JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Sept. 2016. Vol. **10**(3), p. 1817-1824

Occurrence of Hemolytic Fungi Mounted on Wheat Grains in the main Silo of Sakaka, Saudi Arabia

S.M.N. Moustafa[1,2]* and H.M.A. Abdelzaher[1]

[1]Department of Biology, College of Science, Aljouf University, Sakaka, 42421, Saudi Arabia.
[2]Department of Botany and Microbiology, Faculty of Science, Minia University, El-Minia, 61511, Egypt.

(Received: 10 June 2016; accepted: 14 August 2016)

A lot of countries around the world are seeking to strategic storage of large quantities of wheat grain in giant silos for use in the food industry for humans, animals and poultry. Length of the period of transport and storage, which can be exposed to amounts of moisture qualifies them to serious injury of many types of dangerous fungi. Objectives of this study were to identify fungi mounted on wheat grain stored in the main silo in the city of Sakaka, Saudi Arabia and to study the ability of these fungal spores and conidia to cause damage to people exposed to inhalation of large quantities of the dust of wheat grain in causing lyses of human red blood cells. *Aspergillus* was consistently the most frequent genus (100% of places) and contributed by *A. flavus* and *A. niger* were of high frequency (20 places out of 20) contributing 19.3% and 19% (total 38.3%) of the total count, respectively. *Penicillum* was recovered from 18 samples matching 36.5% of total count fungal isolates. It was represented by *P. frequentans* and *P. islandicum* sharing 18.5% and 18%, respectively. *Ulocladium atrum* gave 15.7% of the total count but with low occurrence. *Circinella umbellata* and *Gliocladium* sp. were less frequently isolated representing 7.4% and 2.1% of total count. The tested 7 fungi proved the ability to break down human red blood cells but with varying degrees in varying significant intensity.

Keywords: fungi, hemolytic activity, Sakaka, silo, spores and conidia.

Nowadays, however SURGE in the number of human beings and the hustle and human congestion, became obsessed with the growing pollution levels on the surface of events. It is worth mentioning that the sources of pollution are many and varied, and worse, is that pollution can be up to any place on the earth's surface. Bio-contamination is a kind of pollution sources, and it passed on vital pollutants from one place to another, causing problems for the place which receives pollution.

Wheat is a major source of carbohydrates in many countries of the world. Some countries produce large quantities of wheat which is enough for domestic use and the rest is exported to other countries that suffer from a lack of this product. Wheat flour enters in bread production, which is essential in all diets for humans. Wheat flour is also included in candy industry, biscuits and baby food and so on[1]. Additionally, wheat grains and flour enter as a key ingredient in feed for poultry and the cattle[2]. Therefore, many countries need massive amounts of grains and wheat flour where store it in huge quantities in places such as silos. There are also some countries that import large quantities of wheat which shipped across oceans in giant ships[3].

During storage and transport in wet conditions, those massive stockpiles of grain and flour are affected and encourage growths of various fungi that emit spores and toxins in these foods[4]. It is known that many of fungi have the ability to excrete metabolic mycotoxins within the media that grow in it[5]. Since wheat comes from a

* To whom all correspondence should be addressed.
Tel.: +966537283495;
E-mail: shymaa.nabil@ju.edu.sa

variety of geographical locations to be stored in silos, it makes sense that they carried with it a variety of types of fungi. Sometimes fungi grow in deep places hidden in piles of grain which created fungal growths extremely dangerous as producers of mycotoxins. Subsequently, it is necessary to know fungi situated on grains stored in silos of Sakaka city, Aljouf, Saudi Arabia and ability of these fungi to be factors of human hazard as hemolytic elements.

One of the main objectives of this study is the isolation and identification of fungi located on wheat grain stored in silos of Sakaka city, Saudi Arabia. Study the effect of spores and conidia of the isolated fungi on the decomposition of human red blood cells is one of the objectives of this research.

MATERIALS AND METHODS

Sampling places

Twenty samples (each 200 gm), were collected from inside the heaps of wheat grains (at a depth of 30 cm under the surface) from the silo of Sakaka city, Aljouf governorate, Saudi Arabia, during October, 2015.

