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# Impact of Enzymes and Toxins Potentiality of Four *Aspergillus* Species to Cause Aspergillosis

**Abdel-Nasser Zohri[1]*, M Bassam Aboul-Nasr[2], Mohamed Adam[3], Mohamed A Mustafa[4] and Enas Mahmoud Amer[1]**

[1]*Department of Botany and Microbiology, Faculty of Science, Assiut University, Assiut Governorate, Egypt*

[2]*Department of Botany and Microbiology, Faculty of Science, Sohag University, Sohag Governorate, Egypt*

[3]*Department of Chest Diseases, Faculty of Medicine, Assiut University, Assiut Governorate, Egypt*

[4]*Basic Center of Science, Misr University for Science and Technology, Giza Governorate, Egypt*

***Corresponding author:** Abdel-Nasser Zohri, Department of Botany and Microbiology, Faculty of Science, Assiut University, Assiut Governorate 71515, Egypt, Tel: 01007221923; E-mail: zohriassiut@yahoo.com

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**Abstract**

*Aspergillus* species are the main causing agents of invasive apergillosis chest disease. Eighty isolates of *Aspergillus* species, *A. flavus* (20), *A. fumigatus* (15), *A. niger* (30) and *A. terreus* (15 isolates), previously isolated and identified from aspergillosis suspected patients at our lab in Assiut university hospitals, were assayed for their enzymes and toxins profile. The results revealed that, all of the tested isolates were able to utilize calf lung tissue and produce catalase and peroxidase enzymes. Meanwhile, 82.5-90% of the fungal isolates had the ability to produce protease, lipase, urease and phospholipase, whereas, 70% of isolates exhibited hemolytic activities. Thin layer chromatography (TLC) of the cleaned extracts of the tested isolates exhibited the ability of all *A. flavus* isolates assayed to produce aflatoxins B1 and G1, 53%, moreover, *A. fumigatus* isolates produced fumagillin and gliotoxin. On the other side, 43.3% and 23.3% of *A. niger* isolates produced ochratoxins and gliotoxin respectively. Virulence assay of 10 µl of gliotoxin standard and cleaned extracts of *A. fumigatus* toxic isolates showed necrotic area on Guinea Pigs lungs compared to the control. Thus, opportunistic fungi isolated from aspergillosis patients possess high enzymatic and toxic profile that might play an important role in their mycopathy.

**Keywords:** Aspergillosis; Extracellular enzymes; Mycotoxins; Pathogenicity

## Introduction

Fungal infections have recently emerged as a world-wide health care problem, owing to extensive use of broad spectrum antibiotics, immunosuppressive agents and increasing population of terminally ill and debilitated patients [1]. The incidence of infection with *Aspergillus* has increased in recent years, primarily due to the increasing number of immunosuppressed patients encountered in clinical practice with the advent of solid organ and bone marrow transplantation, the increased use of cortico-steroids and other immune-modulating drugs, and the epidemic of infection with the human immunodeficiency virus (HIV) [2]. *Aspergillus fumigatus* is responsible for more than 90% of invasive disease in some reviews, while the species *Flavus, Niger,* and *Terreus* causing the majority of the remaining invasive aspergillosis cases [3-5].

Mycopathy is a collective term used for diseases caused by fungi either living or dead or their metabolic products (toxins, allergins or enzymes). All these metabolites make this eukaryotic group successful for the survival in a host thereby interacting and overcoming the host immune system. Immunity to fungal infections consists of nonspecific barriers, inflammation and cell-mediated immune responses [6]. Pathogenicity of a fungus depends on its ability to adapt to the tissue environment and to withstand the lytic activity of the host's defenses [7]. Several hydrolytic enzymes such as proteinases (elastinase), lipases and phospholipases are produced by fungi in culture media. These enzymes play a key role in fungal metabolism and may be involved in fungal pathogenesis, causing damage to the host tissues and overcoming the host immune system and strongly contribute to fungal pathogenicity [8]. These enzymes were also found to be helpful in fungal nutritional uptake, tissue invasion, adherence, dissemination inside the host, providing nutrients in a restricted environment [9]. Fungal proteases are active over a wide pH range (pH 4 to 11) and exhibit broad substrate specificity. Several reports are available on production of proteases by fungi belonging to the genus *Aspergillus* [10-17].

