Detection of Aflatoxins and Use of Scanning Electron Microscope for the Identification of Fungal species in Some Commonly Used Spices

Zafar Alam Mahmood*, Najma Shaheen, Farhana Tasleem, Shahlla Imam, Safia Abidi and Iqbal Azhar

Department of Pharmacognosy, Faculty of Pharmacy and Pharmaceutical Sciences, University of Karachi, Pakistan

ABSTRACT

There is a possibility for the presence of fungi as well as mycotoxins in spices due to the improper methods used before and after harvesting, without controlling temperature and moisture during prolong storage period or may be during transportation. The purpose of this study was to detect and identify fungal contamination in some commonly used spices in Pakistan. Spices are available in Pakistan markets in loose packing and they are easily contaminated with dust, waste water and human/animal excreta. In present study five different spices such as black pepper (Piper nigrum), cardamom large (Amomum subulatum), cinnamon (Cinnamomum zeylanicum), coriander (Coriandrum sativum) and red chili (Capsicum annuum) were used for detection of fungus as well as aflatoxins. During fungal contamination test out of five spices, with the exception cinnamon all four spices were found contaminated with various fungal species. The most common organisms identified were Aspergillus niger, Aspergillus flavus, Aspergillus parasiticus, Aspergillus foetidus, Aspergillus fumigatus and Aspergillus ellipticus. In total, twelve different fungal species have been detected and also identified through scanning electron microscope (SEM). Thin layer chromatography (TLC) assay was performed for aflatoxins detection and the results revealed that red chili was heavily contaminated with aflatoxins than other selected spices.

Keywords: Spices, Fungi, Aflatoxins, SEM, TLC, Contamination

INTRODUCTION

Spices represent different parts of plants and herbs, mostly in dried form and consumed for aroma (such as black pepper, cardamom small, cinnamon bark and coriander), color (such as red chili and turmeric) and as a preservative (such as cinnamon, clove, red chili and turmeric) in food and around 50 are being used in cuisine globally. Spices can be obtained from different parts of the plant, such as bark (cinnamon) buds (clove), flowers (lavender), fruits (red chili and cardamom small), leaves (coriander) rhizomes (turmeric), roots (heeng), seeds (cumin seed), etc. [1-3]. Most of the spices are highly valuable in medication due to their associated health benefits such as anti-allergens, anti-microbial, anti-diabetics, anti-cancers and anti-oxidants effects, etc. [4].

Black pepper (Piper nigrum L., family Piperaceae) is a smooth woody flowering plant [5,6]. Cultivated in Bengal, Malaysia, Assam, Vietnam, China, Thailand, Srilanka and India [6-8]. Piperine is a major constituent of black pepper have anti-pyretic, anti-inflammatory, analgesic, anti-fungal, anti-depressant, anti-spasmodic, diaphoretic, anti-septic, anti-toxic, aphrodisiac, diuretic, febrifuge and rubefacient activities [7,9]. Oil of black pepper also used for relief in rheumatism pain, flu, cold, exhaustion, fever, emotional and physical coldness, muscular aches, nerve tonic, stimulate appetite, increase the flow of saliva and encourages peristalsis tones [6,10]. *Amomum subulatum* Roxb. L. is the second largest genus of the family Zingiberaceae.

It is a perennial spice cultivated in moist and shady areas. This well-known ancient spice also known as cardamom large and cultivated in sub Himalayan and North Easter Himalayan of India, Sikkim, Darjeeling hills, hills of Nepal.
and Bhutan. The seed of Anomum subulatum Roxb. L. contain essential oil with 1-8 cineol as a main constituent. Other important constituents are limonene Sabine, β-pine, α-terpineol, spathulenol, α-pinene, β-selinene, etc. The seed is used as an analgesic, alexipharmic, anti-diabetic, anti-malarial, anti-emetic, anti-microbial, in teeth and gum infections, snake venom and scorpion venom, remedy for throat and respiratory troubles. Seeds are also used as a flavoring agent and as a spice produces pleasant odor in foods [11-16].

The ever green small plant cinnamon (Cinnamomum zeylanicum L.) belongs to the family Lauraceae. Cinnamon spice achieved by drying the center portion of bark and it is available in market as quills and powder form. This plant commonly cultivated in Indonesia, South Asia, Sri Lanka, South India and Madagascar. The major constituents of cinnamon bark oil are cinnamaldehyde and eugenol and other important constituents are cinnamic acid, coumarin cinnamyl acetate, borneol, β-caryophyllene etc. The nutritional phytochemical constituents are carbohydrates, proteins, flavonoids, saponins, tannins, glycosides and tri terpenoids, etc. [17-19]. Bark is used as an anti-allergic, anti-microbial, anti-inflammatory, anti-septic, anti-diabetic, anti-obesity, anti-oxidant, also effective in Alzheimer’s disease, liver disease and in tooth ache treatment [15,17,18].

Coriander (Coriander sativum L.) is an annual spice belongs to the family Apiaceae. This ancient spice also known as Chinese parsley and Dhania. Coriander is extensively cultivated in Europe, South America, Pakistan, Turkey, China, India, Malaysia, France, Spain, North Africa, Thailand, Italy, Morocco [20,21]. The major phytochemical constituents of coriander seeds are linalool, α-pinene, granio1, camphor, geranyl acetate, oleic acid, linoleic acid and palmitic acid [22]. The major and nutritional components are flavonoids, amino acid, saponins, tannins, steroids, carbohydrates and cardiac glycosides [23,24]. It has many pharmacological activities such as anti-microbial, anti-cancer, anti-convulsant, anti-diarrheal, anti-hypertensive, anti-oxidant, anti-pyretic and anti-ulcer.

It also useful in heavy metal detoxification, rheumatism, insomnia, nausea and headache [20,22,25-28]. Capsicum annuum L. is an annual spice and belongs to the family Solanaceae. Commonly known as bell pepper, green pepper, sweet pepper, cherry pepper and chili pepper. It is widely grown in America, Mexico, India, Indonesia, China, Ethiopia, Spain, Portugal, South Africa and Central Europe. The active component of red chili are capsaicin (C₁₈H₂₇NO₃) (trans-8 methyl-N-vanillyl-6-nonenamide) and di hydro capsaicin (8 methyl-N vanillyl nonan amide [29-31]. Nutritional compounds are also investigated such as carbohydrates, protein, alkaloids, phenol compounds, saponins, tannins and flavonoids, etc. [32]. Red chili have analgesic, anti-carcinogenic, anti-coagulant, anti-microbial, anti-oxidant, anti-tumor, arteriosclerosis, bronchitis and carminative activities. It is also effective in cough, otalgia and increased blood circulation as well as in rheumatism [15,30-35].

Fungi produce toxic substances such as mycotoxin that can be distinguished according to their chemical structure, fungal origin and biological activity. More than 200 mycotoxins have been reported in which aflatoxins considered to the most dangerous and widely studied mycotoxin. The aflatoxins were firstly isolated and recognized when more than 100,000 turkeys died in the United Kingdom and high incidence in Kenya and hatchery trout in the United States. Aflatoxins are potent carcinogen mainly produced by secondary metabolism of Aspergillus flavus, Aspergillus nomius or Aspergillus parasiticus. Aspergillus flavus is common and prevalent in nature. There are four major aflatoxins B₁, B₂, G₁ and G₂. Metabolic derivative of AFB₁ and AFB₂ are AFM₁ and AFM₂ respectively and both originated from metabolism of few animals and normally found in milk and urine. Aflatoxins have a wide presence in various kinds of sample matrices, such as vegetables, meat, fruits, spices, milk, cereals, oils, etc.

Spices are commonly used as natural preservative for food. Mustard and ginger may accelerate the growth of aflatoxigenic fungi whereas few other spices such as pepper, cinnamon act as anti-aflatoxigenic or antifungal [36-40]. The spices mostly grown in tropical and sub-tropical regions, which have extreme ranges of humidity, rain fall and temperature, provide favorable condition for fungal growth [41]. The high temperature and steam treatment used to reduce the microbial contamination is the major cause of loss of volatile oils from spice which effects level of the flavor, aroma and color of spices as well. Further, steam is also responsible for increase the moisture levels of spices which affects the quality of spice or long term storage and may allow the fungi to grow and produces aflatoxins toxic substances such as mycotoxin that can be distinguished according to their chemical structure, fungal origin and biological activity.

