African Journal of Biotechnology Vol. 11(24), pp. 6566-6577, 22 March, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.4136
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Protective role of *Aspergillus fumigatus* melanin against ultraviolet (UV) irradiation and *Bjerkandera adusta* melanin as a candidate vaccine against systemic candidiasis

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Accepted 23 February, 2012

Melanin protects pigmented cells from physical and biological stresses which are associated with virulence in several important human pathogens, but little is known about the immune response to this ubiquitous biologic compound. Melanin content increased in *Aspergillus fumigatus* mycelium exposed to ultraviolet for 10 min but gradually decreased after 60 min of UV exposure. So, it can be concluded that melanin protects fungus for survival until 60 min which was obvious after studying conidia and mycelia by transmission electron microscope (TEM). This research hypothesized that melanin produced by a higher fungus, *Bjerkandera adusta*, is immunogenic against fungal infection. Melanin was purified from melanized fungal hymenium layer and used in mice immunization, then the actively immunized mice were challenged 1 day later with 10^7 candidal spores. The antibody (Ab) response was evaluated by radio-immunodiffusion diffco-plate of IgM for accurate quantitative measurements of immunoglobulin in biological fluids. Candidasis was detected in mice target organs after the challenge and urea was assayed to detect the degree of kidney damage. The results demonstrate that melanin could be immunogenic; this is indicated through IgM follow-up and its development might suggests that this amorphous insoluble polymer can stimulate the immune system against the latter challenge, thus reducing the degree of infection. This was observed through the reduction of candidiasis and the enhancement of kidney function.

Key words: Melanin, ultraviolet (UV) irradiation, active immunization.

INTRODUCTION

Melanins are complex black polymers of resonance stabilized cyclic subunits (including indoles, phenols, hydroxynaphthalenes) and are notoriously intractable to chemical analysis. Melanin deposition protects the pigmented cell from physical and biological stresses (Nicolaus et al., 1964). Melanin is also a structural component of the conidial wall that is required for correct assembly of the cell wall layers and the expression at the conidial surface of adhesions and other virulence factors (Pihet et al., 2009). The primary (evolutionary) function of melanin in microorganisms is to reduce the detrimental effects of UV radiation, for example, DNA damage. The correlation between concentration of melanin and UV tolerance is debated. Sunlight consists of about 95% UVA radiation. Melanin provides a fine example of physiological value of both inducible and constitutive defense against UV radiations (Cockell and Knowland, 1999; Majerus, 1998; Prota, 1992). Prota (1992) evaluated the survival of spores, spore aggregates, sclerotia and pycnidia of fungi after exposure to darkness, or exposure to ultraviolet (UV) radiation or sunlight. Under most conditions, survival decreased from the most resistant *Sclerotium rolfsii* to *Alternaria macrospora*, *Mycosphaerella pinodes*, *Aspergillus niger* and *Botrytis cinerea*. Exposure to UV reduced survival much more than exposure to darkness. Survival of sclerotia cut into

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slices and exposed to UV increased with thickness, irrespective of exposure to UV with the outer pigmented or inner non-pigmented side.

Melanins protect microorganisms, such as bacteria and fungi, against stresses that involve cell damage such as UV radiation from the sun and reactive oxygen species. Melanin also protects microorganisms against damage from high temperatures, chemical stresses (such as heavy metals and oxidizing agents), and biochemical threats (such as host defenses against invading microbes). Therefore, in many pathogenic microbes (for example, in *Cryptococcus neoformans*), melanins appear to play important roles in virulence and pathogenicity by protecting the microbe against immune responses of its host (Casadevall et al., 2000; Nosanchuk et al., 2000; Rosas et al., 2000 a, b; Hamilton and Gomez, 2002).. *Aspergillus fumigatus* conidia are known to produce a bluish-green pigment by using the dihydroxynaphthalene (DHN)-melanin pathway (Tsai et al., 1997, 1998, 1999; Langfelder et al., 1998; Watanabe et al., 2000). *Candida albicans* is usually a harmless commensal in normal hosts. However, in immunodeficient or immune-suppressed patients, invasive candidiasis can become a life-threatening condition *(*Kullberg and Oude-Lashof, 2002). *Candida* spp. and *C. albicans*, in particular, are among the leading causes of nosocomial infections (Pittet et al., 1994; Safdar and Maki, 2002) including potentially fatal fungal peritonitis in renal patients undergoing peritoneal dialysis (Bibashi et al., 2003).

Developments in vaccines and antibody-based immunotherapy are key importance in treatment of invasive fungal infections (Ostrosky-Zeichner et al., 2010). Given that fungal surfaces are highly complex, there are numerous antigens that can elicit antibody responses, and protective antibodies have been described that target polysaccharide, protein, lipid and melanin antigens (Dromer et al., 1987; Mukherjee et al., 1992;Fleuridor et al., 1998).

Sepsis, due to *Candida* spp., is a worldwide problem. In the United States, *Candida* spp. are the fourth most common cause of nosocomial bloodstream infections, and approximately half of these are due to a single species, *C. albicans* (Spellberg and Edwards, 2002).

In the first part of this study, the protective effect of melanin was investigated against UV irradiation produced from *A. fumigatus* mycelia. Both chemical and physical characters of *A. fumigatus* melanin were covered. Also, morphological changes were observed in the cell wall before and after treatment of the UV ray by transmission electron microscope (TEM). In the second part, melanin was extracted from *Bjerkandera adusta* fruiting bodies and used in active immunization of mice against the latter challenge of *C. albicans.*

MATERIALS AND METHODS

A. fumigatus was isolated from the air by exposing several Petri dishes containing Czapek's agar media and was identified according to the method of Moubasher (1993), then sub cultured in Czapek's media for 7 days at 28°C.

Exposure of mycelia of *A. fumigatus* to UV radiation

Fungal discs, sized 0.5 mm of *A. fumigatus*, were inoculated in the center of Petri dishes containing 20 ml Czapek's Dox solid media. Each group of three plates was then placed under UV irradiation (UV-A- 4w lamp tube with a wave length of 365 nm) to receive doses at different times for 10, 30 and 60 min and one group of plates was untreated and used as the control. The length between the UV Lamp and exposed Petri dishes was constant (10 cm) (Osman and Metwally, 1991).

Effect of UV radiation on dry weight and melanin production of *A. fumigatus*

Two discs from irradiated fungal mycelia were inoculated into 250 ml conical flasks containing 100 ml of sterile culture of Czapek's medium at pH 6 to 6.2. Three replicates were prepared from each exposure time. All flasks were incubated at 28°C for 15 days, after which every flask was filtered and the produced mats were oven dried at 40°C until they attained constant weights (Jimenez and Shafizadeh, 1985).

Extraction of melanin from *A. fumigatus*

Extraction of melanin was carried out according to Gadd (1982) from fungal mycelium of *A. fumigatus;* after washing fungal mycelia with distilled sterilized water and centrifuging it, the pigments were extracted by autoclaving the pellet and then dissolved in 3 ml IM NaOH. The alkaline melanin extract was acidified to pH 2.0 with concentrated HCl to precipitate the melanin, while the extraction of crude melanin from *Bjerkandera adusta* fruiting body (hymenium layer) was carried out according to the method of Saiz-Jimenez (1983). The melanins were extracted from the hymenia that were ground with sand, after which they were washed in 1N HCl, distilled H_2O and pre-sterilized in autoclave for 20 min.

