

Fungal contamination in smokeless tobacco products traditionally consumed in Pakistan

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Abstract

Objective: To isolate potential pathogenic fungi from smokeless tobacco products.

Methods: The study was conducted from January 2015 to February 2017 during which samples of smokeless tobacco products such as Mainpuri, Tambako, Khiwam, Gutkha, Naswar and Mawa etc. were collected from different cities of Pakistan. The samples were tested for fungal contamination by spread plate method. Different strains of fungi were isolated and identified on the basis of their macroscopic as well as microscopic characteristics. The fungal strains isolated were also screened for their susceptibility to commonly used antifungal drugs by disc diffusion method.

Results: Of the 600 samples collected, 300(50%) were from Sindh, 70(11.7%) Balochistan, 74(12.3%) from Khyber Pakhtunkhwa, 105(17.5%) from Punjab and 51(8.5%) from Azad Kashmir. In terms of products, there were 404(67.3%) samples of Naswar, 69(11.5%) Patti, 40(6.6%) Khiwam, 35(5.8%) Mawa, 32(5.3%) Gutkha, and 20(3.3%) Mainpuri samples. Different species of *Aspergillus* were predominantly isolated followed by *Penicillium*, *Mucor*, *Sepedonium* and *Trichophyton*. The isolated strains of *Aspergillus* also revealed resistance against many commonly-used anti-fungals such as Amphotericin B and Itraconazole.

Conclusion: There was high prevalence of opportunistic fungi in study samples, posing a threat for human health which requires prompt notice and management.

Keywords: Smokeless tobacco products, Opportunistic infections, Mycoses. (JPMA 68: 1471; 2018)

Introduction

Consumption of tobacco in different forms such as cigarettes, snuff and other chewable types is a well-recognised type of addiction throughout the world.¹ In Pakistan, tobacco is consumed nearly by all ages and classes of society either by smoking or by using smokeless tobacco products (STPs). Cigarette smoking is the most popular type of tobacco consumption and is responsible for many health-related issues. The hazards of smoking tobacco are well-recognised as the chemicals, toxins and carcinogens arising from transformation of tobacco leads to a substantial health risk. But the consumption of STPs such as Gutkha, Mawa, Khiwam, Naswar, Patti, Mainpuri etc. are also injurious to health.² The consumption of STPs has been increasing day by day and this rise is due to their low cost and easy availability. Besides, they are available in different flavours which attracts consumers, particularly children. Hence, they are more commonly used by children as well as females compared to cigarette smoking.^{2,3}

Nicotine is considered a major constituent of tobacco and its effects on smokers as well as on passive smokers have

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covered a wide area of research and the results are of a serious nature. But it has been proved that nicotine present in STPs is three times more harmful for an individual compared to tobacco smokers. Therefore, the belief that STPs are harmless is nothing less than an illusion.^{1,4} Nicotine not only forces the adrenal medulla to release adrenaline (epinephrine), but it is also responsible for the release of dopamine. Dopamine is considered to be a neuro-transmitter which initially causes excitation and a feeling of enjoyment, but after the decline of blood concentrations of nicotine, a feeling of tiredness, depression and anxiety overwhelms a person. Drugs that are responsible for the release of dopamine are highly addictive and, therefore, quite hazardous. Moreover, urinary excretion of catecholamine in individuals consuming STPs increases along with an increase in sodium excretion and urine mutagenicity.⁵ Risk of premature labour as well as stillbirth is also a serious consequence of consuming STPs by pregnant mothers. Furthermore, the consumption of STPs by pregnant women also affect the development of embryonic brain.⁵ Recent researches also conclude that children who are prone to using STPs have a higher risk of becoming cigarette smokers within 4 years of consuming the product.⁶