More than 200 mycotoxins have been reported in which aflatoxins considered to the most dangerous and widely studied mycotoxin [1,36]. Spices are available in markets in loose packing and they are easily contaminated with dust,
waste water and human/animal excreta. Many countries like Spain, UK, Australia, Turkey, Morocco and Pakistan have been reported that spices and spices products are commonly contaminated with aflatoxins are the most toxic compounds causing cancer in liver and other body organs in human. Foods after affected by aflatoxins can cause serious human health issues. Malnutrition, impaired immune function and stunted growth in children and a number of disabilities and death have also been reported due to extensive use of aflatoxins. The critical impact of aflatoxins in human ranges from acute hepatic toxicity to chronic disease such as haemorrhages, oedema, hepatocellular carcinoma, hepatitis, rye’s syndrome, depressed immune response, liver cancer, pulmonary interstitial fibrosis, teratogenesis, tumor induction [42-45]. DNA mutation, disorder of cardio vascular and central nervous system [46].

The climate of Karachi (Pakistan) is hot and humid as compared to other city of Pakistan which invites the growth of fungi in food and the production of aflatoxins. In Karachi liver cancer is increasing day by day study shows that 34.6% liver cancer caused by alpha fetoprotein and 60% by hepatitis B and remaining through spices containing aflatoxins often sold in open market without any environmental control. Different countries like Kenya, Uganda and Thailand also indicated the relation between consumption of contaminated food with aflatoxins and the formation of liver cancer [47,48]. The present study therefore attracts the attention to potential risk may be caused as a result of using these contaminated spices.

MATERIALS AND METHODS

Collection of spices
Sample of spices black pepper (*Piper nigrum*), cardamom large (*Amomum subulatum*), cinnamon (*Cinamomum zeylanicum*), coriander (*Coriandrum sativum*) and red chili (*Capsicum annuum*) were purchased from local market Karachi, Pakistan. All ten samples were identified and their voucher specimen numbers are deposited in the department of Pharmacognosy, Faculty of Pharmacy and Pharmaceutical Science.

Preparation of powder
The dried samples were cleaned and ground by grinder separately to get powder samples and stored in dry and air tight containers separately. These powder samples were used in preparation of extracts and in serial dilutions.

Preparation of extracts
Test samples (5.0 g each) were transferred as powdered to a conical flask and 50 ml of hexane, ethanol, chloroform, and distilled water were added as single solvent in each flask and stoppered. The mixtures were allowed to stand at room temperature for 24 h. Finally samples were filtered and used for aflatoxins detection test [49].

Preparation of sample for fungal contamination test
Each spice sample (1 g) was taken in a test tube separately and 9 ml distilled water was added and shaken well. Serial dilutions were prepared by transferring 1 ml of first dilution ($10^{-1}$) from each test tube into other 9 ml distill water containing test tube separately then the mixture of each test tube was shaken and to made $10^{-2}$ dilution. Procedure was repeated to obtain $10^{-3}$ through $10^{-6}$ dilution. For contamination test $10^{-4}$ to $10^{-6}$ dilutions were used [50].

Isolation and identification of fungal
With the help of sterilized pipette serially diluted each sample, i.e., $10^4$, $10^5$, $10^6$ was taken and dropped 1 ml aliquots on to the surface in each Sabouraud Dextrose Agar (SDA) plate separately and the drops spread with the help of wire loop by using streaking method. Then the plates were moved left upright for dry purpose and were incubated at 37°C for 10 days. After 10 days fungal growth on plates were observed by visually and were identified by light microscope and scanning electron microscope [51].

Scanning electron microscopy test
Each dehydrated sample was placed separately on specimen stub with two sided adhesive tape and coated with thin layer of gold using quick auto coater model number JFC 1500. Many images of fungal species were observed and identified [52].

Aflatoxins detection test by TLC

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TLC (20 × 20 cm × 0.2 mm coated with 60F254, Merck Germany) were spotted with sample extracts and placed in a TLC tank contained a solution of chloroform and acetone in the ratio of 88:12 (v/v) for 30 min at room temperature and the TLC plates were observed for the presence of aflatoxins by their characteristics fluorescence properties with the help of ultra violet (UV) light. The blue fluorescence corresponding to authentic aflatoxins B1 and B2 indicated the presence of AFB1 and AFB2 in samples. The green fluorescence corresponds to the authentic aflatoxins G1 and G2 [39].

RESULTS

Fungal contamination

The results revealed that four samples out of five were positive for different fungus species (Table 1). In total, twelve different fungal species have been detected and also identified through SEM (Figure 1). All fungal species were identified on the basis of colony color by light microscope and SEM’s obtained data. The identification was based on the various studies reported by various research workers [52-58].

