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Fungal Contaminants of Selected Commonly Used Spices in Tanzania

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Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

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ABSTRACT

Fifty samples of sixteen spices commonly used in Tanzania were collected randomly from different local markets around Dar es Salaam and fungal contaminants determined using standard microbiological procedures. Forty nine filamentous fungi from 7 genera namely; Aspergillus, Fusarium, Rhizomucor, Rhizopus, Lichtheimia, Cladosporium and Penicillium were encountered. Further characterization of some fungi using nucleotide sequencing of the 5.8S-ITS rRNA gene was done and their phylogeny inferred using Unweighted Pair Group Method with Arithmetic Averages (UPGMA). The fungi isolates were identified as *Lichtheimia ramosa, L. corymbifera, Rhizomucor pusillus, R. tauricus, A. aculeatinus, A. parasiticus, A. flavus, A. tubingensis, A. fumigatus, A. niger* and *A. nomius*. Red chill had high level of fungal contamination (18.37%) followed by ginger (14.28%) while curry powder and coriander seeds were less contaminated (2.08%). These results give baseline information on fungal contamination in spices. Proper spices management that will minimize risks of fungal contamination and their metabolites at all stages from planting, harvesting, processing, packaging, transportation, handling and storage is recommended to avoid health risks to consumers.

Keywords: Aflatoxin; Aspergillus; filamentous fungi; 5.8S-ITS rRNA; spices; Dar es Salaam.

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1. INTRODUCTION

Spices are group of plant substances with strong taste and aroma which are used in small amounts as flavoring agents in various foods [1]. They are valued for their colors, taste and aroma making them most versatile and widely used ingredients in numerous food preparations throughout the world. Most of the spices are also consumed due to their associated health benefits such as antioxidants, anti-allergens and antimicrobial effects [2,3]. Spices are used in small amount in foods and sometimes are eaten raw or half cooked eg black pepper, ginger, red chill and sesame. Microbial contaminants such as filamentous fungi can grow or contaminate spices at various stages during harvest, processing, transportation, storage and handling. They can compromise spices quality and safety since they can produce secondary metabolites (mycotoxins) that are harmful to humans, animals and environment. Several studies reported on fungal contamination of spices and most of the fungal genera reported have potential to produce toxic metabolites known as mycotoxins [4,5]. Fungal contaminants and mycotoxin problem is aggravated by warm humid tropical conditions and inadequate drying which provide optimal conditions for fungal growth and subsequent production of mycotoxins. These toxic metabolites cause serious human health problems such as cancer and DNA mutations. For instance aflatoxins which are produced primarily by Aspergillus fungi are carcinogenic, teratogenic, estrogenic, hemorrhagic, immunotoxic, nephrotoxic, hepatotoxic, dermotoxic and neurotoxic [6]. Other harmful mycotoxins are Ochratoxin A that is produced by numerous species of Aspergillus and Penicillium, citrinin and fumonisins produced by Fusarium species. However, threshold values of mycotoxins such as total aflatoxins in various food products has been set by responsible regulatory authorities in each country so as to maintain health standards of consumers. In Tanzania total aflatoxin levels permitted for most of agricultural products range from 10 – 15 ppb [7] while in other regulatory authorities like European Commission Regulation the limits is 4 ppb for total aflatoxins in cereals and peanuts and 10 – 15 ppb for spices [8]. It is therefore important to observe proper handling of agricultural food products so as to maintain required standards for international trade.

In Tanzania spices are sold in almost all local markets and for various purposes including for supplements, food taste, aroma and food preservative. It is among a food ingredient that is used in almost all households irrespective of their income status. Despite its importance in daily food and food product processes, very little has been done to assess microbial status of spices especially filamentous fungi contamination and its health risk implication. Temu [9] identified a few Aspergillus species from only nine types of spices and found three potential aflatoxin producing strains. Other previous studies in Tanzania have mostly reported risks of fungal and mycotoxin contamination in staple crops like maize [10-12] and cassava [13]. Further research should be extended to other agricultural products such as dried and milled spices. This study therefore intended to determine fungal contaminants associated with commonly consumed spices in Tanzania and give baseline information on their safety to consumers based on fungal genera which are known to produce toxic metabolites to humans and animals.

2. MATERIALS AND METHODS

2.1 Sampling

Fifty samples of 16 types of commonly used spices were bought randomly from local markets and Indian shops around the Dar es Salaam City between January and February, 2015. Each sample was bought in various forms as described in Table 1. All samples were sealed in clean paper envelopes or polythene bags depending on the type of spice. Some spices that were industrially produced were already packed in tins or bottles. All samples were taken to the Microbiology Laboratory of the Department of Molecular Biology and Biotechnology University of Dar es Salaam. The common names, scientific names and used parts of each spice are as presented in Table 1.

2.2 Moisture Content Determination, Culturing and Isolation of Filamentous Fungi

Twenty gram of each sample was weighed and dried at 100℃ in a hot oven for 24 hours and the moisture content was determined. Fungi were isolated by using agar plate method where by 4 g of each sample (non- powder samples were first ground using motor and pestle) were dissolved in 20 ml of Maximum Recovery Diluent (HiMedia®) and 200 µl from the mixture was used to inoculate petri dishes containing 20 ml of Malt extract agar (Difco™), Dichloran Glycerol (DG-

18, HiMedia®) media and Potato Dextrose Agar (HiMedia®). To suppress bacterial growth chloramphenicol (25 mg/l) was added in the medium and the plates were incubated at 30℃ for 3 to 7 days. Fungal colonies emerged were examined virtually and microscopically and colonies counted as colony forming units (CFU)/g. Pure colonies of fungal isolates were obtained by sub-culturing on similar media and were further characterized based on macro and microscopic characteristics.

2.3 DNA Extraction

DNA was extracted from fungal mycelia harvested from 24 hours cultures in malt extract broth as described in Temu [9]. Nano-spectrophotometer (XNanoDrop® ND1000, Thermo Scientific) was used to quantify the DNA and all samples were normalized to 50 ng/ µl before use.

2.4 PCR Amplification and Nucleotide Sequencing

PCR of 5.8S–ITS rRNA gene and DNA nucleotide sequencing (Sanger's method) of 28 fungal isolates was done at Inqaba Biotec ™ , South Africa using ITS1 (5´TCC GTA GGT GAA CCT TGC GG 3´) and ITS4 (5´TCC TCC GCT TAT TGA TAT GC 3´) primers. Nucleotide sequence cleaning and analysis was done as described previously in Temu (2016). Comparison of nucleotide sequences to those available in the National Center for Biotechnological Information (NCBI) using nucleotide basic alignment search tool (nBLAST) identified the fungi to the nearest similar accession. The two most similar accessions to each fungal isolate were taken to infer the phylogenetic relationship using Unweighted Pair Group Method with Arithmetic Averages (UPGMA) method with Kimura 80 parameters and 1000 bootstrap values. Nucleotide sequence alignment was done using CLC workbench 7.6.4 (QIAGEN©) software and phylogenetic trees were developed using the same software.

3. RESULTS

3.1 Moisture Content and Fungal Isolates

Most of the spices had moisture content ranging from 7.0 -15.3% (Table 2). Cloves, cardamom and cinnamon had relative higher moisture content amounting to 15.3, 13% and 14.4% versus 12, 12 and 14% respectively. Spices with relative lower moisture content were mustard (7.7 vs 10%), chill (7.5 vs 11%), coriander seeds (8.1 vs 12%) saffron (8.4 vs 10%), black pepper (8 vs 12%), caraway (10.1 vs 13%) and cumin (9 vs 13%). Forty nine (49) fungal isolates were found contaminating 81.25% of all spices tested (Table 2). Differentiation based on the growth characteristics and morphological examination under the microscope (B-350 OPTIKA) at 400 magnifications placed the isolates into seven genera namely Aspergillus, Rhizopus, Fusarium, Rhizomucor, Lichtheimia, Penicillium and Cladosporium. About sixty two percent (62.5 %) of all spices tested were contaminated by *A. niger* and *Rhizopus* spp, 37.5% with *A. fumigatus* while 25% were contaminated with *A. flavus*. The least contaminants were Lichtheimia (11.8%) and Cladosporium (5.8%) spp. Red chill and ginger had higher number of fungal genera while no fungal was isolated from cloves, nutmeg and saffron (Table 2). Fig. 1. depicts some morphological and colony characteristics of filamentous fungi from representative spices.