These enzymes can result in destabilization and destruction of the membranes and lung surfactant, cell lysis and release of lipid second messengers [18,19] and enhances the adhesion of *Cryptococcus neoformans* cells to the lung epithelium [20]. It was suggested that urease production by *C. neoformans* facilitates blood capillaries sequestration and disruption of endothelial cells and, in consequence, crossing the blood-brain barrier via a paracellular mechanism. *C. neoformans* is also able to acquire iron from its environment during host infection via enzymatic system. To overcome the host defense mechanism, superoxide dismutase and catalase are produced by *Candida albicans* and *A. fumigatus* when exposed to reactive oxygen species produced by phagocytic cells [21]. Fungi can synthesize also a vast diversity of chemical compounds either of primary or secondary metabolism origin which are not necessary for normal growth or development, but often have potent physiological activities [22]. These metabolites are of a great importance to humankind due to their involvement in pathogenicity. The hyphae of *A. fumigatus* produce a number of low-molecular-mass toxins, including helvolic acid, fumagillin and gliotoxin [23].

This study was directed to examine the potentiality of the most common fungal species isolated from suspected aspergillosis patients at Assiut university hospitals to produce extracellular hydrolytic enzymes and mycotoxins which might play an important factor involved in fungal pathogenicity.

## Materials and Methods

### Fungal isolates

Eighty isolates of *Aspergillus* species, *A. flavus* (20), *A. fumigatus* (15), *A. niger* (30) and *A. terreus* (15 isolates), previously isolated and identified from aspergillosis suspected patients at our lab in Assiut university hospitals, were assayed for their enzymes and toxins profile. Fungal isolates tested included *Aspergillus flavus* (20 isolates), *A. fumigatus* (15), *A. niger* (30) and *A. terreus* (15) (unpublished data).

### Enzyme analysis

**Protease activity:** Protease was determined using a Casein hydrolysis medium in which skim milk gives an opaque final appearance and hydrolysis of the casein resulted in a clear zone around the fungal colony at 30°C for 7 days according to [24].

**Lipase activity:** Lipase was measured using Ullman and Blasins [25] method with some modification using Tween 80 instead of Tween 20. The lipolytic producing ability was observed as a visible precipitate due to the formation of crystals of calcium salt of the oleic acid liberated by the enzyme at 30°C for 7 days.

**Urease activity:** Urease activity was measured using urease medium described by Paterson and Bridge [24]. Isolates capable of producing urease turned the yellow color of the acidic medium to purple-red or deep pink color at 30°C for 7 days.

**Hemolytic activity:** The ability of fungal isolates to hemolysis of human blood was measured using human blood agar medium with 7% human blood was added per liter of the medium at 7 days at 30°C [26].

**Phospholipase activity:** It was determined according to the method described by Price et al. [27]. After 7 days at 30°C, the production of the enzyme observed as a visible precipitate.

**Peroxidase activity:** Egger [28] assay was used to evaluate peroxidase activity. Development of golden yellow to brown color indicates peroxidase activity.

**Catalase activity:** The isolate possess catalase, when a small amount of fungal isolate is added to hydrogen peroxide, bubbles of oxygen are observed according to the method of Tadayuki et al. [29].

### Lung tissue lytic ability

Amended Czapek's-agar medium containing 40 g lyophilized ground calf lung tissue as a sole carbon source was used. Lung utilization was noticed as a zone of clearing 3 mm around a growing colony after 5 days of incubation.

### Toxin analysis

**Cultivation of fungal isolates:** An inoculum of each of tested fungal isolates was transferred to 250 ml flasks, containing 50 ml potato dextrose broth. Three replicates were used, and the flasks were incubated at 30°C for 10 days as still cultures.

**Extraction and purification of mycotoxins:** After the incubation period, the content of each flask (medium+mycelium) was transferred into a blender jar and homogenized with 50 ml chloroform for 2 minutes. The extraction procedure was repeated again with the same volume of chloroform. Combined, filtered, dried over anhydrous sodium sulfate, and then evaporated to near dryness by flash evaporator [30]. The dry crude extract was suspended in 50 ml chloroform and applied to a silica gel column (200 mesh, Merck) according to the method of AOAC [31]. The column was washed with 50 ml n hexane, and toxins were eluted with 50 ml of chloroform acetone (9:1 v/v) solvents system. The elute was collected and evaporated to near dryness.

**Detection of mycotoxins:** Thin-layer chromatography (TLC) analysis. 10 µl of each cleaned extract were spotted on TLC along with its standard, all standards were purchased from Sigma Aldrich Company.

Aflatoxins: Chloroform: methyl alchohl (97: 3 v/v). Aflatoxins B1 fluoresces bright blue at Rf 0.78 and Aflatoxins G1 fluoresces green at Rf 0.60 under long waves UV light [32].

Gliotoxin and fumagillin: Benzene: ethyl acetate (70:30, v/v). Bluish-green spot under UV 366 nm at Rf value 0.59, 0.60 then appeared as a brown spot in visible light after spraying with freshly prepared 10% (m/V) silver nitrate in 80% (V/V) ethanol [32].

Ochratoxin: toluene: ethyl acetate: formic acid (5:4:1), blue spot at Rf 0.46 [33].

### Virulence assay

Guinea Pigs (animal house, Cairo University, Egypt) were sacrificed by cervical dislocation; lungs were quickly removed and washed in (0.1 M) phosphate buffer (pH 7.4) then transferred into sterilized Petri dishes containing preserving solution. Ten µl of each [fumagillin and gliotoxin standard, spore suspension of *A. fumigatus* (conc. $2 \times 10^7$) and cleaned crude extract in addition to control (ethyl acetate and water)] were spotted on the lung tissue and kept in 30°C for 24 h and 48 h.

## Results and Discussion

Pathogenic fungi secrete enzymes which are considered to be integral to their pathogenesis; these are categorized into two main types; proteinases, which hydrolyze peptide bonds, and phospholipases, which hydrolyze phospholipids. The potentiality of eighty fungal isolates belonged to four genera *Aspergillus flavus* (20), *A. fumigatus* (15), *A. niger* (30) and *A. terreus* (15) isolates isolated and identified from patients suspected with aspergillosis were tested for their enzyme properties (Table 1 and Figure 1). The data showed that all the tested isolates had the ability to utilize calf lung tissue in addition to produce catalase, peroxidase whereas 82.5-90% of the tested isolates produced protease, lipase, urease, phospholipase. Meanwhile, 70 of the tested isolates exhibited hemolytic activities.

| Fungal isolates | No. of tested | Protease | | Tissue utilization | | Lipase | | Urease | | Hemolysis | | Phospholipase | | Catalase | | Peroxidase | | Toxins produced | No. of strain positive |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | +ve | -ve | +ve | -ve | +ve | -ve | +ve | -ve | +ve | -ve | +ve | -ve | +ve | -ve | +ve | -ve | | |
| *Aspergillus flavus* | 20 | 16 | 4 | 20 | - | 16 | 4 | 19 | 1 | 20 | - | 20 | - | 20 | - | 20 | - | Aflatoxins B1, B2 | 20 |
| *Aspergillus fumigatus* | 15 | 13 | 2 | 15 | - | 14 | 2 | 15 | - | 15 | - | 12 | 3 | 15 | - | 15 | - | Fumagillin | 7 |
| | | | | | | | | | | | | | | | | | | Gliotoxin | 8 |
| *Aspergillus niger* | 30 | 26 | 4 | 30 | - | 27 | 4 | 19 | 11 | 20 | 10 | 30 | - | 30 | - | 30 | - | Fumagillin | 7 |
| | | | | | | | | | | | | | | | | | | Gliotoxin | 5 |
| | | | | | | | | | | | | | | | | | | Ochratoxin | 13 |
| *Aspergillus terreus* | 15 | 11 | 4 | 15 | - | 9 | 4 | 15 | - | 15 | 5 | 10 | 5 | 15 | - | 15 | - | Territrem | |
| Total | 80 | 66 | 14 | 80 | - | 66 | 14 | 68 | 12 | 70 | 15 | 72 | 8 | 80 | - | 80 | - | | |

**Table 1:** Extracellular enzymes and mycotoxigenicity produced by the tested fungal isolates collected from suspected aspergillosis patients at Assiut university hospitals.

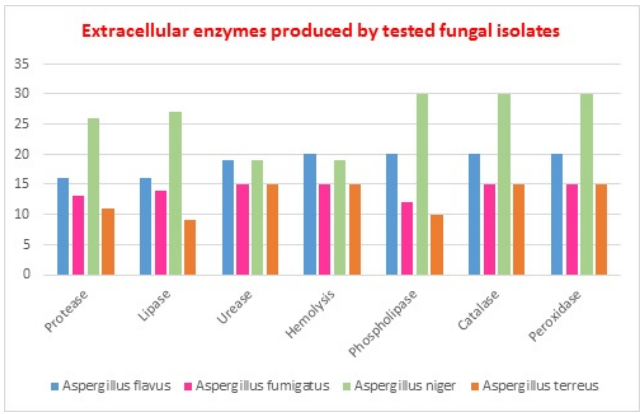


**Figure 1:** Extracellular enzymes produced by tested fungal isolates.

*Aspergillus fumigatus* secretes an aspartic proteinase (aspergillopepsin F) that can catalyze hydrolysis of the major structural proteins of basement membrane, elastin, collagen and laminin in the lung of a host [34]. Lipases play essential roles in lipid metabolism, including digestion, transport, and the processing of dietary lipids [35]. The putative roles of microbial extracellular lipases include digestion of lipids for nutrient acquisition, adhesion to host cells and host tissues, synergistic interactions with other enzymes, nonspecific hydrolysis due to additional phospholipolytic activities, initiation of inflammatory processes by affecting immune cells, and self-defense mediated by lysing competing microbiota [36].

Birch et al. [37] and Koul et al. [38] reported the ability of *A. fumigatus* to produce extracellular phospholipases. Aspergillosis is a disease almost exclusively acquired by inhalation of airborne conidia which penetrate deep into the alveolar spaces that are lined with lung surfactant which is composed of up to 80% phospholipid. Degradation of lung surfactant and subsequent breakdown in oxygen tension may prove beneficial to colonization by fungi [39]. Increased phospholipase activity has also been correlated to increased mucosal pathogenicity in the opportunistic yeast *Candida albicans*. Various pathogenic microbes are able to utilize urea as a nitrogen source through the activity of the enzyme urease that converts urea into ammonia and carbamic acid, with the spontaneous hydrolysis of carbamic acid to carbonic acid generating a further ammonia molecule [40]. *Aspergillus* and other fungi pathogenic to humans have urease activity are saprophytic yeast that infect humans via the lungs and cause disease, with the severity of infection correlating with a loss of host immune function, and in both cases, the disseminated form of infection is potentially life threatening [41,42].

Mono-functional heme-catalase (catalase) dismutates hydrogen peroxide ($H_2O_2$) into water and dioxygen. $H_2O_2$ is both toxic to cells and used as a second messenger for cellular regulation [43]. The use of oxygen as the respiratory substrate is frequently reported to lead to the development of oxidative stress, mainly due to oxygen-derived free radicals, which are collectively termed as reactive oxygen species (ROS). The involvement of oxygen in metabolic processes in fungi is coupled to its activation and formation of number of highly reactive compounds such as superoxide anion radical ($O_2$-), hydrogen peroxide ($H_2O_2$), hydroxyl radical OH-. Activation of this multi-subunit enzyme is tightly regulated [44]. Reactive oxygen species (ROS) produced by alveolar macrophages play an essential role in the killing of *A. fumigatus* conidia [45]. Moreover, *in vitro* studies of neutrophil function have shown that hydrogen peroxide effectively kills fungal hyphae [46] and that neutrophil-mediated damage is blocked by addition of a commercial catalase [47]. Accordingly, catalase, which is a good scavenger of $H_2O_2$, was considered to be a putative virulence factor of *A. fumigatus* [48]. Previous studies have shown that the mycelium of *A. fumigatus* produces two mycelial catalases, one that is monofunctional and one that is a bifunctional catalase-peroxidase [49]. Sophie et al. [50] presented that *A. fumigatus* expresses three active catalases, one that is present in the conidia and two that are

present in the mycelium, which are encoded by three separate structural genes CATA, CAT1, and CAT2 and both mycelial catalases, Cat1p and Cat2p, are involved in the protection of the fungus against the oxidative burst in their experimental rat model.

Hemolysins have been classically defined as exotoxins that are capable of lysing red blood cells as well as nucleated cells. Current knowledge suggests that hemolysins are pore-forming toxins that interact with specific ligands on the surface of various target cells. Although extensively studied in various bacterial species, hemolysins have also been reported in fungi, plants, invertebrates, and mammals (perforins) [51]. In 1939, Henrici reported the first hemolytic activity in filamentous fungi while investigating the pathogenic fungal species, *Aspergillus fumigatus* and *A. flavus* [52]. The extracts collected from the mycelium of the fungus grown for 2-4 days were heat sensitive, hemolytic, and produced necrosis and edema when introduced in experimental animals. It is certain that numerous pathogenic microorganisms grow in the host by using hemin or hemoglobin as a source of iron [53-55]. Several fungal hemolysins have been proposed as virulence factors [56,57]. The hemolysin, which enables the fungus to disrupt blood cells, contains negatively charged domains and can also be detected in infected patients. The hemolysin produced by *A. fumigatus* (asp-hemolysin) promotes Aspergillosis, and may also promote opportunistic infections [58]. Aboul-Nasr et al. isolated 77 fungal isolates from Assiut University Hospitals exhibited some hemolytic activities and mentioned that those isolates represent a risk factors on patients in Assiut university hospitals [59].

Mycotoxins as well as other toxic metabolites are very important virulence factors of fungal infection. The potentiality of the most common fungal isolates recovered from the aspergillosis suspected patients were assayed for toxin/s production.

Chromatographic analysis (TLC) of cleaned extracts of the tested isolates exhibited the ability of all isolates of *A. flavus* had the ability to produce aflatoxins B1 and G1, whereas, 53% of *A. fumigatus* isolates produced fumagillin and gliotoxin. *Aspergillus niger* isolates produced ochratoxins and gliotoxin (43.3% and 23.3% respectively, Table 1). Fungi cause human illness in different ways. Exposure to mycotoxins as a result of infection by a toxigenic fungus is a serious risk to human health in developing countries where the effects of poverty and malnutrition lead to an exacerbation of the detrimental effects of these toxins by circumscribing biochemical detoxification mechanisms [60]. Mycoses are the best-known diseases of fungal etiology, but toxic secondary metabolites produced by saprophytic species are also an important health hazard. Mycotoxins are fungal metabolites which when ingested, inhaled or absorbed through the skin, cause lowered performance sickness or death in man or animals.

Aflatoxins, produced primarily by *A. flavus* and *A. parasiticus*, are among the most toxic and carcinogenic naturally occurring compounds [61]. Aflatoxins also show a wide range of immunotoxic effects, they depress phagocytosis, intracellular killing and spontaneous superoxide production of macrophages. Aflatoxin B1 also inhibits the production of the tumor necrosis factor (TNF), interleukin-1 (IL-1) and IL-6 by lipopolysaccharide-stimulated macrophages [62]. Aflatoxins in the lungs were found in all children diagnosed to have pneumonia, irrespective of the presence of kwashiorkor. This could be due to a reduced clearing ability of the lungs in pulmonary diseases or to exposure via the respiratory route [63].

*Aspergillus fumigatus* produces a range of mycotoxins, such as gliotoxin, helvolic acid and fumagillin, which may facilitate its growth and persistence in the lung [64]. Fumagillin can retard the ciliary beat frequency of pulmonary epithelial cells and thus may facilitate the continued persistence of *A. fumigatus* conidia in the lung. Fumagillin inhibits the growth of colorectal cancer cells and retards metastasis in mice; however, the molecular mechanism of the various activities of fumagillin is ill defined. Gliotoxin possesses a number of powerful bioactivities, including inhibition of activation of the NADPH oxidase of human neutrophils. It has been detected in tissue samples from animals and humans, where it may facilitate fungal persistence and colonization of tissue. In addition, gliotoxin has been implicated in the destruction of lung parenchyma in invasive aspergillosis and the penetration of blood vessels in angioinvasive aspergillosis [65]. Gliotoxin is a potent immunosuppressive mycotoxin and belongs to the epipolythiodioxopiperazine family with an active disulfide bridge in its structure. Gliotoxin is abundantly produced by *A. fumigatus* and is the only toxin isolated *in vivo* from invasive aspergillosis [65,66]. In the *in vitro* assays, gliotoxin inhibits phagocotosis by thymocytes, macrophages, induction of cytotoxic T cells, and stimulation of lymphocytes with mitogen. Gliotoxin can undergo redox cycling, generating oxygen radicals that cause oxidative damage to isolated DNA and induce apoptosis [67]. Production of gliotoxin from clinical isolates of various Aspergillus species indicated that most of the *A. fumigatus* isolates produced gliotoxin (95%) in comparison with other *Aspergillus* species [68]. *In vitro* studies gliotoxin showed immunosuppressive activities including an inhibition of macrophage phagocytosis, mast cell activation, cytotoxic T-cell responses, and mitogen-activated T-cell proliferation [69]. The production of gliotoxin was at highest concentrations in *A. fumigatus*, indicating a link between gliotoxin production and their role in immunosuppression of the host, thus contributing to pathogenesis by diminishing the effect of cellular effector functions [70]. Gliotoxin is able dramatically to modulate lung cell functions such as attachment of epithelial cells and fibroblasts as well as inhibiting phagocytosis by macrophages; other important functions of the host immune defence are also impaired by gliotoxin, including induction of cytotoxic and alloreactive T cells [71]. Hyphal toxins are believed to be an important factor in allowing the hyphae to grow in tissue [72]. Fumagillin can retard the ciliary beat frequency of pulmonary epithelial cells and thus may facilitate the continued persistence of *A. fumigatus* conidia in the lung [73].