The consumption of ST product not only cause

absorption of nicotine through mouth but also through the gastrointestinal tract, therefore excessive and repeated use of STPs contributes to different cardiovascular, gastrointestinal and respiratory tract diseases.^{4,7} There is an established fact that STPs cause noticeable side effects, including poor oral hygiene, staining of teeth, painful sore gums and bleeding gums etc. Social problems, such as bad breath, and the need to spit the excessive saliva every now and then makes a person devilish not only in family but also in society. Additionally, there is 3.6 times greater risk of osteoporosis in men consuming tobacco compared to non-tobacco users.⁸ The more dangerous aspects of STPs consumption is the presence of carcinogens such as nitrosamine, formaldehyde, hydrazine, arsenic, nickel, cadmium, crotonaldehyd etc.^{4,9} Therefore, there is not only an increased risk of cancer of oral cavity in people consuming STPs but evidences of pharyngeal, laryngeal and oesophageal carcinoma are also reported in such individuals.¹⁰

Different constituents of these STPs include agricultural products which are exposed to wide range of microbial contamination. Factors such as climatic conditions, poor storage practices etc. are responsible for the growth of different microorganisms, particularly fungi, in these products. Their presence has been reported to cause microbial infections among consumers. Researchers have clearly concluded by conducting numerous studies the presence of microorganisms in these products. Cases related to the presence of bacterial species, *Bacillus (B.) licheniformis* and *B. pumilus* in these STPs and their involvement in causing inflammation of lungs have been reported.¹¹ Moreover, infections by certain *bacillus* species present in STPs causing outbreaks of diarrhoea and vomiting are also reported.¹² Isolation of *staphylococcus (S.) epidermidis* and *S. hominis* in these STPs is also alarming due to their property of reducing nitrates to nitrites, leading to the formation of tobacco-specific *N-nitrosamines* which are potential carcinogens.¹² Besides, the presence of fungal species such as toxic molds of *Aspergillusglaucus (A.)*, *A.niger*, *A. fumigates* and *A. flavus* have been reported in tobacco products. The fungal contamination in STPs not only causes fungal infections among consumers, but it can also cause mycotoxicoses due to the consumption of such products contaminated with mycotoxins produced by mycotoxigenic fungi. The presence of aflatoxin in these products have been confirmed in many researches showing their potential to cause aflatoxicosis.^{9,13} However, literature appraisals indicate the scarcity of information on the presence of fungal strains in STPs

consumed in our society.

The current study was planned to evaluate the natural existence of different fungal strains in indigenous STPs available in the markets of different cities of Pakistan. Besides, the antifungal susceptibility profile of the isolated fungal strains was also planned to be determined.

Materials and Methods

The sample frame of the study is all possible focused group of Pakistan under significant demographic characteristics. It has been observed that very few related studies are found with reference to the prevalence of the Fungal Contamination in Smokeless tobacco products.

In this connection, a cross sectional study was designed for desired data collection and 600 samples were taken under the Convenience Non-Probability Sampling Procedure, adopted with Stratified sampling method. The sampling frame was stratified into 8 strata such as Lahore, Quetta, Karachi, Peshawar etc. for data collection.

The cross-sectional study was conducted from January 2015 to February 2017 during which samples of STPs were collected from different cities of Pakistan. Convenience non-probability sampling procedure was adopted with stratified sampling method to select areas and their stratification. Karachi was selected from Sindh, Quetta from Balochistan, Peshawar and Mardan from Khyber Pakhtunkhwa (KP), Lahore, Sargodha and Chichawatni from Punjab, and Azad Kashmir. STP samples collected were related to Naswar, Patti, Khiwam, Mawa, Gutkha and Mainpuri from pan shops, vendors, cobblers, wholesalers and different manufacturing places. These samples were collected in sterile containers with appropriate labels. The samples were processed immediately or stored at cold temperature (4°C) until the time of processing.