The extraction was made by 0.5N NaOH solution and the mixture was filtered and centrifuged at 10000 rpm for 10 min; the supernatant was acidified to pH 1.5 with 1N HCl and the melanin precipitates were recovered by centrifugation at 5000 rpm. The melanins were redissolved in 0.1N NaOH and centrifuged at 10000 rpm to eliminate cellular debris. The supernatant was again acidified with HCl to precipitate melanin in which *B. adusta* was selected from Trunk base of *Eucalyptus* trees, taking into consideration their rich melanin contents. Fruiting bodies, belonging to order Aphylloporales, family polyporaceae and identified according to Phillips (1981) and Breitenbach and Kranzlin (1986), were recovered from Shabsheer El Hessavillage, EL Gharbia Governorate (Egypt).

Physical and chemical properties of melanin extracted from *A. fumigatus*

According to Thomas (1955) and Ellis and Griffiths (1974), the dried extracted melanin from each fungus was subjected to various physical and chemical tests as follows: color of extracted solution in NaOH, solubility in water, solubility in organic solvents (acetone, ethanol, chloroform, ethyl acetate and hexane), reaction to polyphenol test, $FeCl_3$ (1% w/v), precipitation in 3N-HC1 and solubility in 1 M KOH (100°C for 2 h), and was compared with the standard melanin purchased from Sigma company.

Infrared (IR) spectrum of melanin from *A. fumigatus* before and after UV stress

For IR spectrum, the pigment was hydrolysed with 5 ml of 7 N HCl in sealed glass vial and kept for 2 h at 100°C (Bell and Wheeler, 1986). The purified pigments were ground with KBr. The spectrum was recorded in Perkin–Elmer 377 spectrophotometer (Ravishankar et al., 1995).

The location of melanin in the cell wall of *A. fumigatus* by transmission electron microscope before and after UV stress

In this experiment, transmission electron microscope was applied to detect the location of melanin in the cell walls of the conidia and mycelia of *A. fumigatus*. Extracellular melanin was apparent after 15 days of incubation period in the culture of Czapek's agar medium. After this period, a small portion of the fungal mat was fixed at room temperature in 2% (v/v) glutaraldehyde mixed with potassium in 2% (w/v) osmium tetraoxide buffered in 0.005 M sodium cacodylate at pH 6.5 for 40 min. After fixation, the material was washed overnight in the appropriate buffer, dehydrated at room temperature in acetone, and embedded overnight at 65°C in low viscosity epoxy resin (Spuur, 1969). At these conditions, the material was polymerized; ultrathin sections were cut by glass knives of an ULKD ultramicrotome. Sections were collected each on stabilized copper grids, stained with lead citrate and examined in a GOL 100 CX electron microscope. This method was carried out according to the instructions of Ellis and Griffiths (1974).

Experimental animals

Mice were bred in the animal facility of the Microbiology Unit, Bacteriological Laboratory, Faculty of Science, Tanta University. Female and male mice aged 8 to 10 weeks and weighing 18 to 20 g were used throughout. They were maintained under a 12 h light-dark cycle at a temperature of 22±2°C and fed with standard diet and water *ad libitum*.

Immunizations

Mice were divided into groups, each of which consisted of six mice. The mice were injected intraperitoneally (i.p.), in three successive doses, with 200 µg\24 h of the following preparation: 600 µg suspension of melanin in 1 ml phosphate-buffered saline (PBS). The respective control animals received PBS (non-immunized).

Challenge and infections

Non-lethal infections with *C. albicans* ATCC 10231 were evaluated in kidneys, liver, lung and spleen of mice, for 5, 10 and 15 days after intraperitoneal (i.p.) infection with 10^7 viable blastoconidia in 1 ml of phosphate-buffered saline (PBS). Prior to infection, the blastoconidia were incubated in Saboroud glucose broth (Difco) for 18 h at 37°C. The two kidneys, liver, lung and spleen were aseptically removed, homogenized in PBS and serially diluted in 1:10 dilutions, respectively. The colony-forming unit (CFU) values of *C. albicans* were counted in duplicate cultures of each serial dilution after 48 h of culture in Saboroud agar (Difco).

Sera

The sera were obtained from mice through the lateral tail vein and stored at -20°C before use.

Agglutination

Melanin particles extracted and purified from *B. adusta* were incubated in 1% BSA overnight to block nonspecific protein binding. Starting with a 1/20 (v/v) dilution of serum, different dilutions of serum in 1% BSA (50 µl/slide) were performed for this examination, and melanin particles in 1% BSA were added to each well. After incubation at 37°C for 1 h, agglutination was assessed by examination of the particle aggregation with a microscope (x400 magnification).

Urea assay

Quantitative determination of urea in plasma was enzymatically assayed and spectrophotometrically measured at 578 nm according to Tietz (1999) and Young (2001).

Antibody detection

The sera were assayed for anti-melanin antibody using radio-immunodiffusion diffco-plate of IgM for accurate quantitative immunoglobulin in biological fluids (Biocientifica S. A).

Statistics

Significance of variation in melanin production and fungal growth under different UV doses was determined. Statistical presentation and analysis of the present study was conducted using the mean, standard deviation [ANOVA] tests by SPSS.V.16. (Pipkin, 1984*)*. Verification of melanin efficiency as fungal protecting agent against UV irradiation was also determined.

RESULTS

Characters of melanin extracted from *A. fumigatus*

A. fumigatus mycelial melanin formed a black pigment in mycelium and conidia. The extracted melanin was not soluble in water but soluble in 1 M KOH for 2 h at 100°C and there was no effect of UV in its characters. Precipitation by 3 NHCl gave reddish brown precipitate with 1% $FeCl_3$, decolorized with H_2O_2 and gave positive result with silver nitrate. It has a resemblance with the standard melanin characters, and the same chemical characters were achieved for melanin extracted from hymenia of *B. adusta* according to the study of Eman (2005) which shows a resemblance with the standard melanin. Quantitative examination of melanin from two fungal species was performed and it was found that melanin extracted from *A. fumigatus* was less than melanin from *B. adusta* fruiting body, in which 5 g of hymenia fruiting body gave 24 mg/ml as compared to 21.25 mg/ml harvested from *A. fumigatus*. The ratio between melanin production and mycelial dry weight of *A. fumigatus* was 42.5, 43.9 and 42.2% at 10, 30 and 60 min, respectively after UV exposure compared to the

Table 1. Effect of UV radiation at different times on dry weight of mycelia and melanin pigment produced from *Aspergillus fumigates.*

Time/min	Mycelial dry weight (mg/ml)	Melanin dry weight (mg/ml)	P. value
Control	8.0 ±0.1	3.4 ± 0.1	0.003*
10	8.2 ± 0.2	3.6 ± 0.1	0.006
30	7.5 ± 0.5	3.2 ± 0.1	0.015
60	7.0 ± 0.1	1.7 ± 0.1	0.035

Value is the mean ± SD of three replicates. * Significant <0.05.

Table 2. IR analysis of extracted melanin from *Aspergillus fumigatus* before and after 60 min of UV stress compared with the standard melanin.

Character	UV stress		Standard melanin (cm^{-1})
	Before	After 60 min	
- IR analysis (wave no.)	3889, 3808, 3381 2927, 2363,1867, 1638, 1531, 1073, 651, 583 and 463	3887, 3818, 3719, 3383, 2926, 2620, 2363,1868, 1984,1632 ,1531, 1849, 1696, 1626, 1206, 817, 780, 581 and 457	3395, 2928, 2358, 1984, 1712, 1627, 1395, 779, 648, 582and 221 cm^{-1}.

control.

Effect of UV on fungal growth and melanin production in *A. fumigatus*

In the present experiment, short time exposure to UV radiation produced stimulatory effects and the dry weight increased with increasing the time of exposure to UV radiation.

Table 1 shows that the dry weight of *A. fumigatus* mycelia increased with increase in the time of exposure and the highest recorded dry weight was 8.2 mg/ml after 10 min of UV exposure, and then decreased to 7 mg/ml after 60 min of UV exposure.

The highest melanin dry weight was 3.6 mg/ml at 10 min but decreased to 1.7 mg/mL at 60 min of UV exposure.

It was noticed that melanin dry weight and mycelial dry weight increased significantly after UV illumination for 10 min at a P- value of 0.006 when compared with the control value, but decreased significantly at a P- value of 0.035 after 60 min of UV exposure when compared with the control value as shown in Table 1.

Crude melanin extracted from *B. adusta* was used in immunization of mice because *B. adusta* was nonpathogenic to higher fungi and produced high quantity of melanin.

Infrared analysis of melanin extracted from *A. fumigatus* before and after UV-stress

A. fumigatus melanin before UV stress exhibited wave numbers at 3381, 2927, 1867, 1531, 1404, 1073 and 651 cm^{-1}.

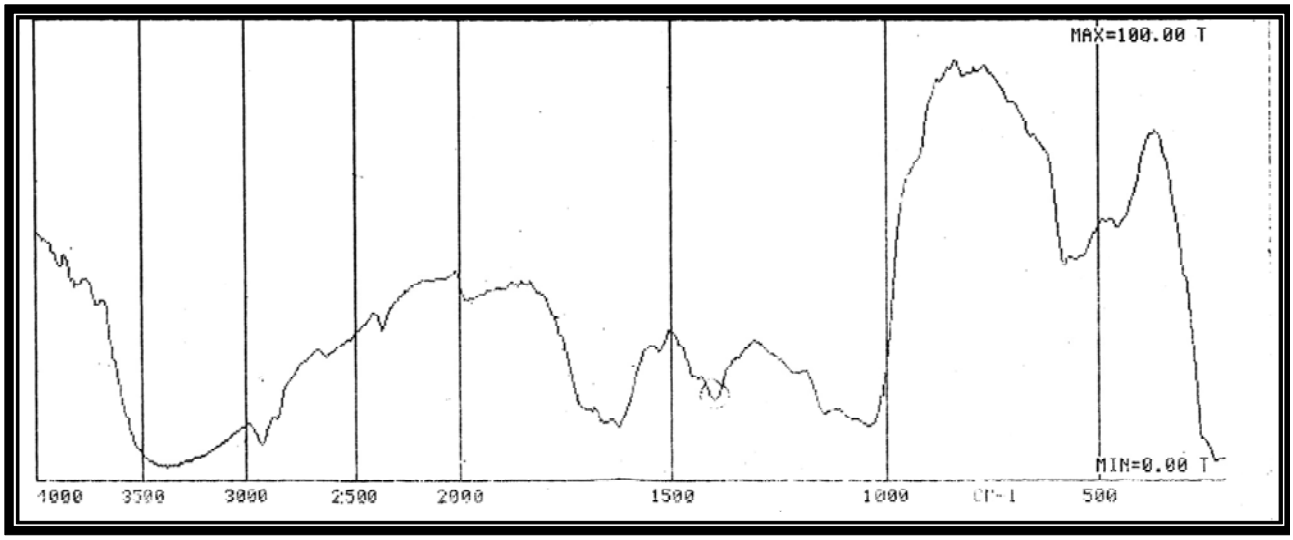
The wave band can be ascribed to the following chemical groups: 3381 cm^{-1} was attributed to OH bonds, 2927 cm^{-1} to H-C or H-C=0 bonds 1404 (C-CH_3), and 651 cm^{-1} to (acyclic) CH_2 bonds (Table 2). IR spectrum of melanin extracted from *A. fumigatus* after 60 min exposure to UV lights had little difference compared with IR spectrum of melanin extracted from *A. fumigatus* before exposure to UV stress. IR band of melanin at 1073 cm^{-1} after exposure to UV lights at 60 min disappeared, but new IR bands appeared at 3719, 2620, 1984, 1849, 1206 and 1696 cm^{-1}. So, UV light in our study had little effect on IR spectrum as shown in Figure 1 (A, B and C).

Transmission electron microscope of melanin from *A. fumigatus*

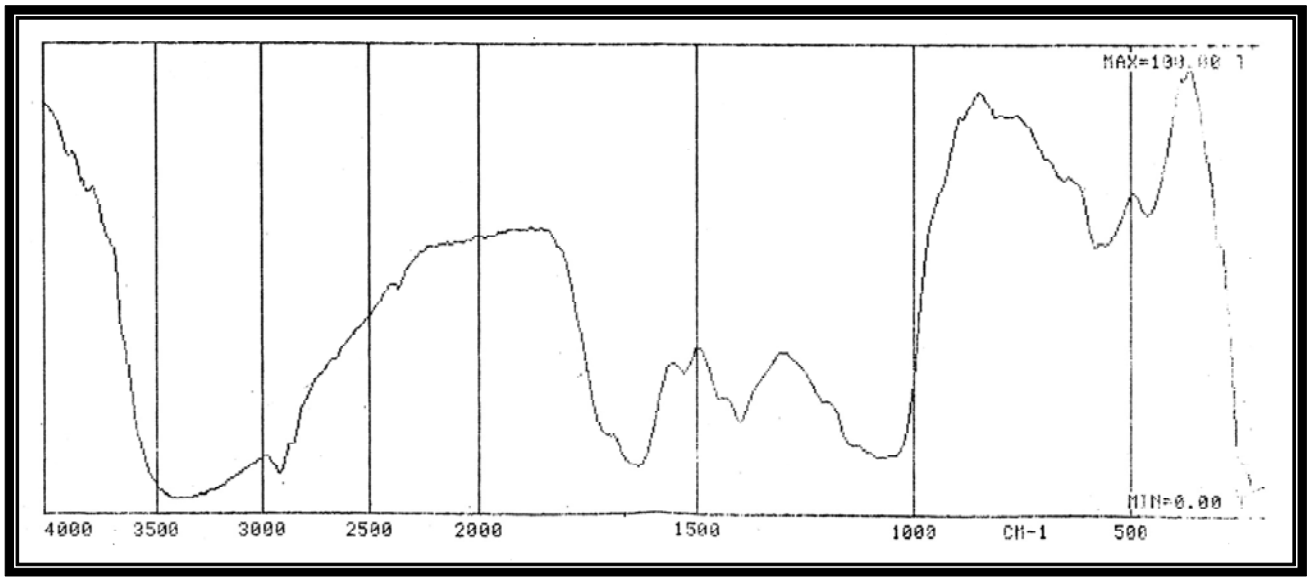
In the control sample, the cell wall of mycelia contains melanins that were found in granular-verrucose surface and in conidia as droplets, and secreted outer droplets external to the conidial wall (Figure 2A). Increase in quantity of melanin after exposure to UV light for 30 min caused an increase in fungal melanin cell wall volume (Figure 2B). In Figure 2C, melanin quantity may be decreased, but still present as melanin droplets in degenerated mycelia and are found in the surrounding conidial wall. Melanin quantity in *A. fumigatus* after 10 and 30 min of UV exposure increased but after 60 min of UV exposure, melanin quantity decreased but still present in attempts made to protect cell content in fungal mycelia or conidia to reduce fungal conidia and mycelia death.

Estimation of urea, IgM and candidiasis CFU in kidney after and before challenge with 10^7 blastoconidia as a result of pre-treatment with melanin

To determine whether antibody (Ab) elicited in response



(A) After 60 minutes



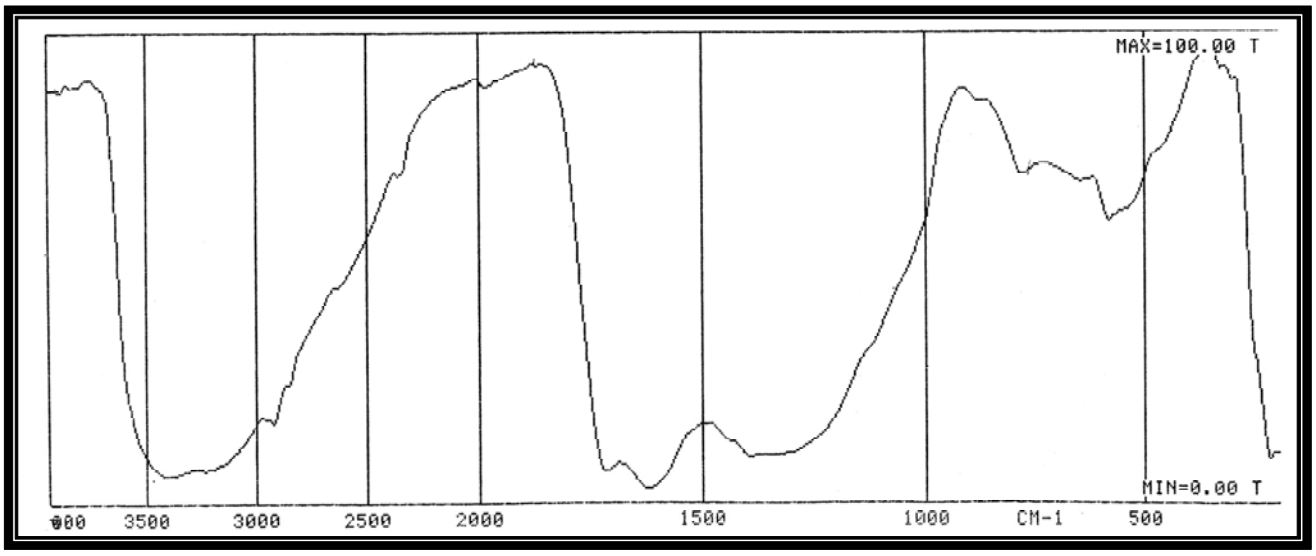
(B) Control

Figure 1. (A, B and C): Infrared analysis of melanin extracted from *A. fumigatus* before and after UV-stress.

to immunization with melanin suspension, the reactivity of immune sera with melanin was detected through agglutination test. The obtained positive result was followed by measuring other parameters.

Induced resistance to systemic candidiasis in mice immunized with melanin against pathogenic *C. albicans*

To evaluate the importance of fungal pathogenicity on immunized mice with melanin, mice were infected with 10^7 pathogenic blastoconidia 1 day later after the animals were immunized with 600 µg/ml of melanin suspension i.p., on three successive doses at 24 h time intervals. The numbers of *C. albicans* C.F.U. recovered from the kidneys were markedly reduced with a P value of 0.002 in i.p immunized mice as shown in Table 3, when compared to the heavily infected non-immunized mice, 5 days after i.p. infection with the large inoculum of pathogenic *Candida* blastoconidia. All non-immunized mice were colonized to a similar extent or were even more colonized at 15 days after infection than at 5 days after the infection. In contrast, all immunized mice were clear of



(C) Standard melanin

Figure 1. Contd.

fungus at 15 days after infection or challenge. The colonization of *Candida* in target organs was seen in the kidney, lung and liver, then spleen. Urea was significantly reduced in immunized mice with melanin than in non-immunized mice with a P-value of 0.001.

Immunoprotection against challenge with *C. albicans* infection associated with a high ratio of IgM antibodies

To investigate the role of antibodies in immunoprotection against systemic candidiasis, the serum antibodies were determined in the non-treated (reference value), immunized and non-immunized (control) mice after challenge or fungal infection. Serum IgM antibodies in the control mice or non-immunized mice were markedly lower than those observed in mice with melanin after being challenged with a large fungal infection with a P-value of 0.025 when compared with the reference value (Table 3).

DISCUSSION

This study assessed the protective efficacy of melanin against UV stress in *A. fumigatus* and against the systemic candidiasis in an experimental mouse model using *B. adusta* melanin as the immunogenic substance against intra-peritoneal (I/P) challenge with the sub-lethal dose of *C. albicans*.

Characters of melanin extracted from *A. fumigatus*

Gadd and Derome (1988) recorded that cultures of *Cladosporium resinae* and *Aspergillus pullulans* contained extracellular melanin and probably other extracellular materials. In the meantime, black fungal pigments were extracted and identified as melanin from the mycelia of *Cladosporium mansoni* (Sussman et al.,1963). In culture, *Cirrenalia pygmea* formed a dark-pigmented mycelium and conidia. The dark brown pigment in the hyphae could not be extracted with organic solvents such as acetone, chloroform, benzene, hexane methanol or petroleum ether (Ravishankar et al., 1995).

Effect of UV on fungal growth and melanin production from *A. fumigatus*

The results of the present study are in agreement with those showing that light inhibited growth, for example, black UV and light inhibited growth of *C. albicans* (Saltarelli and Coppola, 1979) and *Aspegillus ornatus* (Hill, 1976) respectively. It was noticed that the reduction in growth of fungal mycelia and the reduction in melanin production may be the reason for the cells' death due to the reduction provided by the photo protection of melanin.

The effect of UV-A irradiation (366 nm) on growth and lipid composition in *Alternaria alternata* and *Fusarium oxysporum* was investigated. UV-A was found to retard colony size significantly, while biomass production increased, especially at longer frequencies of 1.920 Jm^{-2} (Osman and Metwally, 1991). In general, colony size was inversely related to biomass and total lipids and this could be seen clearly at a frequency of 1.920 Jm^{-2}. This may result from a general induction of enzymes which may lead to a higher metabolic activity resulting in the

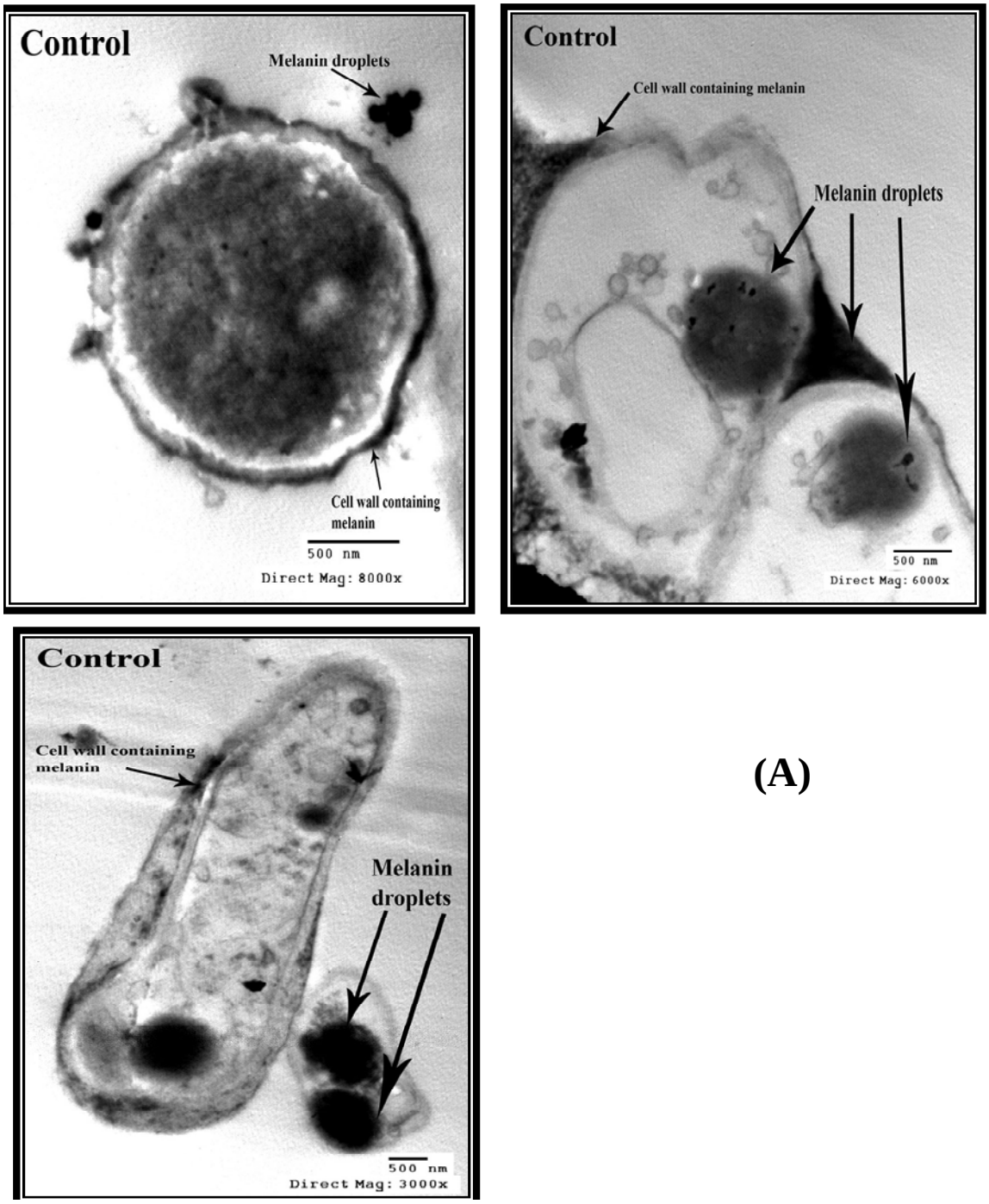


Figure 2. Melanin production by *A. fumigatus* before (A; control) and after exposure to UV (for 30 min; B and for 60 min; C)

production of more reserve materials such as protein (Saltarelli and Coppola, 1979; Abo-Zeid, 1986), carbohydrates (Goldstein and Cantino, 1962) and lipids (Osman, 1980).

Growth was inhibited in *Verticillium agaricinum* (Osman and Valadon, 1979) after UV-A irradiation, while both UV-A and UV-B irradiations inhibited colony size in *Fusarium Solani and A. alternata* (Osman and Abo-Zeid, 1984). Direct illumination resulted in total suppression of growth of *Monascus purpureus*, and incubation in total darkness increased dry weight of mycelia (Babitha et al., 2008). Rate of fungal growth and melanin production decreased because UV-A irradiation causes cellular damage by generating reactive oxygen species, like singlet oxygen,

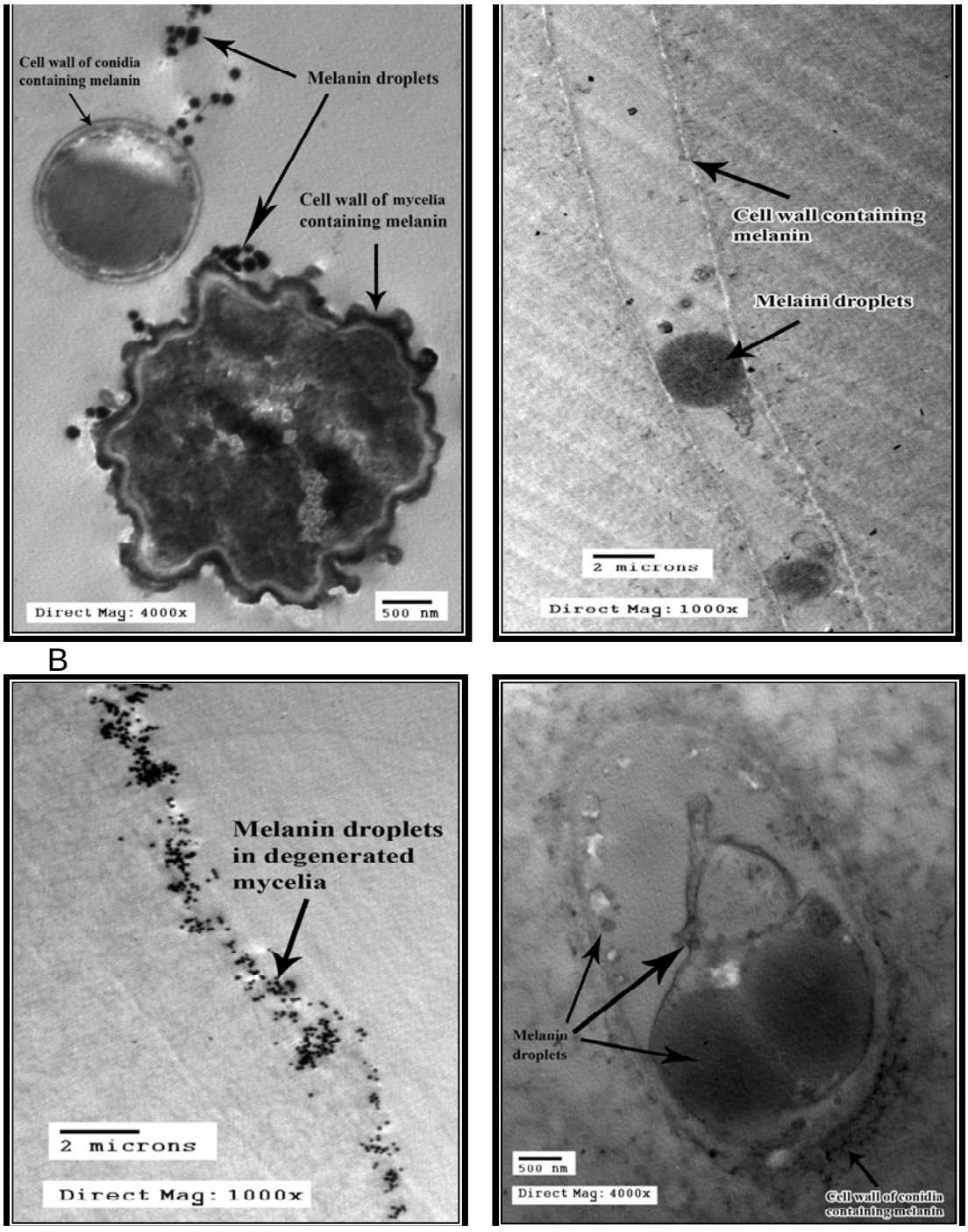


Figure 2. Contd.

which interact with intracellular chromophores. There is every possibility that melanin delayed the effect of UV-A on fungal mycelia and gave it more tolerance against UV-A irradiation. Many fungi produced melanins which are complex black polymers of resonance-stabilized cyclic molecules, such as hydroxynaphthalenes. Fungal melanins, through environmental factors, protect cells against attack (Butler et al., 2009). Also, melanin protects fungal mycelia against UV-A irradiation by the mechanism that has a resemblance with that used by pigmented *Parrcoidedes brasiliensis* yeast cell. Some fungi like *C. neoformans* and several molds can produce melanin in the soil, and this characteristic may provide increased resistance to the environmental stressors

Table 3. Estimation of urea, IgM and candidiasis CFU in kidney after challenge with 10^7 blastoconidia as a result of immunization with melanin.

Time (day)	Non-immunized			Immunized		
	Urea (mg/dl)	IgM (mg/dcl)	CFU x 10^2	Urea (mg/dl)	IgM (mg/dcl)	CFU x 10^2
5	107	130	550	80	152	110
10	130	160	860	90	180	54
15	130	145	1000	88	170	20
Reference value	40	75	0	40	75	0

All data represented means of three replica. * Significant <0.05. P value, 0.001 urea. P value, 0.025 IgM. P value, 0.002 CFU.

(Nosanchuk and Casadevall, 2006). Melanized *C. neoformans* is more resistant to ingestion by environmental amoeboid or nematode species. Melanins also protect fungi from hydrolytic enzymes, UV, solar or gamma radiation, extreme temperatures, and heavy metals and several other toxic compounds (Steenbergen and Casadevall, 2003).

The effect of light (especially UV-A) on fungal growth has been reported by many workers. Tan and Epton (1973) observed that the retardation of growth of *B. cinerea* was caused by black light, and light also inhibited the growth of *Monilinia moli*. Osman and Valadon (1979) recorded a marked inhibition of growth of *V. agaricinum* by UV-A and UV-B, and UV-B was found to inhibit growth in *A. alternate, Aspergillus flavus, F. solani* and *Penicillium notatum (*Abo-Zeid, 1986).

Infrared analysis of melanin extracted from *A. fumigatus* before and after UV-stress

The obtained results revealed that *A. fumigatus* melanin before UV-stress showed characteristic absorption bands that would be expected from an aromatic substance and their wave number was similar to that of the melanin precursors.

In the meantime, Bonner and Duncan (1962) concluded that the infrared absorption spectra of melanin from several biological sources were similar. However, the infrared spectra in the present study supported this view. Also, it was concluded by Korzhova et al. (1989) that the illumination at different wave lengths of DOPA-melanin to UV-light caused significant bleaching of diluted aqueous solution of the pigment.

Transmission electron microscope of melanin from *A. fumigatus*

Dematiaceous fungi are characterized by the production of dark pigments associated with the cell wall (Ainsworth, 1971). San et al. (1996) found that melanin accumulated under the cell wall of *Cladosporium carrionnii and H. resinae.*

The same observation was made for *Amorphotheca resinae* by Ellis and Griffiths (1974), but they found that melanin arose from the rupture and fragmentation of the outer covering caused by the increase in volume of the conidial wall during development and maturations. So, this may be the reason why the fungus was able to hold on to the long time exposure (60 min) to UV light. It may be hypothesized that the presence of melanin protects fungus against any stress found in the surrounding environment.

Melanins confer resistance to UV light by absorbing a broad range of the electromagnetic spectrum and preventing photoinduced damage (Hill, 1992). Consequently, melanins are used commercially in photoprotective creams and eye glasses. Melanin protects fungal and bacterial species from UV, solar or gamma radiation (Nosanchuk and Casadevall, 2003). Increased melanin production is associated with the greater resistance of pigmented fungi to radiation (Vasilevskaya et al., 1970; Zhdanova et al., 1973). The protective properties of melanin against radiation injury could account for the growth of black fungi in the highly contaminated Chernobyl reactor No. 4 (Mironeko et al., 2000).

Active immunization against *C. albicans* challenge using melanin particles of *B. adusta*

In the present study, mice immunized with melanin induced a highly significant IgM response with high protective response against colonization of *C. albicans* in target organs, especially kidneys, and recorded significant improvement for urea compared to the non-immunized group. These results are in accordance with those obtained by Torosantucci et al. (2005), in which a novel conjugated vaccine (laminarin with the diphtheria toxoid CRM197; Lam-CRM conjugate) was generated. This conjugate vaccine can efficiently immunize and protect cells against two major fungal pathogens, *A. fumigatus* and *C. albicans*, by mechanisms that may include direct antifungal properties of anti-glucan antibodies.

Vaccination with the Lam-CRM conjugate induces antibody-mediated anti-Candida protection in a murine

experimental model of disseminated infection. Anti-β-glucan IgG and IgM titers in Lam-CRM–vaccinated mice were measured against the antigens antibodies raised by the vaccination with the Lam-CRM conjugate. A number of fungal CFU in kidneys after a lethal systemic challenge with *C. albicans* revealed that the Lam-CRM-immunized mice, but not the CRM- or Lam-immunized ones, were significantly protected from the lethal systemic challenge by *C. albicans*, as compared to the non-immunized (adjuvant-treated) mice.

The success of immunization with melanin had been investigated and explained by many authors. Rosas et al. (2001) stated that passive immunization with monoclonal antibodies (MAbs) to melanin prolonged the survival and reduced the fungal burden in *C. neoformans*-infected mice in comparison to the controls. MAbs to melanin reduced the growth rate of *in vitro*-melanized *C. neoformans* cells. Nosanchuk et al. (1998) hypothesized that melanin produced by the fungus, *C. neoformans*, was immunogenic. *C. neoformans* melanin was purified from melanized fungal cells and was used to immunize C57BL/6, BALB/c and T cell-deficient (nude) BALB/c mice. The Ab response (including Abs of IgM and IgG isotypes, 3) and the results demonstrate that melanin can be immunogenic, and the humoral immune response can be T cell independent.

The present data suggesting that immunization with melanin elicit antibody response which may interfere with the ability of *C. albicans* to produce melanin particles consequentially reduced its virulence. This result supported by Morris-Jones et al. (2005) who confirmed the presence of melanin particles in *C. albicans in vitro*, and during infection, it played a role in the pathogenesis of the target organs (heart, lungs, liver, spleen and kidneys). Digestion of infected murine kidneys resulted in isolation of melanin particles which reacted with anti-melanin monoclonal antibodies (MAb). *In vivo* data demonstrate the compounds which inhibit melanization, and which are capable of reducing the virulence of *C. neoformans* and other fungi. The administration of monoclonal antibodies to melanin or glyphosate (which inhibits the melanization of *C. neoformans*) prolongs the survival of mice lethally infected with *C. neoformans* (Nosanchuk et al., 2001; Rosas et al., 2001). The development of drugs that interfere with melanin polymerization or rearrangement may be seen as useful therapeutic compounds for the treatment of these melanotic fungi and other pathogens that produce melanin (Nosanchuk et al., 2003). The present data revealed that, immunization with *B. adusta* melanin was able to reduce candidiasis in kidney to 20 fold when compared with non-immunized mice after 10 days of challenge. In accordance with this result, Vilanova et al. (2004) found that classical immunization with native aspartic pro-teinase 2 (Sap2) and alum as adjuvant induced a strong specific humoral response associated with a 20-fold decrease in *C. albicans* load in the kidney upon infection when compared with unimmunized animals.

The role of antibody mediated immunity during immunization or vaccination could be explained as follows: antibody-mediated immunity can contribute to host defense by enhancing the effectiveness of innate immunity through complement activation, by direct antimicrobial effects on fungal cells (Rosas et al., 2001). Chai et al. (2010) found that melanin pigments on the surface of resting *A. fumigatus* conidia may serve to mask pathogen-associated molecular patterns (PAMPs)-induced cytokine response. The albino conidia induced significantly more proinflammatory cytokines in human peripheral blood mononuclear cells as compared to melanised wild-type conidia. Melanin may play a modulatory role by impeding the capability of host immune cells to respond to specific ligands on *A. fumigatus*.

It can be concluded that melanin occurrence in *A. fumigatus* may give more protection to the mycelial and conidia of the fungus against UV radiation. Also, this study may assume that melanin, due to its success, can be used as a candidate vaccine against melanised fungal pathogens such as *C. albicans*.

REFERENCES

Abo-Zeid AM (1986). Studies on the effects of UV-radiation on activities of certain fungi. MSC, Tanta University, Egypt.

Ainsworth GC (1971). Ainsworth and Bisby's Dictionary of the fungi 6th ed. Commonwealth mycological Institute. Kew, Surrey, England.

Babitha S, Carvahlo JC, Soccol CR, Pandey A (2008). Effect of light on growth, pigment production and culture morphology of *Monascus purpureus* in solid-state fermentation. World J. Microbiol. Biotechnol. 24(11): 2671-2675.

Bibashi E, Memmos D, Kokolina E, Tsakiris D, Sofianou D, Papadimitrou M (2003). Fungal peritonitis complicationg peritoneal dialysis during an 11-year period: report of 46 cases. Clin. Infect. Dis. 36: 927-931.

Bonner TG, Duncan A (1962). Infrared spectra of some melanins, Nature London. 194: 1078-1079.

Breitenbach J, Kränzlin F (1986). Fungi of Switzerland a contribution to knowledge of the fungal flora of Switzerland. Verlage Mykologia, Ch-6000 Luzern 9 Switzerland. 328-423

Butler JM, Gardiner RB, Day AW (2009). Melanin synthesis by *Sclerotinia sclerotiorum*. Mycol. 101(3): 296-304.

Casadevall A, Rosas AL, Nosanchuk JD (2000). Melanin and virulence in *Cryptococcus neoformans*. Curr. Opin. Microbiol. 3: 354-358.

Chai LY, Netea MG, Sugui J, Vonk AG, van de Sande WW, Warris A, Kwon- Chung KJ, Kullberg BJ (2010). *Aspergillus fumigatus* conidial melanin modulates host cytokine response. Immunobiol. 215(11): 915-920.

Cockell CS, Knowland J (1999). Ultraviolet radiation screening compounds. Biol. Rev. 74: 311-345.

Dromer F, Salamero J, Contrepois A, Carbon C, Yeni P (1987). Production, characterization, and antibody specificity of a mouse monoclonal antibody reactive with *Cryptococcus neoformans* capsular polysaccharide. Infect. Immun. 55: 742-748.

Ellis PH, Griffiths DA (1974). The location and analysis of melanins in the cell walls of some soil fungi. Can. J. Microbiol. 20: 1379-1386.

Eman HF Abd El-Zaher (2005). Studies of melanin and polysaccharides from some fungi. PhD. thesis. Microbiology. Faculty of Science, Tanta University. Tanta, Egypt.

Fleuridor R, Zhong Z, Pirofski L (1998). A human IgM monoclonal antibody prolongs survival of mice with lethal cryptococcosis. J. Infect. Dis. 178: 1213-1216.

Gadd GM (1982). Effects of media composition and light on colony differentiation and melanin synthesis in *Microdochium bolleyi*. Trans. Br. Mycol. Soc. 78(1): 115-122.
Gadd GM, Derome L (1988). Biosorption of copper by fungal melanin. Appl. Microbiol. Biotechnol. 29: 610-617.
Goldstein A, Cantino EC (1962). Light stimulated polysaccharide and protein synthesis by synchronized single generations of *Blastocladiella emersonii*. J. Gen Microbiol. 29: 689-99.
Hamilton AJ, Gomez BL (2002). Melanins in fungal pathogens. J. Med. Microbiol. 53 (3): 189-191.
Hill EP (1976). Effect of light on growth and sporulation of *Aspergillus ornatus*. J. Gen. Microbiol. 95: 39-44.
Hill HZ (1992). The function of melanin or six blind people examine an elephant. Bioess. 14: 49-56.
Jimenez SC, Shafizadeh F (1985). Electron spin resonance spectrometry of fungal melanins. Soil Sci. 139(4): 319-325.
Korzhova LP, Frolova EV, Romakov Lu A and Kuznetova NA (1989). Photo-induced destruction of DOPA melanin. 54(6): 992-998.
Kullberg BJ, Oude-Lashof AML (2002). Epidemiology of opportunistic invasive mycoses. Eur. J. Med. Res. 7: 183-191.
Kuo MJ, Alexander M. (1967). Inhibition of the lysis of fungi by melanins. J. Bacteriol. 94: 624-629.
Langfelder K, Jahn B, Gehringer H, Schmidt A, Wanner G, Brakhage AA (1998). Identification of a polyketide synthase gene (pksP) of *Aspergillus fumigatus* involved in conidial pigment biosynthesis and virulence. Med Microbiol. Immunol. 187: 79-89.
Majerus MEN (1998). Melanism evolution in action. Oxford: Oxford University Press.
Mironenko NV, Alekhina IA, Zhdanova NN, Bulat SA (2000). Intraspecific variation in gamma-radiation resistance and genomic structure in the filamentous fungus *Alternaria alternata*: a case study of strains inhabiting Chernobyl Reactor No. 4. Ecotoxicol. Environ. Saf. 45: 177-187.
Morris-Jones R, Gomez BL, Diez S, Uran M, Morris-Jones SD, Casadevall A. Nosanchuk JD, Hamilton AJ (2005). Synthesis of Melanin Pigment by *Candida albicans In Vitro* and during Infection. Infect Immun. 73(9): 6147-6150.
Moubasher AH (1993). Soil fungi in Qatar and other Arab Countries. University of Qatar, 1st ed. Center for Scientific, Appl. Res. Qatar. 78-81.
Mukherjee J, Scharff MD, Casadevall (1992). A Protective murine monoclonal antibodies to *Cryptococcus neoformans*. Infect. Immun. 60: 4534-4541.
Nicolaus RA, Piatelli M, Fattorusso E (1964). The structure of melanins and melanogensis. IV on some natural melanins. Tetrahed, 20: 1163-1172.
Nosanchuk JD, Casadevall A (2003). The contribution of melanin to microbial pathogenesis. Cell. Microbiol. 5: 203-223.
Nosanchuk JD, Casadevall A (2006). Impact of melanin on microbial virulence and clinical resistance to antimicrobial compounds. Antimicrob. Agents Chemother. 50: 3519-3528.
Nosanchuk JD, Casadevall A, Ovalle R (2003). Method for inhibiting melanogenesis and uses thereof. U.S. Patent. 6: 509-325.92
Nosanchuk JD, Ovalle R, Casadevall A. (2001). Glyphosate inhibits melanization of *Cryptococcus neoformans* and prolongs survival of mice after systemic infection. J. Infect. Dis. 183: 1093-1099.
Nosanchuk JD, Rosas AL, Lee SC, Casadevall A (2000). Melanization of *Cryptococcus neoformans* in human brain tissue. Lancet. 355: 2049-2050.
Nosanchuk JD, Rosas AL, Casadevall A (1998). The antibody response to fungal melanin in mice. J. Immunol. 15; 160(12): 6026-6031.
Osman M (1980). Effect of light on lipids (with emphasis on carotenoids). PhD thesis, University of London.
Osman M, Abo-Zeid AM (1984). Effect of UV-irradiation on spore germination and growth of *Alternaria alternate* and *Fusarium solani*. Delta J. Sci. 8: 722-32.
Osman M, Metwally M (1991). Influence of UV-A irradiation on growth and lipid composition in *Alternaria alternate* and *Fusarium oxysporum*. Microbios 68: 147-155.
Osman M, Valadon LRG (1979). Effect of light quality on growth and sporulation in *Verticillium agaricinum*. Trans. Br Mycol Soc. 72: 145-146.
Ostrosky-Zeichner L, Casadevall A, Galgiani JN, Odds FkC, Rex JH (2010). An insight into the antifungal pipeline: selected new molecules and beyond. Nat. Rev. Drug Discovery. 9: 719-727.
Phillips R (1981). Mushrooms and Other Fungi of Great Britain and Europe. (Russula LS and Rayner eds) New Interlitho SPS Milan, Italy. pp. 249-262.
Pihet M, Vandeputte P, Tronchin G, Renier G, Saulnier P, Georgeault S, Mallet R, Chabasse D, Symoens F, Bouchara JP (2009). Melanin is an essential component for the integrity of the cell wall of *Aspergillus fumigatus* conidia. BMC Microbiol. 24(9): p. 177.
Pipkin FB (1984). Medical statistics made easy. Churchill livingstone. Edinburgh London Melpourne and New York, p. 137.
Pittet D, Monod M, Suter PM, Frenk E, Auckenthaler R (1994). Candida colonization and subsequent infections in critically ill surgical patients. Ann. Surg. 220: 751-758.
Prota G (1992). Melanin and melanogenesis. New York: Academic Press.
Ravishankar JP, Suryanarayanan TS, Muruganandam V (1995). Isolation and characterization of melanin from a marine fungus. Botanica Marina. 38: 413-416.
Rosas AL, Nosanchuk JD, Casadevall A (2001). Passive immunization with melanin-binding monoclonal antibodies prolongs survival of mice with lethal *Cryptococcus neoformans* infection. Infect. Immun. 69(5): 3410-3412.
Rosas AL, Nosanchuk JD, Feldmesser M, Cox GM, McDade HC, Casadevall A (2000a). Synthesis of polymerized melanin by *Cryptococcus neoformans* in infected rodents. Infect. Immun. 68: 2845-2853.
Rosas AL, Nosanchuk JD, Gómez BL, Edens WA, Henson JM, Casadevall A (2000b). Isolation and serological analyses of fungal melanins. J. Immunol. Methods. 244: 69-80.
Safdar N, Maki DG (2002). The commonality of risk factors for nosocomial colonization and infection with antimicrobial-resistant *Staphylococcus aureus*, Enterococcus, Gram-negative bacilli, *Clostridium difficile*, and *Candida*. Ann. Int. Med. 136: 834-44.
Saiz-Jimenez C (1983). The chemical nature of the melanins from *Coprinus* Sp Soil Sci. 136(2): 65-74.
Saltarelli CC, Coppola CP (1979). Effect of light on growth and metabolite synthesis in *Candida albicans*. Mycologia, 71: 773-785.
San BG, San BF, Guanipa O, Moreno B , Pekerar S (1996). *Cladosporium carrionii* and *Hormoconis resinae* cell wall and melanin studies. Curr. Microbiol. 32(1): 11-16.
Spellberg B, Edwards JE (2002). The pathophysiology and treatment of *Candida* sepsis. Curr. Infect. Dis. Rep. 4: 387-399.
Spurr AR (1969). A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultra Res. 26: 31-41.
Steenbergen JN, Casadevall A (2003). The origin and maintenance of virulence for the human pathogenic fungus *Cryptococcus neoformans*. Microbes Infect. 5: 667-675.
Sussman AS, Lingappa Y, Bernstein LA (1963). Effect of light and media upon growth and melanin formation in *Cladosporium mansoni*. Mycopathol. Mycol. Appl. 10: 809-814.
Tan KK , Epton HAS (1973). Effect of light of *Botrytis cinerea*. Trans. Br. Mycol. Soc. 61: 145-157.
Thomas M (1955). Melanins (Paech K and Tracey MU eds) Modern methods of plant analysis. Springer Verlag-Berlin. pp. 661-675.
Tietz NW (1999). Text Book of Clinical Chemistry, 3rd eds. AACC.
Torosantucci AC, Bromuro P, Chiani FD, Bernardis F, Berti C, Galli F, Norelli C, Bellucci L, Polonelli P, Costantino R, Rappuoli , Cassone A (2005). A novel glyco-conjugate vaccine against fungal pathogens. J. Exp. Med. 5: 597-606
Tsai HF, Chang YC, Washburn RG, Wheeler MH, Kwong-Chung KJ (1998). The developmentally regulated alb1 gene of *Aspergillus fumigatus*: its role in modulation of conidial morphology and virulence. J. Bacteriol. 180: 3031-3038.
Tsai HF, Washburn RG, Chang YC, Kwon-Chung KJ (1997). *Aspergillus fumigatus* arp1 modulates conidial pigmentation and complement deposition. Mol. Microbiol. 26: 175-183.
Tsai HF, Wheeler MH, Chang YC, Kwon-Chung KJ (1999). A developmentally regulated gene cluster involved in conidial pigment biosynthesis in *Aspergillus fumigatus*. J. Bacteriol. 181: 6469-6477.
Vasilevskaya A, Zhdanova NM, Pokhodenko VD (1970). Character of survival of some gamma irradiated species of dark-colored

Hyphomycetes. Mikrobiol. Zh. 33: 438-441.
Vilanova ML, Teixeira Í, Caramalho E, Torrado A, Mar ques P, Madureira A, Ribeiro P, Ferreira M, Gama, Demengeot J (2004). Protection against systemic candidiasis in mice immunized with secreted aspartic proteinase 2. Immunol. 111(3): 334-342.
Watanabe A, Fujii I, Tsai HF, Chang YC, Kwon-Chung KJ, Ebizuka Y (2000). *Aspergillus fumigatus* alb1 encodes naphthopyrone synthase when expressed in *Aspergillus oryzae*. FEMS. Microbiol. Lett. 192: 39-44.
Young DS (2001). Effect of disease on clinical lab. Tests, 4th eds.
Zhdanova NN, Gavriushina AI, Vasilevskaia AI (1973). