Pak. J. Bot., 51(4): 1523-1529, 2019. DOI: http://dx.doi.org/10.30848/PJB2019-4(31)

FUNGAL CONTAMINATION IN DRIED FRUITS AND NUTS: A POSSIBLE SOURCE OF MYCOSIS AND MYCOTOXICOSIS

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Abstract

Fungi, being an integral constituent of this earth, cause contamination in many food stuffs including dried fruits and nuts. This fungal contamination not only leads to spoilage of these nutritive substances but also responsible for mycoses and mycotoxicoses among consumers especially immune compromised individuals. Keeping in view this aspect, this study was designed to investigate the fungal contamination in dried fruits and nuts sold in local markets of Karachi city. For this purpose, a total of eighty-four samples of dried fruits and nuts were collected from different local vendors in Karachi. These samples were crushed and screened for the presence of fungal contamination by streaking on Sabouraud`s dextrose agar. The fungal colonies appeared were identified by macroscopic and microscopic study. The isolated strains were further tested for their susceptibility to antifungal agents by using disc diffusion method. The aflatoxigenic strains among isolated *Aspergillus* species were also detected by using cultural methods (Ammonium hydroxide technique and Ultra Violet Photography technique).The overall results exhibited presence of high fungal load in dried fruits and nuts particularly in raisins and apricots. The samples collected from local markets (Karachi) were found more contaminated as compared to those obtained from their sites of origin (Gilgit). *Aspergillus niger* was isolated as the most predominant species (25.8%) followed by *A. flavus* (19.35%) among all isolated fungal strains. Among all *A. flavus* strains, 18% were found aflatoxigenic as detected by cultural method. Furthermore, the isolated fungal strains exhibited 100% resistance against fluconazole, while high resistance against Amphotericin B was also recorded by many fungal strains. High burden of drug resistant and aflatoxigenic fungi in edible items such as dried fruits and nuts pose an upcoming threat for human population therefore needs prompt management.

Key words: Aflatoxin, Mycoses, Mycotoxicoses, Fungal contamination.

Introduction

Dried fruits and nuts are considered as best source of essential nutrients as they provide protein, fatty acids, potassium, dietary fibers and bioactive compounds. They also provide benefits to human health by reducing the chances of obesity, cardiovascular illnesses as well as they overcome the possibilities of diabetes (Carughi *et al.,* 2015). Dried fruits and nuts have high shelf life as compared to fresh fruits so they are best alternative of fresh fruits for long time usage (Masood *et al*., 2015). The economy of a country is mostly dependent on their export and most of the developing countries export agricultural products and these are considered as backbone of the country's economy (Alghalibi & Shater, 2004). The dried fruits and nuts also play an important role in this regard, as observed, United States earn more than $3 billion income to export dried fruits and nuts annually (Johnson *et al.,* 2009). Pakistan being an agricultural country, exports wide range of dried fruits and nuts to other countries and stands 3rd among the apricot producing countries (Hussian *et al.,* 2015).

Unfortunately, these valuable food stuffs can be contaminated and spoiled by microorganisms. As reported by USDA-Economic Research Service, 18.9 billion pounds of fruits and vegetables are lost annually due to microbial spoilage. Bacteria and fungi are mainly involved in spoilage of these food stuffs. In case of bacteria, *Erwinia carotovora, Pseudomonas* spp. i.e., *Pseudomonas fluorescence* and *Pseudomonas viridiflava* are majorly responsible to cause soft rot across a wide range of vegetables and some fruits (Barth *et al*., 2009). Many fungi such as *Alternaria, Aspergillus, Candida, Fusarium, Mucor, Rhizopus, Penicillium* etc. not only cause spoilage of different food stuffs but their growth in food stuff and their ingestion may leads to develop many health-related issues such as mycoses which may range from mild to life threatening clinical conditions, particularly in immuno compromised patients (Tournas *et al.,* 2015).

There are almost 300 fungal species among 1.5 million total species of fungi which can cause diseases in humans and animals. These diseases may range from mild allergic reactions to severe invasive life-threatening infections. Mycoses associated with contaminated food could be among 600 million food borne diseases which occur every year worldwide. These food borne infections are usually caused by mold fungi, however few cases of yeast infections associated with dairy products are reported. Generally, these infections are not common in normal healthy population but immune compromised individuals or those with medical risk factors are more susceptible to acquire such infections. As observed, food contaminated with opportunistic pathogens such as *Zygomycetes* and *Aspergillus* posed a major risk of morbidity and mortality among immune suppressed population. In addition to mycoses, fungal growth on food stuffs may also cause mycotoxicoses, a condition caused due to ingestion of mycotoxins resulting in severe manifestations such as acute poisoning, liver diseases, cancer and neural tube defects

(Benedict *et al*., 2016). Mycotoxins are produced as secondary metabolite by different fungi during their growth cycle and this ability of toxin production is a genetically controlled phenomenon. There are 400 known mycotoxins discovered up till now in which most of them are responsible for causing acute and chronic mycotoxicoses (Peraica *et al*., 1995).

Keeping in view the hazards of fungal growth in food stuffs particularly those which are sold openly in local markets, this study was designed to evaluate the fungal load in dried fruits and nuts sold by local vendors in different areas of Karachi. Moreover, the aflatoxigenic potential of isolated fungal strains along with their susceptibility to antifungal agents was also determined.

Materials and Methods

Collection and processing of samples: A total of 84 different dried fruits and nut samples were collected by convenience sampling method in clean polyethylene bags from the different areas of Karachi. The bags were properly labeled, sealed and kept in refrigerator until use. The samples were processed for the isolation of fungi by crushing them and transferring one gram of sample in ten ml. of sterile distilled water and vortexed to homogenize suspension. The serial dilutions of homogenized suspension were made and by the help of sterile cotton swab streaked on Sabouraud`s Dextrose agar (Oxoid) and incubated at 25ºC for at least 1 week. After incubation, the colonies appeared on Sabouraud`s Dextrose agar (Oxoid) plates were counted and colony forming unit per gram (cfu/g) was calculated. The isolated fungal strains were identified by macroscopic and microscopic characteristics.

Macroscopic characteristics: The macroscopic characteristics (color, texture, pigmentation and reverse of colony) of fungal colonies observed after incubation were thoroughly studied and observations were recorded.

Microscopic characteristics: Microscopic examination of fungal strains was done by using the following methods.

Wet mount technique: In this technique, a drop of lacto phenol cotton blue (LPCB) was placed on a clean grease free slide, then with the help of sterile fungus needle a tiny piece of fungal growth was transferred into the lacto phenol cotton blue drop on slide. The growth was gently teased with the help of sterile fungus needle. A cover slip was placed and finally examined under the microscope in low power 10X and high power 40X magnification (Larone, 1995).

Slide culture technique: The fungal strains which were difficult to identify by wet mount technique were identified by slide culture technique. In this technique, the bottom of sterile petri dish was covered with a piece of filter paper and placed on a bend rod in the petri dish. A clean grease free slide was placed on the glass rod and then from the plate of SDA a block of 1x1cm agar was cut with a sterile scalpel and transferred into the center of the glass slide. The fungal culture was inoculated with the help of sterile fungus needle on the center of the agar block. Then a coverslip was placed over the block and pressed to ensure adherence. 1.5ml of sterile distilled water was added in the bottom of the petri plate and incubated at room temperature for 2 to 5 days. After the appearance of fungal growth, the coverslip was removed with the help of forcep and placed it on a drop of lacto phenol cotton blue on another clean glass slide and then observed under microscope in low power 10X and high power 40X magnification (Larone, 1995).

Antifungal susceptibility profile of fungal species isolated from dried fruits and nuts: The antifungal susceptibility of selected fungal strains isolated from dried fruits and nuts was determined by using disc diffusion method (Naz *et al*., 2017; Mubeen *et al.,* 2006). Briefly, a uniform lawn was made from freshly grown culture of fungal strain on Muller`s Hinton agar plate. Commercially prepared antifungal discs were placed aseptically on agar surface and incubated at room temperature for 2-4 days. After incubation, the size of zone of inhibition was measured.

Detection of Aflatoxin producing strains of *Aspergillus*: Aflatoxin producing strains of *Aspergillus* spp. can be detected by two methods i.e. Analytical methods and Cultural methods. In the present study, cultural technique (Ammonium hydroxide vapor test and UV photographs) were used to detect the aflatoxin producing strains of *Aspergillus flavus.*

Ammonium hydroxide vapor test: This technique was introduced by Saito & Machida (1999) and it is very rapid and sensitive method used for the identification of aflatoxin producing and non-producing strains of *A. flavus* and *A. parasiticus.* In this method, a single fungal colony was grown in the center of potato dextrose agar containing Petri plate. The plate was then inverted and added 1 or 2 drops of concentrated ammonium hydroxide on inside the lid. The reverse of aflatoxin producing colonies quickly converted into plum red color and no color change occurs on the reverse of the non-producing colonies (Abbas *et al.,* 2004).

Ultra violet (UV) photography: It is a very rapid technique used to detect the aflatoxin producing fungal strains. In this technique fungal colonies grown on Potato Dextrose agar were exposed to UV radiation at 365 nm for the detection of aflatoxin producing strains. Aflatoxigenic strains produced fluorescence under UV light while negative strains showed no fluorescence (Nair *et al.,* 2014).

Statistical analysis: Statistical analysis was done by using Graph Pad Prism 5.0. to determine the significant variation in the level of contamination found in different nuts and dried fruits samples collected from different regions by applying the Two-way ANOVA test.

Results and Discussion

Isolation and identification of fungal strains from dried fruit and nuts samples: Karachi is one of the biggest city of Pakistan and it is inhabited by more than 20 million people. This dreadful situation has increased the incidence of water and food borne diseases caused by microorganisms in the city. The microorganisms such as fungi, bacteria, viruses and some protozoa, are increasingly reported to cause many health-related issues in the population. Particularly contamination of bacteria and fungi in food, water and air has been causing many human and animal diseases (Ahmed *et al.,* 2011). Different hazards associated with consumption of contaminated food are usually because of open selling of different food items in markets especially in areas of low socio-economic groups. Among food items, dried fruits and nuts are also contaminated due to improper storage, transportation and open selling on local street vendors (Redhi, Thaila). Especially fungi, because of their low nutrients and moisture requirement, can flourish well in these dried fruits and nuts. The present study was focused on the isolation of fungal strains from dried fruits and nuts sold by vendors in open local markets of Karachi. The mycological profile of dried fruits collected in this study revealed isolation of a total of 540 fungal strains were isolated including *Aspergillus, Penicillium, Rhizopus, Candida* spp. etc. (Table 1, Fig. 1). Among them the most predominant fungal spp. isolated were *A. niger* (25.8%) *A. flavus* (19.3%) followed by *A. fumigatus* (11.8%) (Fig. 2). These results coincide with the previous study which also reported, *Aspergillus* spp., such as *A. niger* and *A. flavus* as the most predominant spp. from dried fruits and nuts samples (Alhussaini, 2012).

Among the dried fruit samples, raisins and apricot revealed highest load of fungal strains as compared to other samples. The most predominant fungal species isolated from raisins were *A. niger, A. flavus* and *A. fumigatus* (Table 1). A similar result was reported from a study conducted in Yemen where the mycological examination of raisins showed contamination of *A. flavus A. niger A. fumigatus* (Alghalibi & Shater, 2004). In terms of fungal contamination, apricot was found heavily contaminated with of *A. terreus* followed by *A. niger* and *A. fumigatus* (Table 1) which simulates with a study conducted in Iraq in which *A. niger, A. carbonarius* and *A. flavus* were the most frequently isolated fungal spp. (Saadullah & Abdullah, 2014). Other dried fruits such as dry dates also showed *A. flavus, A. niger* and *Penicillium* sp., as the most predominant fungal spp., isolated (Table 1). These findings also coincide with previous study conducted in Saudi Arabia where dry date samples were collected from different markets exhibited isolation of *A. flavus, A. niger, Penicillium* and *Rhizopus* spp. (Gherbawy *et al.,* 2012). The most frequently isolated species from fig samples in present study were *A. niger* and *A. flavus* (Table 1). While similar results were reported from Iran where investigation on fungal contamination in dried figs demonstrated isolation of *A. niger* and *A. flavus* as the most predominant fungal strains (Javanmard, 2010). Among all, pistachio was found to be the least contaminated sample the data regarding pistachio sample showed isolation of *A. niger* and *A. nidulans* as the most frequently isolated fungal strains (Table 1) which is in contrast with previous literature where *A. flavus* was the most predominant sp. isolated (Taghi *et al.,* 2010). The present study also revealed contamination of potentially pathogenic fungal strains such as *A. fumigatus, A. glaucus, Absidia* spp. and *Candida* spp. in pine nut samples (Table 1) which also differed from results of another study where *A. niger, A. flavus* and *Penicillium* spp., were isolated from pine nuts . Similarly, peanut samples collected in present study showed high incidence of *A. flavus, A. fumigatus* and *Rhizopus* spp. (Table 1) which was in accordance with previous findings (Rossetto *et al.,* 2005). While *A. flavus* and *A. niger* were isolated as the most predominantly isolated fungal spp., from walnut samples (Table 1) which also correlates with previous literature (Abdulla, 2013). Interestingly, cashew nut showed minimal fungal contamination (Table 1) whichis in contrast with past studies which reported presence of various pathogenic molds in these samples (Adebajo & Diyaolu, 2003).

In the view of basic statistical test after fungal isolation from different dry fruits and nuts, we have found that the nuts included Almond and Pistachio were shown less difference (SEM ± 0.577) while in case of fruits raisins and apricot has high standard error mean ±1.612 and ±1.592 were observed (Table 1).

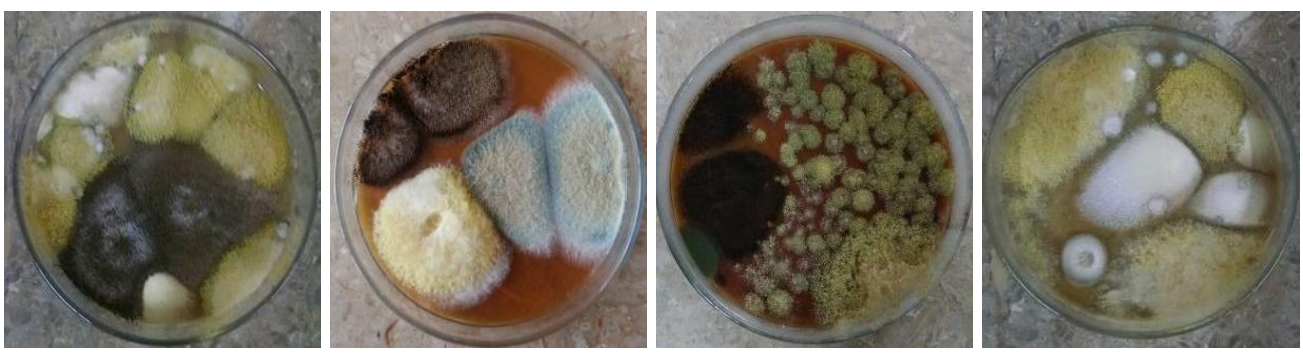

Fig. 1. Fungal isolation from dried fruit and nuts samples.

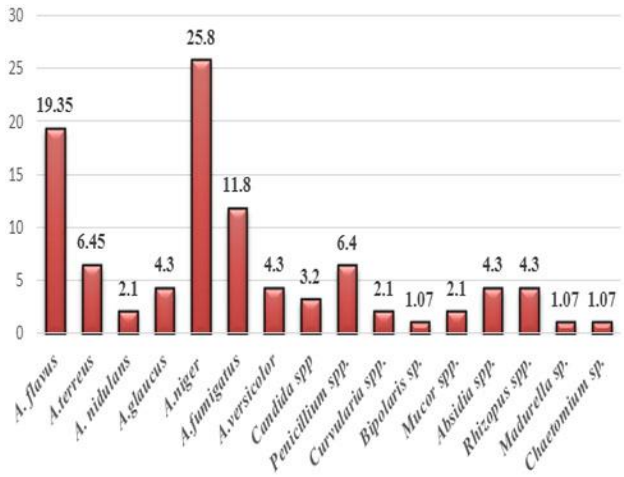

Fig. 2. Distribution of different fungal species isolated from dried fruits and nuts.

Comparison between fungal load of samples obtained from production region and local markets: The comparative study between the isolation of fungal strains from the samples obtained from production region (Gilgit) and those collected from the local markets (Karachi) was also conducted. In this study, equal number of nuts and dried fruits samples were collected from both regions. The samples collected from local markets of Karachi were found more contaminated as compared to the samples collected from the production region (Gilgit). Because a total of 195 fungal colonies were obtained from samples collected from local markets however, a total of 76 fungal colonies were isolated from the samples collected from the production region. In both cases apricot and almond samples exhibited more contamination. Among all the isolated fungal spp. *Aspergillus flavus* and *Aspergillus niger* and *Aspergillus fumigatus* were the most predominantly isolated spp. from the samples collected from the local markets of Karachi while *Aspergillus flavus,* and *Aspergillus niger* were the most commonly isolated fungal spp. from the production region (Gilgit) (Table 2).

Comparative statistical analysis between the contamination level in production region (Gilgit) and local markets of Karachi were obtained by using 2way ANOVA (variance analysis). The total variance between two regions were found 65.06% with F=33.1 whereas the P-value was 0.0104, which is significant at alpha (α=0.05). These results indicated the notable variation among different dried fruit and nut samples collected from two regions.

Detection of Aflatoxin producing *Aspergillus* spp.: *Aspergillus flavus,* a known producer of aflatoxin, was one of the predominant sp. isolated in the present study. There are different analytical as well as cultural methods which are used to detect aflatoxin. Analytical methods include, thin layer chromatography, High performance liquid chromatography, Liquid chromatography/mass spectroscopy, Enzyme linked immune sorbent Assay, Immune affinity with florescence. These methods are sensitive, reliable, definitive and quantitative but they are very expensive and are not easily available in developing countries. However, Aflatoxin producing strains can also be detected by different cultural methods such as Blue florescence technique, Yellow pigmentation technique, Ammonium hydroxide vapor test, UV photography. These methods are very cost effective and do not require major equipment's and expertise. But these techniques are considered as less sensitive than analytical methods. However, these methods are also used in many developing countries to detect aflatoxin producing fungal strains in different food stuffs (Abbas *et al.,* 2004). As the production of this aflatoxin is a potential risk of severe health hazards to consumers therefore in this study the presence of aflatoxigenic strains among isolated *Aspergillus* spp., were also detected by using cultural method such as Ammonium Hydroxide vapor test and UV photography test which are well-known technique for the detection of aflatoxin producing strains (Figs. 3&4). The result demonstrated that 14% of the total *Aspergillus flavus* strains isolated were found aflatoxigenic. These strains were mainly isolated from dried apricots and almonds. By using the same technique, the aflatoxigenic strains were detected from 70% peanuts samples in a previous study (Afsah-Hejri *et al.,* 2013).

Antifungal susceptibility profile: There is a constant elevation in morbidity and mortality due to mycoses despite of the availability of antifungal treatments. This might be due to emerging trends of antifungal resistance. This resistance has been increasingly reported from most of the fungi against virtually most of the antifungal agent. The same pattern was observed in this study where isolated (selected) strains showed remarkable resistance against antifungal drugs. *Aspergillus* spp., which were the major fungi isolated in present study showed high resistance against fluconazole and Amphotericin B (Table 3). Among different antifungals, fluconazole is the major triazole used commonly to treat fungal infections revealed high resistance which might be due to excessive use of agricultural azole in crop production (Mortensen *et al.,* 2010; Sanglard, 2016). Rhizopus and other members of zygomycetes are usually susceptible to Amphotericin B and resistant to Azoles. But in the present study these species were found resistant to Amphotericin B as well as fluconazole (Pfaller & Diekema, 2004).

The present study highlighted that dried fruits and nuts which are considered as nutrients rich foods may cause serious health problems due to fungal contamination during their transportation, storage and selling in open environments, Moreover, isolation of antifungal resistant and aflatoxigenic strains from these contaminants also pose a threat for consumers and require strategies to control fungal contamination at pre- and post-harvest levels.

Table 1. Isolation of fungal strains from different dried fruits and nuts samples.

Name of samples	Total number of fungal isolates cfu/g	Mean	Standard error mean	Isolated fungal strain
Walnut (n=6)	36	6.000	0.683	*A. flavus, A. terreus, A. niger, A. fumigatus, A. versicolor, Absidia* spp.
Almond (n=6)	48	8.000	0.577	*A. flavus, A. niger, A. glaucus, A. fumigatus, Penicillium* spp. *Rhizopus* spp.
Apricot kernel (n=6)	24	4.000	0.856	*A. fumigatus, Mucor* spp., *Absidia* spp.
Cashew nut (n=6)	36	6.000	0.816	*A. niger, Penicillium* spp., *Curvalaria* spp., *Absidia* spp.
Coconut (n=6)	48	8.000	0.775	*A. flavus, A. niger, A. versicolor*
Pine nut (n=6)	36	6.000	0.856	*A. glaucus, A. fumigatus, Candida* spp., *Absidia* spp.
Peanut (n=6)	30	5.000	0.632	*A. flavus, A. fumigatus, Rhizopus* spp.
Pistachio (n=6)	12	2.000	0.683	*A. flavus, A. nidulans, A. niger, Bipolaris* spp.
Prunes (n=6)	30	5.000	0.577	*A. glaucus, A. niger, Rhizopus* spp.
Dry dates (n=6)	30	5.000	1.238	*A. flavus, A. terreus, A. niger, A. fumigatus, A. versicolor, Penicillium* spp., *Mucor* spp., *Rhizopus* spp., *Madurella* spp.
Fig (n=6)	42	7.000	1.461	*A. flavus, A. niger, A. fumigatus*
Mulberry (n=6)	48	8.000	1.291	*A. flavus, A. niger, Aspergillus fumigatus, Candida* spp.
Raisins (n=6)	60	10.000	1.612	*A. flavus, A. glaucus, A. niger, A. terreus, A. fumigatus, Candida* spp., *Penicillium* spp.
Apricot (n=6)	60	10.000	1.592	*A. flavus, A. terreus A. niger, Curvalaria* spp., *Chaetomium* spp.
14	540			

Table 2. Comparison between fungal load on dried fruits and nuts from the production region (Gilgit) and local markets of (Karachi).

Name of sample	No of samples	Isolation from production region		No of samples	Isolation from local market	
		Total no of colonies cfu/g	Name of fungi		Total no of colonies cfu/g	Name of fungi
Apricot	06	24	*A. flavus, A.fumigatus, A. niger*	06	60	*A. flavus, A. terreus A.niger, Curvalaria* spp., *Chaetomium* spp.
Almond	06	24	*A. flavus, A. niger, A. versicolor, Absidia* spp.	06	42	*A. flavus, A.niger, A. glaucus, A. fumigatus, Penicillium* spp., *Rhizopus* spp.
Mulberry	06	18	*A. niger, A. nidulans, Penicillium* spp., *Rhizopus* spp.	06	48	*A. flavus, A. niger, A. fumigatus, Candida* spp.
Walnut	06	10	*A. flavus A. fumigatus, Candida* spp.	06	36	*A. flavus, A. terreus, A. niger, A. fumigatus, A. versicolor, Absidia* spp.
Total	24	76		24	186	

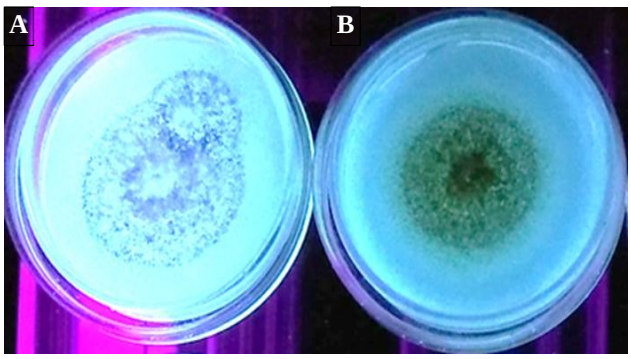

Fig. 3. Detection of aflatoxin producing strains of *A. flavus* by Ultra Violet Photography technique.
(A) Non-aflatoxigenic colony, (B) Aflatoxigenic colony

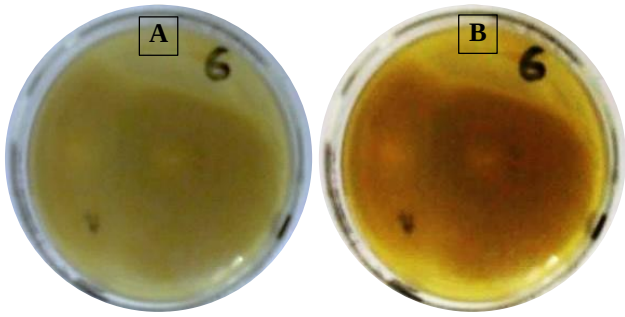

Fig. 4. Detection of aflatoxin producing strains of *A. flavus* by Ammonium Hydroxide Vapor Test.
(A) Aflatoxigenic *A. flavus* colony before exposure to Ammonium hydroxide (B) After exposure

Table 3. Antifungal susceptibility pattern of selected fungal strains.

Name of fungi	Susceptibility against antifungal agents (in percentage)		
	Nystatin	Amphotericin	Fluconazole
Aspergillus flavus (n=5)	100	40	0
Aspergillus niger (n=2)	100	0	0
Aspergillus glaucus (n=1)	100	0	0
Aspergillus fumigatus (n=1)	100	100	0
Aspergillus terreus (n=1)	100	0	0
Aspergillus versicolor (n=1)	100	0	0
Penicillium (n=2)	100	50	0
Rhizopus (n=1)	0	0	0
Absidia (n=1)	0	0	0
Yeast (n=1)	100	100	0
Chaetomium (n=1)	100	0	0
Curvularia (n=1)	100	100	0

Acknowledgement

The authors gratefully acknowledge Higher Education Commission, Pakistan for the partial support in this study through NRPU Grant No: 4497 (awarded to corresponding author).

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(Received for publication 6 April 2018)