Fungal isolation and identification

In a 300 ml bottle of sterile water-packed, each bottle was opened next to the rallies in wheat grain silos Sakaka city and half of the bottle was discarded and then each bottle was placed filled with wheat grains. Aliquots of one ml water containing washing grains were added to milted (50ºC) Potato dextrose agar (PDA), which is most suitable culture media for the isolation of seed borne fungi, with Rose-bengal [1/15000 ml / media / l medium][6]. Plates were incubated at 28ºC for 7 days or until emergence of colonies and then purified to be examined. The number of the identified fungi was counted. Fungal genera and species were identified using colonial and microscopical structures with the aid of taxonomic keys of[7-12].

Study the ability of isolated fungi on the hemolytic activity to the human red blood cells

To study the toxicity of different types of fungi that have been isolated used human red blood cells through the method of[13,14] was followed. Solutions of conidia or spores of the tested fungi were prepared using neutral osmotic solution (0.9 % NaCl). One hundred µl of human blood, after washing with saline solution (0.9 % NaCl) three times, was incubated with 900 µl of each of the tested spore suspension or sodium chloride solution, which represents a negative control sample, as well as, or distilled water (positive control sample), under aseptic conditions, for comparison. After incubation at 27 ºC, for 24 hours, in the dark, components of the mixture were separated using a centrifuge and absorbance of the supernatant was then measured using UV visdspectrophotometer (spectro uv-2505) at 540 nm to calculate percentage of dissolution and cracking of red blood cells according to the following formula (each treatment was repeated 3 times and subjected to statistical analysis):

% Hemolytic activity = absorbance of sample - absorbance of saline / absorbance of dist. water X 100.

Statistical analysis

ANOVA was used to assess data using Minitab statistical software (version 12) unless somewhere else mentioned.

RESULTS

Numbers in Table (1) reveal that 7 species belonging to 5 genera were isolated from 20 samples placed in the main silo of Sakaka city, Aljouf governorate, Saudi Arabia. *Aspergillus* was consistently the most frequent genus (100% of places) and contributed by *A. flavus* and *A. niger* were of high frequency (20 places out of 20) contributing 19.3% and 19% (total 38.3%) of the total count, respectively, by the used isolation method (Figs. 1&2). *Penicillum* came second which was recovered from 18 samples matching 36.5% of total count fungal isolates. It was represented by *P. frequentans* and *P. islandicum* sharing 18.5% and 18%, respectively, (Figs. 5&6). *Ulocladium atrum* gave 15.7% of the total count but with low occurrence (Fig. 7). *Circinella umbellata* and *Gliocladium* sp. were less frequently isolated representing 7.4% and 2.1% of total count (Figs. 3&4).

Study the ability of fungi isolated on the hemolytic activity of the red blood cells of the human Hemolytic activity

Results revealed that all of the tested fungi had the ability to analyze and break down

human red blood cells but with varying degrees in intensity, as shown in Figs (8 & 9).

P. frequentans appeared the highest ability to break down human red blood cells (by analyzing 63%), followed by *Gliocladium* sp. (Analysis by 51%), then *A. niger* (analysis by 50%). Spore suspension of *Ulocladium atrum*, *Circinella umbellata*, *Aspergillus flavus* and *Penicillium islandicum* gave effects ratio of 23, 22, 20 and 19%, respectively (Fig. 8).

DISCUSSION

Search results showed, the presence of fungi *Aspergillus flavus*, *Aspergillus niger*, *Circinella umbellate*, *Gliocladium* sp., *Penicillium frequentans*, *Penicillium islandicum*, and *Ulocladium atrum*, mounted on surfaces of wheat grains collected and stored in the main silo of the city of Sakaka, Aljouf governorate, Saudi Arabia, in October, 2015.

Table 1. Total counts of fungal genera and species recovered from 20 samples of wheat grains collected from the main silo, Sakaka, Aljouf, Saudi Arabia by germs derived from soaked grains in sterilized water, number of cases of isolation (NCl; out of 20 cases), occurrence remarks (OR), percentage of total counts (TC%) on PDA agar at 28°C

Genera and species	Wheat grains		
	NCl	OR	TC (%)
Aspergillus	145	20H	38.3
A. flavus	73	20H	19.3
A. niger	72	20H	19.0
Circinella umbellata	28	5L	7.4
Gliocladium sp.	8	4L	2.1
Penicillium	138	18H	36.5
P. frequentans	70	18H	18.5
P. islandicum	68	17H	18.0
Ulocladium atrum	59	3L	15.7
Gross total counts	378		
No. of genera	5		
No. of species	7		

OR = Occurrence remarks; H = 60% -100.0%, M = 33 - 59.0% , L = 20 - 32%, and R = 7 – 19%

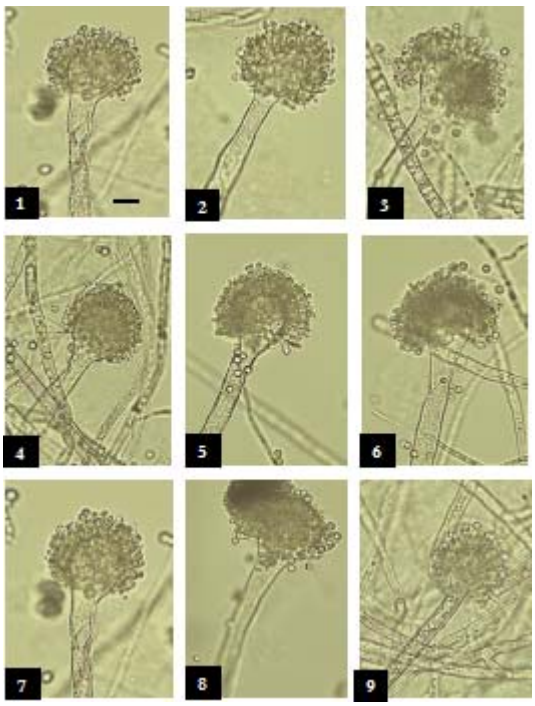

Fig. 1. Mycelial growth of *Aspergillus flavus* on PDA at 28ºC in the dark. Images from 1-9 were taken using normal compound microscope. Aseptate conidiophores and mostly radiate heads with mono and biserriate sterigmata mounted on flask shaped vesicles. Bar 10 µm in the pictures (1) is the same for all images

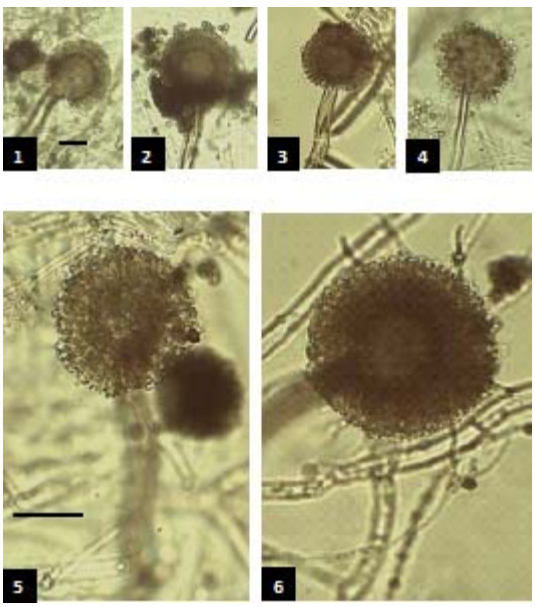

Fig. 2. Mycelial growth of *Aspergillus niger* PDA at 28ºC in the dark. Images from 1-6 were taken using normal compound microscope. Aseptate conidiophores and radiate heads with biserriate sterigmata . Bar 10 µm in the pictures (1) are the same for photos 2-4, and in picture 5 is the same as the image of No. 6

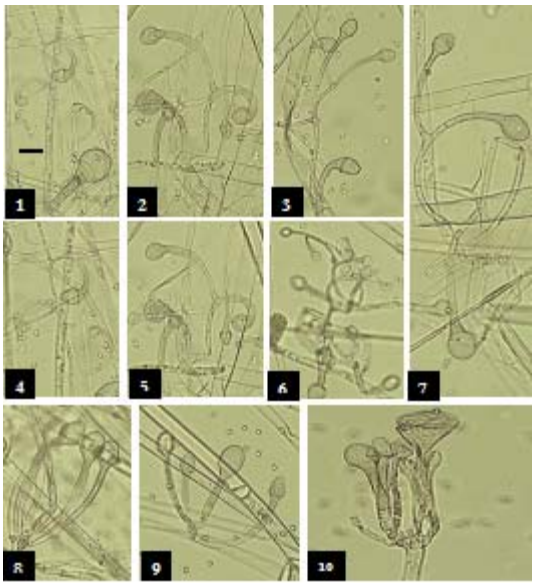

Fig. 3. Mycelial growth of *Circinella umbellate* on PDA at 28ºC in the dark. Images from 1-10 were taken using normal compound microscope. 1-7 Branched conidiophores with curved side branches ended by sporangia. 8-10 gatherings tent form sporangiophores. Bar 10 µm in the pictures (1) are the same for all images

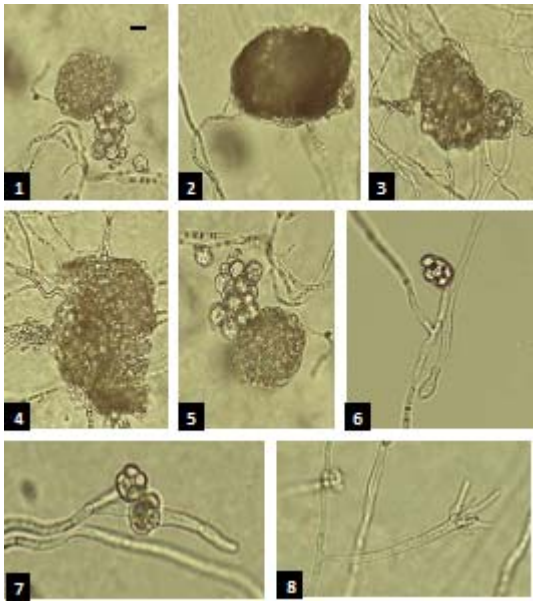

Fig. 4. Mycelial growth of *Gliocladium* sp. on PDA at 28ºC in the dark. Images from 1-8 were taken using normal compound microscope. 1-7 gelatinous aggregations of conidia. 8 A conidiophore carries a distinctive sterigmata characteristic of this fungus. Bar 10 µm in the pictures (1) is the same for all images

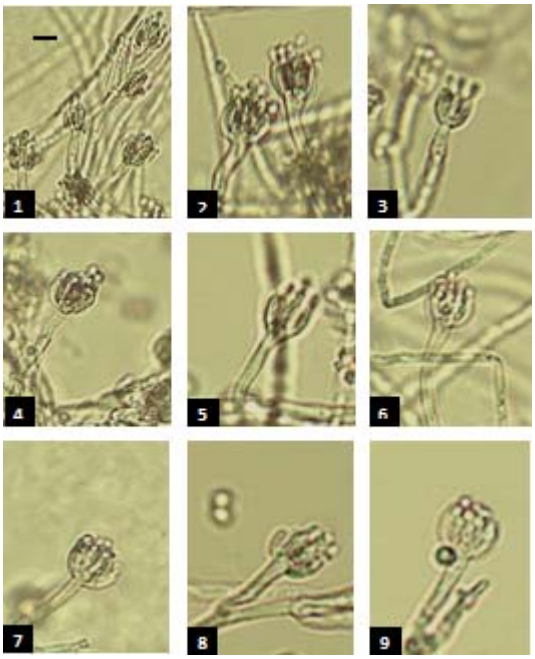

Fig. 5. Mycelial growth of *Penicillium frequentns* on PDA at 28ºC in the dark. Images from 1-9 were taken using normal compound microscope. 1-9 septate conidiophores mounted monoserriate sterigmata. Bar 10 µm in the pictures (1) are the same for all images

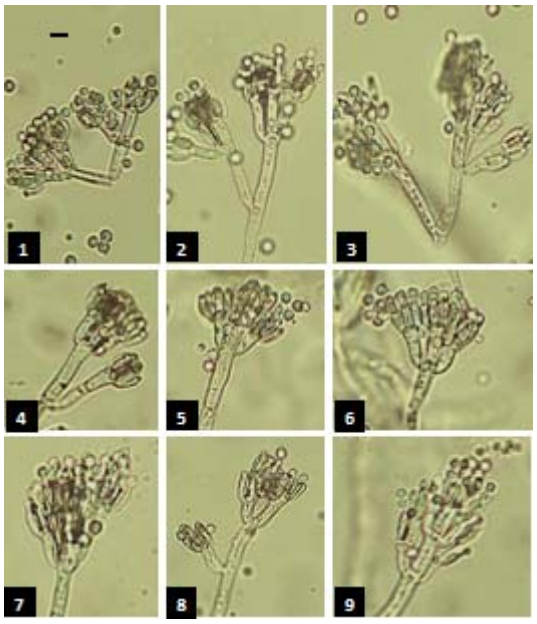

Fig. 6. Mycelial growth of *Penicillium islandicum* on PDA at 28ºC in the dark. Images from 1-9 were taken using normal compound microscope. 1-9 septate conidiophores mounted biserriate sterigmata and symmetrical. Bar 10 µm in the pictures (1) are the same for all images

It is known that fungi isolated in this study have precedents in the cause of serious diseases to humans and animals[15-27]. Furthermore, many previous studies have demonstrated is no doubt that exposure to grains agricultural dust was one of the important causes of many of respiratory and allergic diseases for people exposed to the dust[28-31]. In detailed terms, it found that penicillin, such as, *Penicillium fequentans* and *Penicillium islandicum* were the cause of pathogenesis of pulmonary disease, hypersensitivity, alveolitis allergic (alveoli), skin allergies and emphysema, as well as the production of dangerous toxins[32,33]. *Aspergillus niger* is also the huge producer of conidia was the biggest causes of disease resembling tuberculosis and called Aspergillosis, which is spread in poorly ventilated and heavy pollution and dust places[34]. Previous studies have also postulated the involvement of *Ulocladium atrum* and *Circinella umbellate* in chest allergy[35,36]. It is worth mentioning that *Aspergillus flavus* is a type of saprophytic and parasitic factor, with the global distribution of its presence. Colonization of many grains, legumes, and nuts by this fungus, may cause rot and maceration of grains during the harvest season, storage or transport, especially in the slow ships that travel long distances through

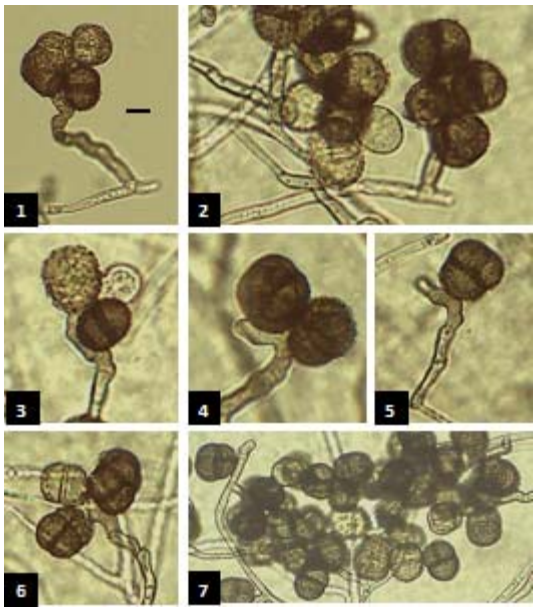

Fig. 7. Mycelial growth of *Ulocladium atrum* on PDA at 28ºC in the dark. Images from 1-7 were taken using normal compound microscope. 1-7 septate conidiophores mounted solitarily broad conidia with rough wall has transverse septa (1-3 septa) and longitudinal septum (one or more), somewhat narrow at the base and broad at the top. Bar 10 µm in the pictures (1) are the same for all images

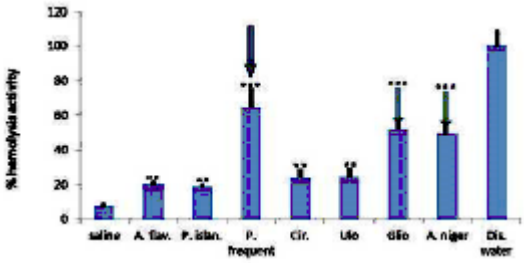

Fig. 8. Effect of spores suspension of 7 tested fungi on decomposition of the red blood cells of a human sample. Bars above all drawing column, represent the standard error of the average data from three replicates and reflect differences between averages of the samples compared to the control sample. Significant values against control represent: ** = highly significant at $p < 0.01$, *** = very significant at p Â 0.001

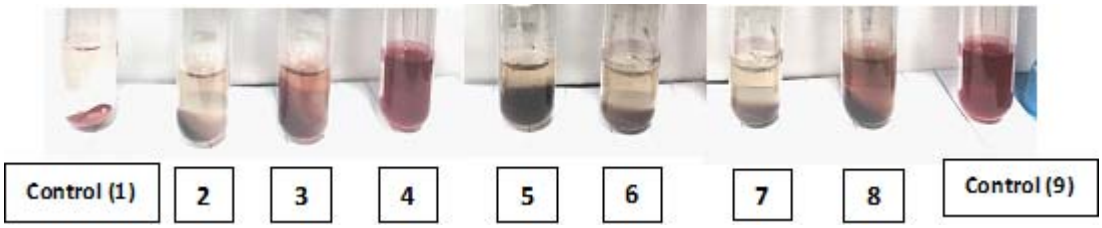

Fig. 9. Effect of fungal spores suspension of each of *Circinella umbellata* (sample No. 2), *Gliocladium* sp. (sample No. 3), *Penicillium frequentans* (sample No. 4), *Ulocladium atrum* (sample No. 5), *Penicillium islandicum* (sample No. 6), *Aspergillus flavus* (sample No. 7), and *Aspergillus niger* (sample No. 8) on the breakdown of red blood cells of a human sample. Sample No. 1, representing the negative control sample [human blood+saline solution (0.9% NaCl)], and Sample No. 9, representing the positive control sample [human blood+distilled H_2O]

humid environments. And for sure, it was found that many of strains of this type produce large amounts of known biological toxins called mycotoxins, which when consumed becomes very toxic to mammals, causing very serious diseases such as liver cancer and diseases of the respiratory system to humans and animals especially for people with weakened immune systems[37]. Although many previous studies have put *Gliocladium* in a rank of "non-pathogenic fungi", but recent studies have shown the involvement of this organism in the secretion of a kind of fungal toxins called Gliotoxin[38].

And where the fungus, mentioned above, have been isolated in this study, so their presence in wheat grains stored in the main silo in Sakaka city, constitutes a serious source of pollution, especially when there is moisture and the growth of this fungus inside piles of wheat grains, without appears to the consumers, causing a disaster disturbing the excretion of mycotoxins and produced huge amounts of spores and conidia that can cause diseases of the respiratory system of mammals consuming those foodstuffs. The exposure of workers in the storage areas and silos to dust raised from grains movement poses a significant risk to public health. Breathing in wheat grains dust can affect the health and overall comfort for workers who work in silos.

Results of this study showed that fungi of *Penicillium frequentans*, *Gliocladium* sp. and *Aspergillus niger* were caused severe decomposition of red blood cells in humans at high rates and impressive, and the rest of tested fungi gave significant impact, too, which cast a stern warning on exposure to these fungi. These results are harmonious with previous studies documenting a relationship that fungal disease to humans, although previous studies have not carried out the experiment of Hemolysis that conducted in our research.

Therefore, we recommend the reality of the results of this research to wear masks covering the nose and mouth such as influenza masks in order to reduce the entry of dust laden with fungal germs and conidia to the respiratory tract for those people. Additionally, it is recommend making sure to dry the environment of stored grains to prevent encouraging fungal growth by placing dissecting materials in places of conservation.

Grain dust is a complex soup that is made up of both organic and inorganic particles. Some of these can be inhaled easily, and depending on their size, can find their way deep into various parts of the respiratory system causing a range of adverse health effects. Entry of Fungal spores to the inside of the human body and the possibility of interaction with the blood can not be given by this study and needs further investigation.

ACKNOWLEDGEMENTS

Sincere thanks and gratitude are due to the Vice-presidency for Graduate Studies and Scientific Research, Aljouf University, Saudi Arabia, for supporting this study through the grant of the research project No. 296/35.

REFERENCES

1. FAO. Biofuel co-products as livestock feed - *Opportunities and challenges* 2012; edited by Harinder P.S. Makkar. Rome.
2. ADM Ingredients catalog: *feed & Pet food*, 2016; © 2016 Archer Daniels Midland Company.
3. Blonigen, B. and Wilson, W. "New Measures of Port Efficiency Using International Trade Data" *NBER Working Paper* 2006; 12052.
4. Lawley, R. Aflatoxins. *Food safety watch (The science of safe foods,)* 2013; *e-books.*
5. Abbas, H. K. Aflatoxin and Food Safety. *CRC Press* 2005; ISBN 0-8247-2303-1.
6. Maghazy, S. M. N., Abdelzaher, H. M. A. and El-Gendy, Z. K. Studies on mycobiota of monumental elements in Minia governorate. *EL-Minia Science Bulletin* 2013; 24 (1): 83-115.
7. Raper, K. B. and Fennell, P. I. 1965. The genus *Aspergillus* . Williams and Willims, Baltimore. USA.
8. Pitt, J. I. The genus *Penicillium* and its telemorphic states *Eupenicillium* and *Talaromyces*. Commonwealth Scientific and Industrial Research Organisation Division of Food Research, North Ryde, N. S. W. 2113 *Australia Academic Press* 1979; A subsidiary of Harcourt Brace Javanovich, Publishers London. New York. Toronto. Sydney.San Fransisco.
9. Sivaneasan, A. Graminicolous species of *Bipolaris*, *Curvularia*, *Derechslera*, *Exserohilu* and their teleomorphs. *Mycological Papers*; 1987; 158: 1-261, CAB International Mycological Institute, Ferry Lane, Kew, Surrey,

UK.
10. Moubasher, A. H. Soil fungi in Qatar and other Arab Countries. *Environmental studies center* 1993; University of Qatar.
11. Leslie, J. and Summerll, B. A. *Fusarium*. Laboratory Manual. *Blackwell publishing professional* 2006; 1st edition, Iowa USA. pp.388.
12. Domsch, K. H., Gams, W. and Anderson, T. H. *Compendium of soil fungi* 2007; London. New York Academic press.
13. Tasca, T. and Adecarrli, G. Hemolytic activity of fresh isolates and clones of *Trichomonas gallinae*. *Parasitol. Día* 1999; v.23 n.3-4 Santiago jul.
14. Mukherjee, A. and Rajasekaran, C. 2010. In-vitro hemolytic activity of *Allium stracheyi* Baker. *Journal of Pharmacy Research* 2010; 3(5), 1160-1162.
15. Paivi, M. S., Samuel, A. Jr., Michelle, S., Renee, J., Richard, D. C., Stephanie, J. L. and Darryl, C. Z. Exposure to *Alternaria alternata* in US homes is associated with asthma symptoms. *J Allergy Clin Immunol.* 2007; 118 (4): 892-898.
16. Bush, R. K. and Prochnau, J. J. *Alternaria*-induced asthma. *J Allergy Clin Immunol.* 2004; 113: 227-234.
17. Eaton, M. and Gallagher, J. Mechanisms of aflatoxin carcinogenesis. *Ann. Rev. Pharmacol . Toxicol* . 1994; 34: 135 – 171.
18. Bryden , W. L. Chronic effects of mycotoxins in animals . *mycotoxin symposium* 1988; University of Sydney, N.S.W, Australia. pp11.
19. Karam, G. H. and Griffin, F. M. Jr. Invasive pulmonary aspergillosis in nonimmuno compromiscd, nonneutropenic hosts. *Rev Infect Dis*. 1986; **357**.
20. Abarca, M., Bragulat, M., Castellá, G. and Cabañes, F. Ochratoxin A production by strains of *Aspergillus niger* var. *niger*". *Appl Environ Microbiol.* 1994; **60**(7): 2650-2652.
21. Samson, R. A., Houbraken, J., Summerbell, R. C., Flannigan, B. and Miller, J. D .Common and important species of fungi and actinomycetes in indoor environments. *In: Microogranisms in Home and Indoor Work Environments*.2001; New York: Taylor & Francis, pp 287-292
22. Mohamed, F. and Abdelghafour, T. Production of toxic metabolities by *Penicillium italicum* and *P. digitatum* isolated from citrus fruits. *Journal of food production* 1989; **3**: 194-197.
23. Schuster, E., Dunn-Coleman, N., Frisvad, J. C. and Van Dijck, P. W. On the safety of *Aspergillus niger*—a review. *Applied microbiology and biotechnology* 2002; **59**(4-5): 426-435.
24. Ashiqm, S., Hussain, M. and Ahmad, B. "Natural occurrence of mycotoxins in medicinal plants: a review". *Fungal Genetics and Biology* 2014; 66: 1-10.doi:10.1016/j.fgb.2014.02.005. PMID 24594211.
25. Do, K. H., An, T. J, Oh, S. K. and Moon, Y. "Nation-Based Occurrence and Endogenous Biological Reduction of Mycotoxins in Medicinal Herbs and Spices". *Toxins* 2015; **7**(10): 4111–30. doi:10.3390/toxins7104111. PMC 4626724. PMID 26473926.
26. Veprikova, Z., Zachariasova, M., Dzuman, Z., Zachariasova, A., Fenclova, M., Slavikova, P., Vaclavikova, M., Mastovska, K., Hengst, D. and Hajslova, J. "Mycotoxins in Plant-Based Dietary Supplements: Hidden Health Risk for Consumers". *Journal of Agricultural and Food Chemistry* 2015; **63**(29): 6633–43. doi:10.1021/acs.jafc.5b02105. PMID 26168136.
27. Scallan, E., Griffin, P. M., Angulo, F. J., Tauxe, R. V. and Hoekstra, R. M. "Foodborne illness acquired in the United States—unspecified agents". *Emerging Infectious Diseases* 2011; 17(1): 16–22. doi:10.3201/eid1701.P21101. PMC 3204615. PMID 21192849.
28. Kirkhorn, S. R. and Garry, V. F. Agricultural lung diseases. *Environ Health Perspect*.; 2000, **108** Suppl 4:705-12. Review.
29. Seifert, S. A., Von Essen, S., Jacobitz, K., Crouch, R. and Lintner, C. P. Organic dust toxic syndrome: a review. *J Toxicol Clin Toxicol* 2003; **41**(2): 185-93. Review
30. Donham, K. J. and Thelin, A. Agricultural Medicine: *Occupational and Environmental Health for the Health Professions* 2006; Ames, Iowa: Blackwell Publishing.
31. Girard, M., Lacasse, Y. and Cormier, Y. Hypersensitivity pneumonitis. *Allergy*. 2009; 64(3): 322-34. Epub 2009 Feb 6. Review.
32. Kirk, P. M., Cannon, P. F., Minter, D. W. and Stalpers, J. A. Dictionary of the Fungi (10th ed.). *Wallingford, UK: CABI*. 2008; p. 505.
33. Pitt, J. I., Basílico, J. C., Abarca, M. L. and López, C. "Mycotoxins and toxigenic fungi". *Medical Mycology* 2000; **38**(Suppl 1): 41–46.
34. Ainsworth, G. C., Bisby, G. R., Kirk, P. M. and CABI Bioscience. Ainsworth & Bisby's dictionary of the fungi / by P.M. Kirk ... [et al.] Moustafa & Abdelzaher: Hemolytic Fungi In The Silo Of Sakaka; with the assistance of A. Aptroot ... [et al.]. *Wallingford, Oxon, UK: CABI Pub* . 2001.
35. Sharpe, R. A., Cocq, K. L., Nikolaou, V., Osborne, N. J. and Thornton, C. R. 2015. Identifying risk factors for exposure to culturable allergenic moulds in energy efficient homes by

using highly specific monoclonal antibodies. *Environ Res*. 2016; **144**: 32-42.

36. Joseph, F. Dail and Hammar's Pulmonary Pathology (volume I, nonneoplastic lung disease), 3rd edition, ISBN: 978-0-387-72139-2. Springer Science+Business Media, LLC. 2008

37. Ramírez-Camejo, L. A., Zuluaga-Montero, A., Lázaro-Escudero, M. A., Hernández-Kendall, V. N. and Bayman, P. "Phylogeography of the cosmopolitan fungus Aspergillus flavus: Is everything everywhere?". *Fungal Biology* 2012; **116**(3): 452–463. doi:10.1016/j.funbio.2012.01.006. PMID 22385627.

38. Puri, A., Ahmad, A. and Panda, B. P. Development of an HPTLC-based diagnostic method for invasive aspergillosis. *Biomed. Chromatogr*. 2010; **24**: 887–892. doi: 10.1002/bmc.1382.