As part of its complement of virulence attributes *A. fumigatus* produces a range of toxins, most predominantly the immunosuppressive gliotoxin and enzymes (proteases, elastases and phospholipases) which hinder the host immune response and facilitate tissue penetration, respectively. Furthermore, extracts obtained from aspergillosis patient sputum have been shown to damage human respiratory epithelial cells. Subsequent analysis confirmed that gliotoxin derived from clinical isolates of *A. fumigatus* was the toxic agent and that helvolic acid also caused complete ciliostasis and epithelial cell disruption [73]. A number of protein or non-proteinaceous toxins produced by *A. fumigatus* play a crucial role in assisting the fungus to colonise and penetrate pulmonary tissue and may be detected in blood, urine or sputum specimens [74].

It was demonstrated that ochratoxins disturb blood coagulation and carbohydrate metabolism and is immunosuppressive, teratogenic, genotoxic and carcinogenic. Beside its strong toxic effect on the heart, variable intensity loss of alveolar architecture, distorted appearance of lung parenchyma, intraparenchymal hyperaemic vessels, peribronchial, perivascular and intraparenchymal inflammatory infiltration, respiratory epithelial proliferation, pneumonia, alveolar cell hyperplasia and emphysematous areas were found in the lung of rats

treated with OTA [75]. It is well-known that OTA causes histopathological damage in liver, kidney and testis [76]. Similarly significant histopathological damage in lung and heart of rats treated with OTA were observed in another study [77]. Changes in lung were recorded after feeding with ochratoxin. The changes in the lungs included serous exudates in alveoli, moderate to extensive infiltration, oedema and congestion and hemorrhages at a few places in the interalveolar space [78].

In this study, the virulence assay of 10 µl of each (fumagillin and gliotoxin) standards purchased from Sigma Aldrich Company, spore suspension and cleaned extracts of *A. fumigatus* was spotted on Guinea Pigs lung tissue compared to the control (Figure 2). Gliotoxin in addition to the crude extracts gave necrosis on the lung tissue used whereas the spore suspension and fumagillin gave no necrotic area on the lung tissue assayed after 24 h. Necrosis is caused by various physiological and non-physiological factors. Physiological causes of necrosis include metabolic failure and lack of ATP, acute hypoxia or ischemic injuries such as stroke. Non physiological initiators of necrosis include temperature shock, mechanical damage, and toxins [79]. It has been considered that pathogenicity in *A. fumigatus* is the result of the activity of numerous factors, including adherence systems, toxins, and extracellular enzymes [80]. Gliotoxin is immunosuppressive and causes apoptotic and necrotic cell death *in vitro* [81]. Kyung and Janyce [82] in their study proved that gliotoxin affected lungs as multifocal bronchopneumonia with necrosis, neutrophilic infiltration, and airways filled with necrotic debris and hyphae.

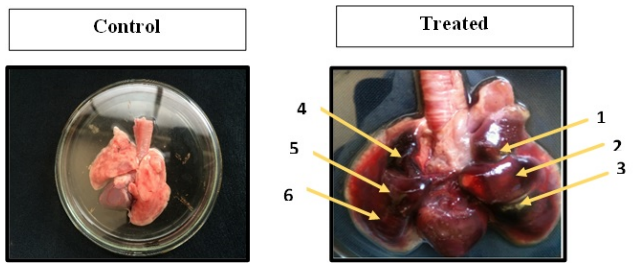


**Figure 2:** Fumagillin and gliotoxin standards, spore suspension and cleaned extract of *A. fumigatus* toxic isolate spotted on Guinea Pigs lung tissue compared to the control. (1- Gliotoxin standard, 2- Cleaned extract, 3- Fumagillin standard, 4-Spore suspension, 5- water, 6- Ethyl acetate).

## Conclusion

In conclusion, in addition to the role that might be played by hydrolytic enzymes produced by opportunistic fungi to cause aspergillosis, toxins might possess the key role in their mycopathy especially gliotoxin that is produced by *A. fumigatus* and *A. niger* (the most incriminated fungal species that cause aspergillosis) due to its tendency to dissolve in water, which may increase the severity of the disease.

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