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Isolation of Fungi and their Mycotoxin Extract from Stored Wheat and Other Grains Importer in Saudi Arabia

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ABSTRACT

Wheat and other grain samples were separately analysed to identify the presence of fungi and their mycotoxins. The mycological profile of the retail in different markets at Riyadh (Kingdom of Saudi Arabia) was studied. Sixteen samples were collected from storage shops importer from Saudi Arabia. The most common genera were *Alternaria* (isolated from 68.96% of the tested samples), *Aspergillus* (24.14%) and in a lesser extent *Fusarium* (6.9%). Mycotoxin production by isolated fungi was subsequently evaluated using the High-Performance Liquid Chromatography Technique (HPLC). The present results indicate that mycotoxins with aflatoxin are widespread fungal contaminants of wheat. Therefore, their presence as well as the occurrence of mycotoxins should be further investigated to assess health risks linked with the consumption of this commodity.

Key words: Aflatoxin, fungal growth, HPLC, *Alternaria*, *Aspergillus*

INTRODUCTION

Wheat is an ingredient used in many foods and is one of the most important foods in European an American culture. Bread, pasta, crackers and many cakes, among other foods and cooking recipes, are made using flour or including this as an ingredient. Wheat (*Triticum* spp.) (Donner *et al*., 2000) is a cereal grain, originally from the Levant region of the Near East and Ethiopian Highlands but now cultivated worldwide. In 2010, world production of wheat was 651 million tons, making it the third most-produced cereal after maize (844 million tons) and rice (672 million tons). However, pathogens that contaminate wheat may survive for extended periods (Berghofer *et al*., 2003; Cabanas *et al*., 2008; Gashgari *et al*., 2010). Wheat were also found to be contaminated in variable amounts by potentially toxigenic fungi including *Aspergillus*, *Alternaria* and *Fusarium* (Halt, 1998; Tournas and Katsoudas, 2008). These fungi are present in soil and plant material, cause the decay of stored grain and food (Herrman, 2002). The word mycotoxin was derived from mycotoxicosis which was a term first used in 1955 to describe diseases of animals caused by toxics metabolic by-products of certain fungi (Herrman, 2002) which includes mushrooms, molds and yeast. Mycotoxins can appear in the food chain as a result of fungal infection of crops, either by being eaten directly by human, or used as livestock feed. Mycotoxins greatly resist decomposition or being broken down in digestion, so they remain in the food chain even after heat treatment, such as cooking and freezing (Urraca *et al*., 2004). The most common mycotoxins are aflatoxins, ochratoxin A, fumonisins, deoxynivalenol, T-2 toxin and zearalenone. Of these, aflatoxins represents the main threat world-widely due to their occurrence and toxicity. Aflatoxins are produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus* (Aycicek *et al*., 2005). Aflatoxins occur naturally in most of the commodities, including wheat, corn, soybean and peanut and other grains which are consumed by human and animal. High moisture and

temperature are two main factors that cause the occurrence of mycotosins at pre-harvest and post harvest stages (Aycicek *et al*., 2005). Aflatoxins may increase stress susceptibility and compromise growth efficiency. The clinical signs of aflatoxicosis are extremely varied. Signs of acute aflatoxicosis include depression, nervousness, abdominal pain, diarrhea and death (Herrman, 2002). Since these toxins have been considered unavoidable contaminants in food chain, the Food and Drug Administration (FDA) of USA has established an action 732 level for total aflatoxins which is at 20 ppb for all foods, including animal feeds (Munkvold *et al*., 2005). Of the currently identified many types of aflatoxins, aflatoxin B1, B2, G1 and G2 occur naturally and are significant contaminants of a wide variety of foods and feeds (Juan *et al*., 2008). Samples of wheat, corn and other grains based product were analysed for the presence of aflatoxins in this study as these food were commonly consumed by saudian without any awareness of their safety from any local organizations. The aim of this study was to screen the content of aflatoxins in samples of wheat, corn and other grains based products available in Saudia Arabia that were widely consumed in huge amounts, as well as providing sensitive, accurate and reproducible analytical method for the detection of aflatoxins to assess the exposure of consumers to the toxins in order to bring them to the attention of the importance of monitoring the levels of aflatoxins in the wheat, corn and other grains based products. So, this study aimed to determine the occurrence and load of fungi in wheat and other grains offered for sale to consumers retail stores at Riyadh region in Saudi Arabia.

MATERIALS AND METHODS

Samples: Fungal infected were collected from different regions such as: Wadi Al-Dawser, Dhurmah, Al-Qassim, Najran, Yemen, Al-Sharjah, Dubai, Turkey, India, Asuteralia, Ethiopia. Wheat samples include brown wheat from Wadi Al-Dawser, Dhurma, Al-Qassim, Najran, Yemen and Dubai, groats from Turkey and Dubai, grains from Austria and Al-Sharjah, millet from India and corn from India and Ethiopia. Sixteen samples were collected from storage shops. The collected samples were put into sterile polythene bags and sealed properly. The samples were stored and analyzed the day after collection. They were brought into laboratory for further processing (Sekar *et al*., 2008).