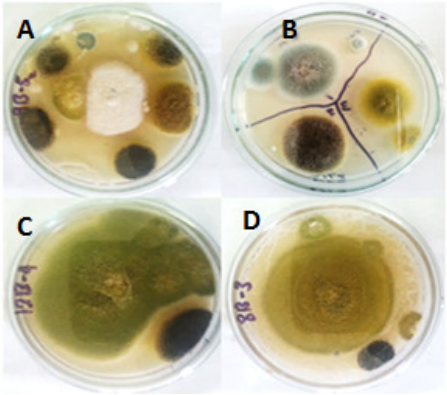

Fig. 1. Representative fungal colonies from A: red chill; B and C: caraway; D: ginger, five days post inoculation on MEA

3.2 Nucleotide Sequencing and Phylogenetic Analysis

Nucleotide sequence analysis of 28 fungal isolates in relation to other nucleotide sequences available in the NCBI database clustered the fungal isolates into six main groups (Fig. 2), supported with 100% bootstrap values. The accession EU293436 which is *Candida tropicalis* was used as an out-group strain. Isolates 12B-3 and 8B-1 isolated from ginger and caraway respectively clustered closely with a human pathogenic fungal *Lichtheimia ramosa* (KP132382) and

L. corymbifera (JN638761). Isolate 9B-6 from red chill clustered with FJ713079 and FJ713094 which are *Rhizomucor pusillus* and *R. tauricus* respectively. Two isolates namely 9B-2 and Co-1 clustered well with *A. aculeatinus* (KM278127) while isolates 9B-3 and 9B-4 from red chill clustered with *A. parasiticus* (AY373859) and *A. flavus* (KP278181) respectively. Samples Co-4, Cu-1, Co-2 and 6B-3 clustered with *A. tubingensis* (JN585944). Other fungal isolates clustered well with their respective accessions as shown in Fig. 2. Based on the nucleotide sequence analysis fungal identities were revealed as *Lichtheimia ramosa, L. corymbifera, Rhizomucor pusillus, R. tauricus, A. aculeatinus, A. parasiticus, A. flavus, A. tubingensis and A. nomius*.

Table 1. Description of spices used in this study

Code	English name	Swahili name	Scientific Name	Part used
Ca	Cardamom	Iliki	*Elleteria cardamomum*	Seeds, powder
BP	Black pepper	Pilipilimanga	*Piper nigrum*	Powder
Cu	Cumin	Jeera	*Cuminum cyminum*	Seeds
Co	Coriander	Pilipili mtama/uzile	*Coriandrum sativum*	Seeds
6B	Turmeric	Manjano	*Curcuma longa*	Powder
7B	Cloves	Karafuu	*Syzygium aromaticum*	Seeds
Ci	Cinnamon	Mdalasini	*Cinnamomum zeylanicum*	Barks
8B	Caraway	Binzari nyeusi	*Carum carvi*	Powder
9B	Red chill	Pilipili nyekundu	*Capsicum annuum*	Dried fruits
10A	Mustards	Haradali	*Brassica nigra*	Seeds
12B	Ginger	Tangawizi	*Zingiber offficinale*	Dried rhizomes powder
13B	Fenugreek	Uwatu	*Trigonella foenum-graecum*	Seeds, powder
19B	Curry	Kare	*Murraya koenegii*	Powder
23A	White pepper	Pilipili nyeupe	*Piper nigrum*	Seeds
24B	Saffron	Zafarani	*Crocus sativus*	Powder
Nt	Nut meg	Kungu	*Myristica fragrans*	Seeds

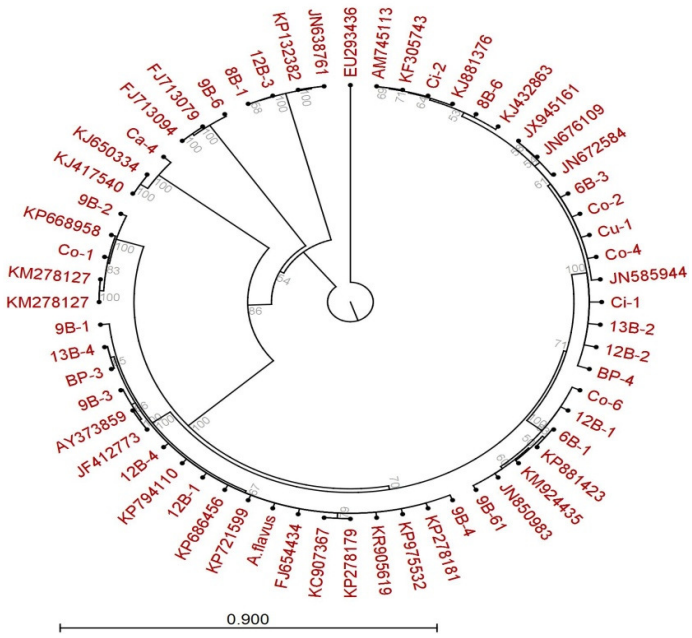

Fig. 2. Phylogenetic tree inferred using the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) with Kimura 80 parameter. Numbers in the nodes are bootstrap values with threshold above 50%. *Candida tropicalis* strain XM11A (EU293436) was used as an out-group

Table 2. Moisture content and fungal isolates from each spice

Sample name	Moisture content (%)	Recommended moisture content by ESA*	Fungi isolated	Fungal isolates (%)
Black pepper	8.0	12	*Yeast, Rhizomucor, Rhizopus, A. flavus, Cladosporium, A. niger*	12.24
Caraway	10.1	13	*A. niger, A. fumigatus, Rhizopus, Lichtheimia*	8.16
Cardamom	13.3	12	*A. niger, A. flavus, yeasts, Rhizopus*	8.16
Cinnamon	14.4	14	*Rhiopus, Rhizomucor*	4.08
Cumin	9	13	*Rhizopus, Yeasts*	4.08
Coriander seeds	8.1	12	*A. aculeatinus, Yeasts, Rhizopus*	6.12
Curry powder	7.0	Not clear	*A. niger*	2.04
Fenugreek powder	7.3	Not clear	*A. niger, A. fumigatus, Rhizopus*	6.12
Fenugreek seeds	11.2	11	*A. niger*	2.04
Ginger powder	10.9	12	*A. fumigatus, A. niger, A. flavus, A. nomius, Rhizomucor, Lichtheimia, Rhizopus*	14.28
Mustard seeds	7.7	10	*Yeasts, A. fumigatus, Aspergillus spp*	6.12
Red chill powder	7.75	11	*A. flavus, A. niger, A. fumigatus, Penicillium, A. aculeatinus, Fusarium, Rhizopus, Rhizomucor, A. parasiticus,*	18.37
Turmeric	9.6	10	*A. niger, A. fumigatus, A. tubingensis*	6.12
White pepper	10.9	12	*A. niger, Rhizopus*	4.08
Nutmeg	Not tested	10	*Nil*	Nil
Cloves	15.3	12	*Nil*	Nil
Saffron	8.4	10	*Nil*	Nil

**ESA= European Spice Association*

4. DISCUSSION

Contamination of spices may arise from poor hygienic conditions during harvesting, processing, transportation and storage. Fungal contamination is further intensified by warm humid tropical conditions and partial drying which provide optimal conditions for fungal growth and consequently production of mycotoxins [5]. Most of the spices had moisture content ranging from 7.0 -15.3%. According to [14] most of the spices were within or below the allowed range of moisture content in spices. Cloves, cardamom and cinnamon had relative higher moisture content while spices with lower moisture content below the recommended were cumin, black pepper, coriander seeds and red chill (Table 2). Complete drying of spices, proper packaging and storage can minimize growth of microorganisms such as fungi, bacteria, and yeasts. Other factors such as presence of antimicrobial activity properties such as essential oils can also limit their growth [15,16]. However, despite of having the highest moisture content, no fungal colonies were isolated from cloves even upon several repeats of culturing. This might be attributed by its antimicrobial properties and essential oils that are effective against certain microbes including fungi [15,16]. In this study curry powder, fenugreek seeds, cumin, cinnamon and white pepper were among less contaminated spices with only one or two fungal genera. These results are in agreement with [17] who also observed low levels of fungal contamination from cloves,

cinnamon and cardamom. Presence of antimicrobial (antifungal) active components in cinnamon and essential oils in cumin spices might have been contributed to lower contamination observed in these spices. Abou [18] also reported low levels of fungal contaminations in cumin while [5] observed lower levels of aflatoxin in curry and nutmeg. No fungus was isolated from nutmeg and saffron probably due to less exposure to fungal contaminations during storage and handling. However, both saffron and nutmeg contain essential oils and or antimicrobial activities that can inhibit fungal growth [19,20]. The most contaminated spices were red chill powder (18.37%) followed by ginger (14.28%) and black pepper (12.24%). Contamination might have occurred during sun drying in the open air, through processing techniques such as grinding and also from storage.