Aflatoxins contamination

The results of aflatoxins revealed (Table 2) that in test spices ethanol and chloroform extract of black pepper showed aflatoxins G2, ethanol extract of cardamom large showed aflatoxins B2 and aflatoxins B2 also showed in ethanol and

<table>
<thead>
<tr>
<th>Name of spices</th>
<th>Fungal species</th>
<th>Colony color</th>
<th>Vesicle serration</th>
<th>Shape</th>
<th>Texture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black pepper</td>
<td>Aspergillus niger</td>
<td>Dark brown to black</td>
<td>Biseriate</td>
<td>Globose</td>
<td>Smooth</td>
</tr>
<tr>
<td></td>
<td>Aspergillus ellipticus</td>
<td>Grayish brown</td>
<td>Biseriate</td>
<td>Globose/Radiate</td>
<td>Rough</td>
</tr>
<tr>
<td></td>
<td>Aspergillus fumigatus</td>
<td>Smoky gray</td>
<td>Uniseriate</td>
<td>Globose to ellipsoidal</td>
<td>Smooth to finely rough</td>
</tr>
<tr>
<td></td>
<td>Aspergillus carbonarius</td>
<td>Brownish black</td>
<td>Biseriate</td>
<td>Globose to radiate</td>
<td>Smooth to rough</td>
</tr>
<tr>
<td></td>
<td>Aspergillus foetidus</td>
<td>Reddish brown</td>
<td>Biseriate</td>
<td>Globose to subglobose</td>
<td>Smooth</td>
</tr>
<tr>
<td></td>
<td>Aspergillus flavus</td>
<td>Green</td>
<td>Biseriate</td>
<td>Elliptical</td>
<td>Smooth to finely rough</td>
</tr>
<tr>
<td></td>
<td>Aspergillus parasiticus</td>
<td>White</td>
<td>Uniseriate</td>
<td>Globose to subglobose</td>
<td>Rough</td>
</tr>
<tr>
<td></td>
<td>Aspergillus niger</td>
<td>Brown</td>
<td>Biseriate</td>
<td>Globose</td>
<td>Rough</td>
</tr>
<tr>
<td></td>
<td>Aspergillus aculeatus</td>
<td>Brown to black</td>
<td>Uniseriate</td>
<td>Ellipsoidal</td>
<td>Rough</td>
</tr>
<tr>
<td></td>
<td>Aspergillus tubingensis</td>
<td>Grayish black</td>
<td>Biseriate</td>
<td>Elliptical</td>
<td>Rough</td>
</tr>
<tr>
<td>Cardamon large</td>
<td>Aspergillus niger</td>
<td>Brown</td>
<td>Biseriate</td>
<td>Globose</td>
<td>Rough</td>
</tr>
<tr>
<td></td>
<td>Aspergillus spp. UFLADC 01</td>
<td>Light brown</td>
<td>Biseriate</td>
<td>Globose to subglobose</td>
<td>Spiny</td>
</tr>
<tr>
<td></td>
<td>Aspergillus sclerotion niger</td>
<td>Black pigments on yellow</td>
<td>Biseriate</td>
<td>Globose</td>
<td>Smooth</td>
</tr>
<tr>
<td></td>
<td>Aspergillus ibericus</td>
<td>Greenish black</td>
<td>Biseriate</td>
<td>Granular</td>
<td>Spine</td>
</tr>
<tr>
<td>Cinnamon</td>
<td>Aspergillus uvarum</td>
<td>Black</td>
<td>Uniseriate</td>
<td>Globose to sub globose</td>
<td>Spine</td>
</tr>
<tr>
<td></td>
<td>Aspergillus flavus</td>
<td>Yellowish green</td>
<td>Biseriate</td>
<td>Elliptical</td>
<td>Rough</td>
</tr>
</tbody>
</table>

Table 1: Spices showing fungal contamination on SDA plates and characteristic features of colonies recorded through light and SEM
chloroform extract of coriander, while aflatoxins G2 showed in hexane, ethanol and chloroform extract of red chili, while all cinnamon extracts showed negative results of aflatoxins.

DISCUSSION

In recent years, consumption of spices has considerably increased in daily food preparation to enhance aroma and flavor. Spices like black pepper, cardamom large, coriander and red chili may be contaminated with fungus and aflatoxins due to the improper methods used before and after harvesting, without controlling temperature and moisture during prolong storage period or may be during transportation.