Isolation and identification of fungi: About 1 g of the collected samples were washed aseptically with ten successive 10 mL volumes of sterile distilled water and was surface sterilized using KOH 10% and then rinsed with sterile distilled water. Czapec (dox) agar media were prepared and chloramphenicol (500 mg L^{-1}) was added to inhibit bacterial growth. About 1 g of the washed samples was inoculated randomly in each of the Czapec (dox) plate. For each sample, three replicates were maintained. The plates were incubated at room temperature and examined daily for growth and sporulation for 5 days. After 5 days of incubation the different fungal colonies were transferred into fresh Czapec (dox) plates. The fungi isolated were identified by a drop of distilled water placed on a clean glass slide and a loopful of the fungal colony was taken and placed on the slide. With the help of sterile needles, the fungal mycelia were teased gently and a cover slip was placed over the drop of distilled water. The slide is then observed under 40X power in microscope and identified based on morphological characteristics (Sekar *et al*., 2008).

Extraction and clean-up: A 30 g of ground sample was mixed with 100 mL of mixture of methanol and distilled water at ratio of 80:20 and shaked for 1 h. The extract was filtered through

Advantec filter paper No.131. A 9 mL of filtrate was then transferred into a test tube and clean-up cartridge which was obtained from Romer Labs. Inc. (Binder *et al*., 2007).

Chromatography: HPLC is the preferred method for analyzing aflatoxins. A variety of separation and quantitation modes using reverse-phase chromatography (RPLC) have been developed. The RPLC employs a nonpolar bonded silica surface and a polar mobile phase. For the analysis of aflatoxins, silica-based HPLC columns bonded with C8 or C18 groups are used with mobile phases consisting of binary or ternary mixtures of polar solvents. Commonly used solvent mixtures include deionized water, methanol and acetonitrile. In the reversed phase mode, the elution order of the common aflatoxins is G2, G1, B2 and B1. Aflatoxins may be separated and detected by UV detection (Sekar *et al*., 2008).

RESULTS AND DISCUSSION

The results of isolation of some species of microorganisms from 16 wheat and small grains samples collected from different Mills in Riyadh, a total of three genera of fungi were isolated (Table 1). Fungi are remarkable organisms that readily produce a wide range of natural products called secondary metabolites. Some are beneficial (e.g., antibiotics) to human kind, while others are deleterious (e.g. mycotoxins). Fungi that exhibit filamentous growth and have a relatively complex morphology produce most secondary metabolites. The production of these secondary metabolites usually commences late in the growth of the fungus, often upon entering the stationary phase (Sekar *et al*., 2008). The collected samples were grown on Czapec (dox) plates. After 5 days incubation, the results of inoculation of the sample on Czapec (dox) plates are tabulated in Table 2. It is shown that some of the following wheat samples contain fungi above or below the

Table 1: Percentage of fungal growth

Fungi	Growth (%)
Alternaria	68.96
Aspergillus	24.14
Fusarium	6.90

Table 2: Growth after 5 days incubation on Czapec (dox) (PFUs g^{-1})

Samples	PFUs g^{-1}
Wadi Al-Dawser brown wheat	0.113
Dhurmah brown wheat	0.121
Al-Qassim brown wheat	0.113
Najran brown wheat	0.121
Yemen brown wheat	0.111
Al-Sharjah grains	0.211
Dubai brown wheat	0.131
Turkey groats	0.110
Dubai groats	0.231
India millet	0.221
Asuteralia grains	0.121
Ethiopia corn	0.131
India corn	0.120
Turkey mash	0.110
Red corn	0.111
Barley	0.212

Table 3: Colony morphology of fungi on Czapec (dox) plates and results of wet mount microscopic observation

	Microscopic observations			
Colony morphology	Mycelium	Spores	Conidiophores/ sterigmata	Probable organism
Velvety, yellow to green or brown	Blue mycelia woolly at first, white to yellow, then turn dark brown to black	Blue spores	Conidiophores variable in length, rough, spiny; sterigmata single and double, pointed in all directions	*A. flavus*
Woolly at first, white, to yellow then turn dark brown to black	Blue/brown mycelium	Blue spores	Sterigmata double, cover entire vesicle, form radiate head	*A. niger*
Usually fast growing, pale or brightly colored	White/pink mycelium	Black spores	Conidiophores may be single or branched with conidia	*Fusarium*
Grow quickly and colony may be gray, brown or black in color	Usually starts white before changing to a darker color	Dark brown to black spores	Conidiophores pale brown to olive brown straight or flexuous	*Alternaria*

permissible number in terms of PFUs per gram of sample. For identification by morphology, LCB wet mount was prepared and the following morphologies were observed. The results of LCB wet mount preparation are shown on Table 3.

Microorganism propagules get on grain in different ways, most often with dust from soil, from the surface of plant remnants during harvesting, transportation, storage and processing (Klich, 2002). Mold spores present in wheat survive for several years and therefore, care should be taken in the storage of wheat (Christensen and Cohen, 1950). Table 2 shows the mean values of total fungal counts obtained with the direct plating technique. These results are in agreement with the results reported by Cabanas *et al.* (2008) in their study on wheat flour from Spanish markets. Dilution plating is the technique recommended for fungal enumeration in flours and direct plating is considered to be the more effective technique for mycological examination of particulate foods such as grains or nuts and wheat samples (ICFM, 2006; Cabanas *et al.*, 2008). Cabanas *et al.* (2008) reported that the total mold counts obtained from wheat flour samples in Spain (<10-1.6; 103 CFU = g) are similar to those reported by other authors. In wheat flours, the fungal counts reported from various countries varied about 102-6; 103 CFU = g (Weidenborner *et al.*, 2000; Berghofer *et al.*, 2003). In Malaysia, total fungal count in wheat flour samples ranged from 102 CFU = g sample to slightly more than 104 CFU = g sample (Abdullah *et al.*, 1998). In Spain, the maximum mold count limit for wheat flour for human consumption is 1; 104 CFU = g (Real Decreto 1286 = 1984). Recently, in the quality guidelines proposed for Australian flour the acceptable quality limit for yeasts and molds was <103 CFU = g (Berghofer *et al.*, 2003). In Germany, 51 species belonging to 14 different genera were isolated from whole wheat flour and white wheat flour and total fungal counts of the whole wheat flour amounted to 1833 molds and the white wheat four contained 1730 CFU/2 g_1 (Weidenborner *et al.*, 2000). In Algeria, total fungal count in wheat flour samples was 275 CFU/g_1 (Riba *et al.*, 2008). Mycotoxins occur and exert their toxic effect in extremely small quantities in food stuffs. Mycotoxin literally means "fungus poison" and the fungi that produce mycotoxins do not have to be present to cause harm (Anonymous, 2003). The mycotoxin produced by each of these fungi may differ from each other in chemical formula, products in which they occur, conditions under which they are produced, their effects on various animals and humans and in degree of toxicity (Agrios, 1978). Several different

Table 4: Validation data for extraction of wheat from HPLC

Samples	Time	Slope (UV/sec)	Offset (UV)
Wadi Al-Dawser brown wheat	2.100	2.05	-5.57
Dhurmah brown wheat	2.000	9.19	-3.07
Al-Qassim brown wheat	2.167	6.11	-1.40
Najran brown wheat	3.733	2.32	-9.06
Yemen brown wheat	2.700	-2.38	1.14
Al-Sharjah grains	3.967	4.42	1.86
Dubai brown wheat	3.933	5.47	-2.25
Turkey groats	4.017	4.93	-2.09
Dubai groats	3.683	3.62	-1.45
India millet	2.000	2.21	-5.14
Asuteralia grains	3.283	2.62	-8.86
Ethiopia corn	2.300	8.15	-1.94
India corn	3.317	2.30	-8.07
Turkey mash	3.617	2.63	-1.01
Barley	3.267	4.71	-4.28
Red corn		Error	

fungi, however, produce some of the same or closely related toxins. *Fuzarium*, *Alternaria* and *Aspergillus* species particularly produce similar toxins (Amadi and Adeniyi, 2009). Many of the isolates produced toxins but were not identified within the scope of this study. Validation data for extraction of wheat from HPLC are given in Table 4. This gives relevant information about the presence of mycotoxins in a run time from 2-4 min.

This study represents the investigation of toxigenic fungi occurrence in wheat and other grains. *Alternaria* were the most common group of encountered fungi and *Aspergillus* and *Fusarium* could be isolated from more than half of the tested samples. Their presence together with that of other potentially toxigenic molds indicates a risk of mycotoxins contamination and demands an exhaustive survey of the mycotoxins occurrence as well as the elaboration of specifc regulations for this commodity (Storari *et al*., 2012). The monitoring and elimination of mycotoxin in consumer products should be implemented and frequently conducted by the industry and government in order to bring the consumers and manufacturers to the concerns of quality and public health (Hong *et al*., 2010).

CONCLUSION

The samples of imported wheat in Saudi Arabia, it was found that samples containing common *Alternaria*, *Aspergillus* and *Fusarium*. It was detected fungi produce toxins device by HPLC and results indicated that the fungi are mostly produced aflatoxin, so verify their existence before consumption in order to preserve the health of the consumer.

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