The analysis of different STP samples for the presence of fungi was done by spread plate method. One gram of each sample was mixed with 10 mL of sterile distilled water and was vortexed to make homogenised suspension. The serial dilutions of homogenised suspension were made and were carefully inoculated on Sabouraud's Dextrose agar (Oxoid, UK) plates. The plates were incubated at room temperature for seven days.¹⁴

The isolated strains of fungi were identified by conventional methods mainly depending on macroscopic and microscopic characteristics. The colonies were examined macroscopically by examining their texture, colour, reverse of plate and pigmentation, while microscopic studies were carried out by using wet mount

technique and slide culture technique. The slides were observed under 10x and 40x of microscope.

Strains of *Aspergillus* isolated from different STPs were tested for antifungal susceptibility by disc diffusion method. Fungal strains were sub-cultured on Sabouraud's Dextrose agar (Oxoid, UK) and incubated at 28°C for 7 days to enhance sporulation. For the preparation of inoculum, these fungal strains were inoculated separately in Sabouraud's Dextrose broth (Oxoid, UK) for 6 hours. Size of the inoculum obtained was approximately 106cfu/ml (0.5 McFarland scale).¹⁴ The fungal suspension was gradually and evenly spread on Mueller-Hinton agar (Oxoid, UK). These plates were dried for approximately 15 minutes. Commercially available discs, fluconazole (10µg), itraconazole (10µg) and amphotericin B (20µg) were used. These discs were applied to each inoculated plate and then incubated at 25°C for up to 48 hours. After incubation, the zones of inhibition were measured for each antifungal agent in millimetres and recorded.¹⁵

Results

Of the 600 samples collected, 300(50%) were from Sindh, 70(11.7%) Balochistan, 74(12.3%) from KP, 105(17.5%) from Punjab and 51(8.5%) from Azad Kashmir. In terms of STPs, there were 404(67.3%) samples of Naswar, 69(11.5%) Patti, 40(6.6%) Khiwam, 35(5.8%) Mawa, 32(5.3%) Gutkha, and 20(3.3%) of Mainpuri (Table-1).

All the samples of STPs were found contaminated with different fungal strains which were identified as

potential pathogenic species (Figure-1). *Aspergillus* was found to be most prevalent fungi and among its species, *A. Fumigatus* (25.51%) was isolated predominantly. However, other species such as *A. Flavus* (24.36%), *A. Terreus* (7.04%), *A. Niger* (16.05%), *A. Glaucus* (6.004%), *A. Nidulans* (9.006%) and *A. Versicolor* (0.46%) were also isolated in remarkable numbers (Figure-2). *Penicillium* (0.92%), *Mucor* (6.58%), *Sepedonium* (3.81%) and *T. Rubrum* (0.23%) species were also isolated from the STPs.

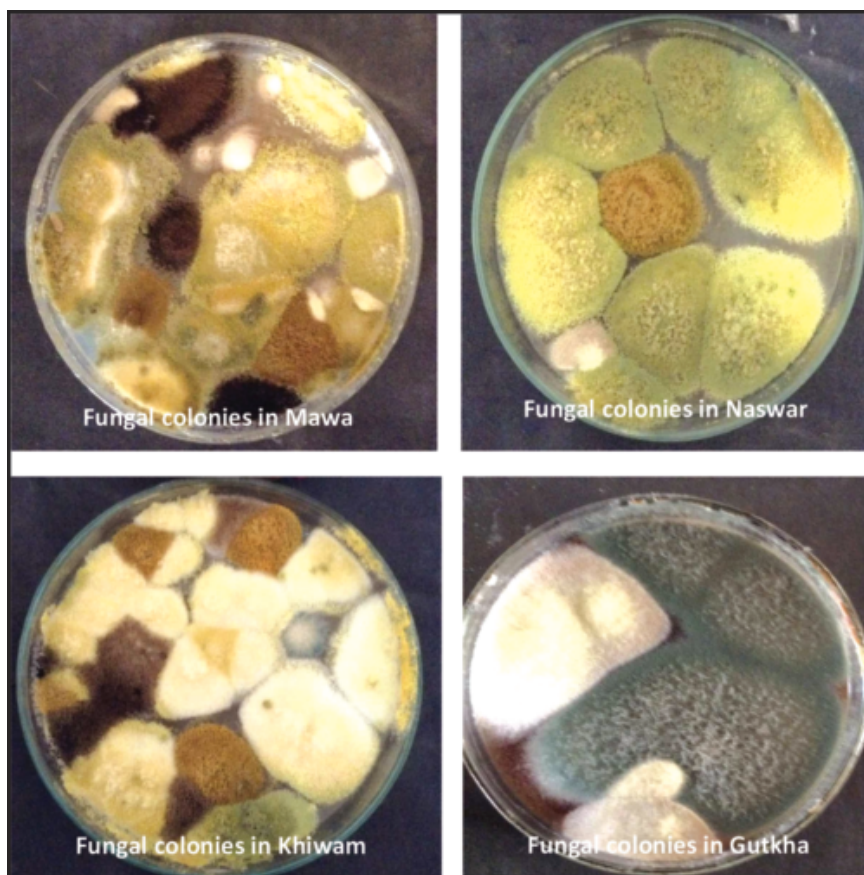


Figure-1: Fungal strains isolated from different smokeless tobacco product.

Table-1: Collection of smokeless tobacco products for the isolation of fungi from different cities of Pakistan (n = 600 samples).

Chewable Tobacco Product	Samples collected from different cities								Total No. of individual samples
	Sindh Karachi	Baluchistan Quetta	Khyber Peshawar	Pakhtunkhwa Mardan	Sargodha	Punjab Chichawatni	Lahore	Azad Kashmir Azad Kashmir	
Naswar	165	50	36	38	30	20	40	25	404
Patti	50	13	0	0	0	0	0	6	69
Khiwam	30	0	0	0	0	0	0	10	40
Mawa	20	0	0	0	0	5	0	10	35
Gutkha	15	7	0	0	0	0	10	0	32
Mainpuri	20	0	0	0	0	0	0	0	20
Total No. of products	300	70	36	38	30	25	50	51	-

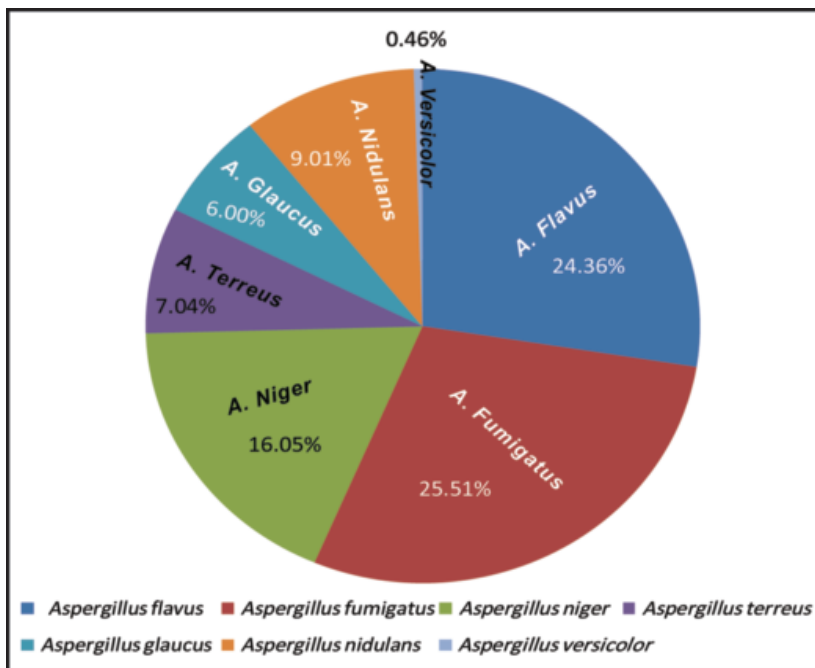


Figure-2: Percentage distribution of *Aspergillus* species among different smokeless tobacco STPs.

Naswar was the highest consumed STP and was collected from all the cities. Naswar samples showed high prevalence of *A. Fumigatus* (25.23%) and *A. Fluvus* (25.09%), while other fungal species of *Mucor*, *Sepedonium* and *T. Rubrum* were also isolated (Figure-3). Gutkha, the second most commonly used smokeless tobacco product observed in our study was found to be inhabited with spores of *Mucor spp.* (77.78%) and *Penicillium spp.* (22.22%) while in case of Mainpuri, the fungal contaminant were *Aspergillusfluvus* (50%) and *Sepedonium spp.* (50%).

The antifungal susceptibility of isolated *Aspergillus* strains from different STPs revealed variable responses to different antifungal agents (Table-2). High rates of resistance were observed for amphotericin B from different species such as *A. Fluvus* and *A. Fumigatus*, while *A. Niger* was found to be 100% susceptible to this drug

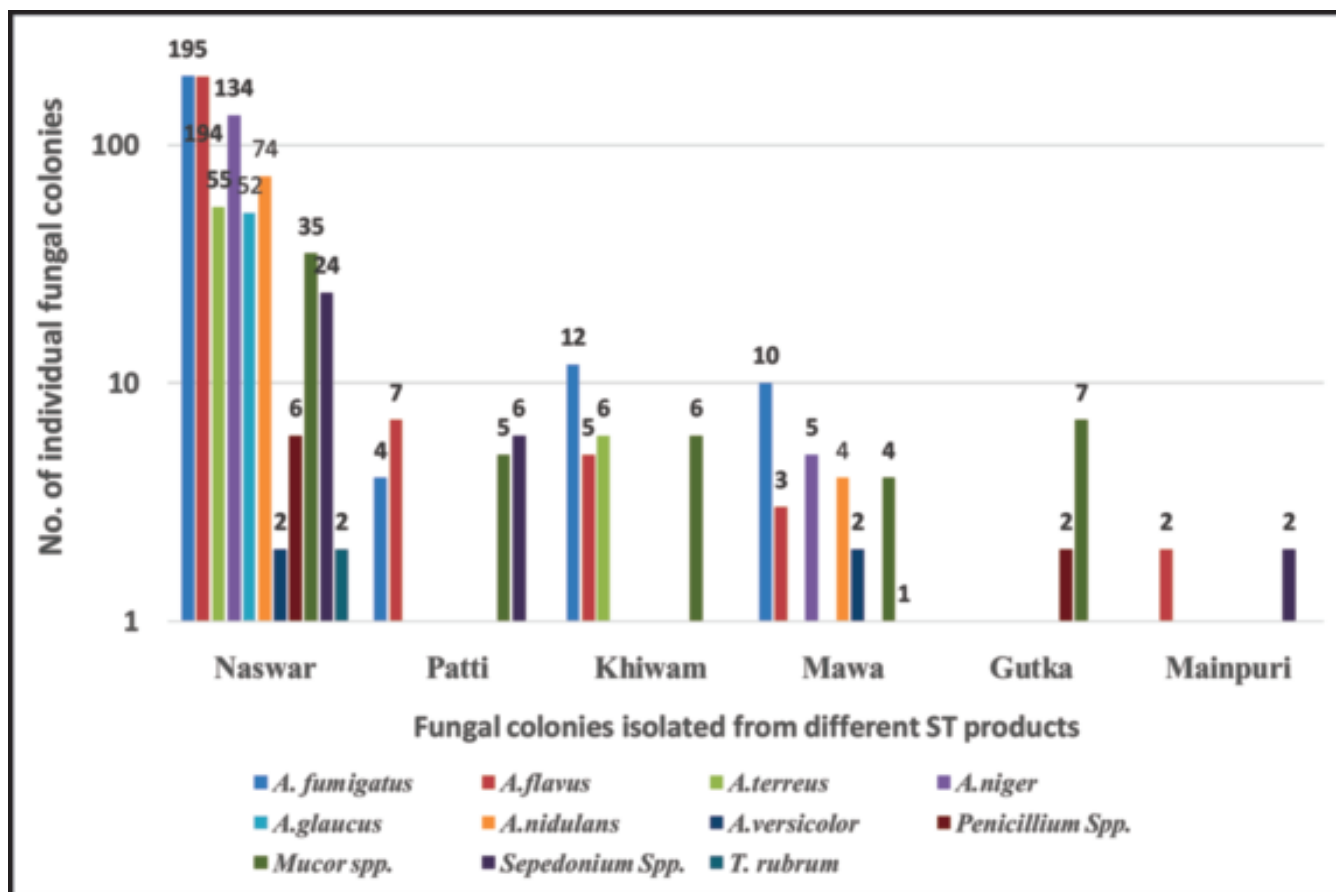


Figure-3: Distribution of Isolated fungal strains among various smokeless tobacco products.

Table-2: Antifungal resistance profile of selected *Aspergillus* species isolated from smokeless tobacco products.

Name of fungi isolated	No. of strains tested	Amphotericin B (20 µg)	Itraconazole (10 µg)	Fluconazole (10 µg)
<i>A. Flavus</i>	20	R (18)	S (13)	R (17)
<i>A. Fumigatus</i>	20	R (16)	S (11)	R (19)
<i>A. Niger</i>	20	S (20)	R (15)	S (16)
<i>A. Terreus</i>	10	R (8)	R (6)	R (7)
<i>A. Glaucus</i>	10	R (7)	R (9)	R (6)
<i>A. Nidulans</i>	16	R (11)	R (13)	R (12)
<i>A. Versicolor</i>	4	R (4)	R (4)	R (4)

* (R) Resistance and (S) susceptible

* Resistant: ≤ 14 mm

* Susceptible: ≥ 19 mm

Reference: Method for antifungal Disk Diffusion susceptibility testing of molds; Approved guidelines - second edition. 2001 NCCLS document M23-A2.

although it exhibited strong resistance against itraconazole. Similarly, high resistance rates were also observed from *A. Terreus*, *A. Glaucus*, *A. Nidulans* and *A. Versicolor* against almost all of these antifungal agents.

Discussion

Evidence of tobacco consumption dates back as far as 5000 to 3000 BC when its cultivation began in Mesoamerica and South America. The ancient civilization of Babylonians, Chinese and the Indians used tobacco by burning it for fragrance during religious events. During that era the smoking of tobacco and various hallucinogenic drugs were used to cast spells and to come into contact with the spiritual world. It was believed that tobacco was a gift from the Creator and that the exhaled tobacco smoke was capable of carrying one's thoughts and prayers to heaven.¹⁶

Nowadays, tobacco has been used worldwide as a more popular type of addiction. It has been consumed in two ways, either by smoking in the form of cigarette, Beri, Hukka etc. or by using chewable tobacco products, such as Gutkha, Naswar, Mawa, Mainpuri etc. The use of tobacco, especially by smoking, has been well-recognised as the leading cause of different cancers as well as chronic lung diseases such as emphysema and bronchitis. It has also been associated with different heart diseases, pregnancy-related problems, and many other serious health issues, including microbial infections.^{4,17} The consumption of tobacco in chewable forms (smokeless) was considered less harmful compared to tobacco smoking, but in reality they are 3-4 times more hazardous compared to smoking tobacco.¹⁸ Being potent hallucinogens, easy availability and their extremely affordable cost increases the rate of consumption of these STPs in our society. The consumers of STPs belong to more or less all classes of society, including both genders and all ages. These products vary greatly in appearance,

toxicant release and in their composition of tobacco and non-tobacco ingredients. STPs range in variety from simple tobacco to rich products with numerous chemical ingredients as well as non-tobacco material that may affect the attractiveness, addictiveness and toxicity of these products. The most commonly used ingredients in these products include alkaline agents such as slaked lime, calcium carbonate, alkaline ashes that boost the potential of hydrogen (pH) of product readily enhancing the absorption of nicotine through mucous membrane.¹⁹ In addition, most STPs contain areca nut rich in arecoline which enhances the addictive property of a product as it acts on the same receptors in brain as nicotine.²⁰