The morphological contamination of spices has great influence upon its quality and its application for any specific purpose. A number of investigators have performed microbiological contamination tests and reported various fungal species in various spices collected from domestic market [59,60]. The most commonly identified fungus organisms were Aspergillus niger, Aspergillus flavus and Aspergillus fumigatus, etc. [60]. Presence of these organisms and including some other fastidious organisms can greatly affect the quality of spices and may produce aflatoxins causing harmful effects upon human body. The European pharmacopoeia specifies that the total viable aerobic count should not be more than $10^2$ fungi per gram of any sample taken for fungal contamination tests. During fungal contamination test out of five spices with the exception cinnamon, all four spices were found contaminated with various fungal species.

The most common organisms identified were Aspergillus niger, Aspergillus flavus, Aspergillus parasiticus, Aspergillus foetidus, Aspergillus fumigatus and Aspergillus ellipticus. The shape and texture of identified fungal species have mentioned along with the colony color (Table 1). In total, twelve different fungal species have been detected and also identified through SEM (Figure 1).

All fungus was identified on the basis of colony color and by light microscope and SEM’s obtained data. On the basis of conidia ornamentation we distinguished the different species for example Aspergillus foetidus, Aspergillus niger and Aspergillus tubingensis species are difficult to distinguish on morphological basis but on the basis of conidial ornamentation Aspergillus foetidus could be distinguished, it has delicately spine in immature stage, when mature its conidia become smooth. Aspergillus tubingensis could be differentiating to Aspergillums niger to its white to pink
Aspergillus ibericus and Aspergillus carbonarius are similar but can be differentiated on the basis of conidia ornamentation. Aspergillus carbonarius has bigger conidia than Aspergillus ibericus while Aspergillus ibericus conidia were found in elliptical shape. Aspergillus spp. UFLA DCA 01 and Aspergillus costaricensis morphologically are similar but on the bases of conidial ornamentation they are distinguished each other. Aspergillus spp. UFLA DCA 01 showed spiny to finely wrinkled conidia while Aspergillus costaricensis has smooth conidia to distinctly wrinkle and has larger vesicle size than Aspergillus spp. UFLA DCA 01 vesicle.

Aspergillus nidulans identified by its colony color with smooth wall and has short conidiophores. Ascospore of A. nidulans surrounded by Hulle cells. Aspergillus japonicus and Aspergillus aculeatus were found similar both have spiny conidia but Aspergillus aculeatus had large vesicle with ellipsoidal conidia while Aspergillus japonicus had globular conidia with small vesicle. Aspergillus niger and Aspergillus lacticoffeatus were found morphologically similar but distinguished morphologically by difference in conidia color and ornamentation. The identification of fungus species was confirmed of the various studies reported by various research workers [52-58]

Aflatoxins are potent carcinogen mainly produced as secondary metabolite of Aspergillus flavus, Aspergillus parasiticus or Aspergillus parasiticus. The most common reported aflatoxins producing organism is Aspergillus flavus. In the present study (Table 2) red chili was found more contaminated with aflatoxins than other selected spices. Many researcher like Yernani et al. [39], Reddy et al. [30], Rajarajan et al. [61], Fufa and Urga [62] have also investigated the high level of aflatoxin in red chili and since it is an essential ingredient of food specially in sub-continent, therefore people who are consuming red chili are more prone to be affected by liver fungus due to the improper methods used before and after harvesting, without controlling temperature and moisture during prolong storage period or may be during transportation.

Figure 1: SEM images of identified fungal species

In present studies the absence of fungus species in cinnamon may be due to the unfavorable condition for the development of microbial species during production, storage and transportation and may be applicable to good manufacturing practice after harvesting like cleaning, drying and packing which may have minimized the growth of mould and production of aflatoxins in spice. Synder [63] investigated that cinnamon has strong anti-microbial effect; coriander seed has moderate anti-microbial effect while black pepper and red chili have weak anti-microbial effects [63]. Among test spices cinnamon was showed negative result of fungus because it contain eugenol, cinnamyldehyde, cinnamic acid, methoxy cinnamaldehyde and cinnamyl alcohol they have inhibitory effects to mycotoxigenic Aspergillus and Aspergillus parasiticus [64,65].

CONCLUSION

Spices are natural products and cultivated in tropical and non-tropical countries. Pre and post harvesting spices implicates a number of hygienic problems and lead to high microbial counts which affect quality of the spices and thus on the basis of microbial contamination results, it is believed that a requirement of a control system to maintain hygienic quality and good sanitary practice during different process of production of spices to improve the quality of spices is highly essential. The fungal contamination test must be performed before these can be used in food products.

REFERENCES


