila and Kuttan, 2004) such as alkaloids and terpenoids (Khan and Siddiqui, 2007).

Plant materials used for medical purposes should be carefully stored and the growth of toxigenic fungi should be inhibited. Since aflatoxins are extremely thermostable, the best remedy is to check production of aflatoxins by anti-aflatoxigenic chemicals, which do not affect taste and odor of raw materials and are also non-mammalian toxic in nature (Dubey *et al.*, 2008). It is advisable to treat plant drugs with non-toxic volatile components like monoterpenes, sesquiterpenes and phenyl propionoids in essential oils such as *Cinnamomum* sp. *and Cymbopogon* sp. at various stages of storage and processing (Paranagama *et al.*, 2003).

This is the first report that such investigation launched in Sri Lanka for isolation and identification of fungal species from selected herbal raw materials. Further investigations needed to discover whether there are any natural antifungal agents which could preserve stored herbal raw materials.

Conclusion

The findings of the present study show that the raw materials were heavily contaminated with different fungal species; some of them are known to be highly toxigenic in nature. The *Terminalia belerica* was identified as most contaminated herbal raw material where as *Piper longum* was relatively free from fungal inhabitants.

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