In terms of production, STPs are either premade or custom-made products. Premade STPs are made for sale and are generally consumed as ready-to-use. They are either commercial products as moist snuff, Khaini etc. that are made in traditional manufacturing settings such as factories.²¹ The other ones are the cottage products as Naswar, Mainpuri, Mawa etc. They are made in non-traditional production environments as markets, stalls shops, houses, etc. and often sold in non-commercial packaging (paper or plastic bags; wrapped in paper). Owing to their ingredients rich in organic substances, the moisture contents of these products and, above all, the manufacturing hazards make them an ideal niche for the growth of microorganisms such as bacteria and fungi. Thus, the fungal contamination in edible items not only predisposes an individual to acquire mycoses but there are also chances of mycotoxicoses. All STPs have tobacco as its main constituent and in turn nicotine as the most important psychoactive agent. This nicotine exerts immune-suppressive effect by altering the levels of cytokines, hence the consumers of STPs are prone to developing infections.

The present study found a variety of fungal strains isolated from almost all types of STPs which is in

accordance with the results of a study conducted for microbial flora of tobacco almost 4 decades back.²² Most of these strains are reported as potential pathogens and are responsible for causing mild to life-threatening infections.^{12,21} Among isolated fungi, aspergillus species were found to be the most abundant and the most important encountered fungal pathogens. *A. fumigatus* was found to be the predominant strain, while its occurrence in Naswar samples was also found. This high load of *A. fumigatus* in Naswar samples is an alarming situation as the specie is considered a potential pathogen and has great clinical importance, particularly in immune-compromised individuals, this specie is responsible for causing aspergilloma and allergic bronchopulmonary aspergilloses.²³

Moreover, *A. flavus* was also isolated in high rates from Naswar samples. This fungal specie is not only a pathogen but also a potential source of aflatoxin.²⁴ Aflatoxin is known to be the most carcinogenic substance and no human and animal species is immune to it. Aflatoxin exposure not only causes liver damage but it is a source of various other cancers, including cancer of the gall bladder. Additionally, aflatoxin causes aflatoxicoses in children, resulting in stunted growth and delayed development.²⁴

A. Niger, another important isolated specie, is known to cause serious otomycosis characterised by severe pain and swelling of the ear with temporary hear loss and in severe cases damage to ear canal and tympanic membrane. Similarly, *A. Glaucus*, which is also isolated, is reported to produce mycotoxin and even fatal brain infection. *A. Versicolor*, an opportunistic pathogen reported to have caused aspergilloses as well as onychomycosis, was also isolated from STPs but not in a very high volume. Moreover, *T. Rubrum*, one of the most common causative agents of dermatophytosis, was also isolated from these products.

Other isolated species from these STPs included *Penicillium*, *Sepedonium* and *Mucor*, which were also reported in previous literature as potential pathogens.²⁵

In the current study, the antifungal susceptibility pattern of isolated aspergillus species showed increasing resistance against commonly-used antifungal drugs such as amphotericin B, itraconazole and fluconazole. Amphotericin B is considered a drug of choice for antifungal systemic infections, but in the present study, certain strains of *A. Flavus*, *A. Fumigates*, *A. Terreus* and *A. Glaucus* (70%) were resistant against this drug which is in accordance with the findings reported earlier.²⁵ Likewise, strains of *A. Niger*, *A. Terreus*, *A. Glaucus*, *A.*

Nidulans and *A. Versicolor* showed resistance against itraconazole, while *A. Flavus* and *A. Fumigates* were found susceptible to this antifungal agent. Fluconazole was found effective for *A. Niger*.

The unavailability of control due to enormous strains is one of the limitations of the study.

Conclusion

The established evidence of occurrence of pathogenic fungi in the most consumed STPs pose a great danger on human health as it is consumed by majority of individuals irrespective of age and class. Prompt measures must be adopted by health authorities to safeguard the health of the general public, especially the upcoming youth.

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