The most contaminating fungal genera were of the Aspergillus group. More than 50% of all spices were at least contaminated with an *Aspergillus* spp. High incidence of *Aspergillus spp* on spices calls for attention since among others, *A. flavus, A. parasiticus* and *A. nomius* are known to produce aflatoxins [21,22]. Aflatoxins are of major concerns in food chains due to their potent effects to human health. Hashem and Alamri [17] also reported *Aspergillus* spp as the main contaminants of spices collected from Saudi Arabia markets. Abou [18,23] also reported *A. niger* and *A. flavus* as dominant fungi in Egyptian and Indian spices respectively. *A. tubingensis*, a wide spread fungi which grows predominantly on dead plant materials, *A. fumigatus* and *A. aculeatinus* were also isolated. *A. niger* and *A. fumigatus* were common fungi isolated from more than 50% of the samples probably due to their opportunistic ability to grow on various hosts. Penicilium and Fusarium were isolated from red chill powder only. Some *Penicillium* spp are known to produce toxic metabolites like ochratoxin and citrinin [23] while some Fusarium strains produce fumonisins. Although not all fungal strains in these two genera are toxin producers, their presence in food stuffs are still of major concerns. Hashem and Alamri [17] also observed Penicilium as among three common genera that contaminated Saud Arabia common spices while Fusarium was detected as the least contaminant. Toma and Abdulla [2] also isolated Penicillium and Cladosporium species from spices and medicinal plants while [22] isolated both Penicillium and its metabolite ochratoxin A in red chill and other various spices. Lichtheimia has rarely reported to contaminate spices but was found in this study. *L. corymbifera* (earlier *Absidia corymbifera*) is a soil-borne fungus, which is an opportunistic human pathogen especially in immune-compromised patients hence can cause life-threatening diseases in humans. However, *L. corymbifera* was among fungal species reported by [24] from spices imported in Bahrain.

Higher levels of fungal contamination observed on red chill and black pepper has also been reported elsewhere [24-26,5]. Mandeel [24] reported that red chill was the most contaminated spice followed by black pepper while [26] also found higher levels of aflatoxin in red chill from Pakistan. Using simple qualitative detection of total aflatoxin, [9] also reported total aflatoxin amounting to or higher than 4 ppb in red chill. Therefore, from literature reports precautions should be taken during processing and handling of black peppers and red chill since they seem more prone to fungal and aflatoxin contaminations compared with other spices. In Tanzania, black pepper is commonly used by people along the Coast and Muslim communities to prepare porridge especially during the holy month of Ramadhan. However, black pepper powder is normally added in ready-to-drink porridge hence it may increase the risk of fungal and their toxic metabolites to consumers. Similarly, red chill is commonly prepared locally and served with meals in almost all restaurants around the cities, rural areas and households.

PCR and sequence analysis of 5.8S-ITS rRNA gene was used as a tool to ascertain the identity of fungal isolates. The six clusters resulted from UPGMA revealed identity and relationship between the isolates and data bank accessions. Six fungi isolates that are in the category of potential aflatoxin producers were identified as *A. flavus* (3), *A. parasiticus* (2) and *A. nomius* (1). These isolates were from black pepper, cardamom, ginger and red chill. Red chill had both *A. flavus* and *A. parasiticus*.

5. CONCLUSION

This study reports fungal contaminants of several commonly used spices in Tanzania. The current results give baseline information and alert on the potential fungi that may associate with spices. Spices can suffer microbial contamination and their additions to ready to-eat foods can result in the proliferation of fungi which can be

pathogenic. Further, most of the spices used in this study were bought from open markets, some packed in old sacs and stored in humid conditions, clustered together with other food commodities such as vegetables and sardines. Some of the spices were already packed in small polythene sacs while others were sold openly. Therefore, proper handling and storage conditions should be observed so that each spice is packed and stored appropriately under hygienic dry conditions to minimize fungal growth. If fungi contamination could be kept at minimal levels would consequently help maintain health standards of spice consumers. Moreover, a need to advocate proper harvesting methods, processing, transportation, storage and handling of spices that will minimize risks of fungal contamination and their metabolites is inevitable.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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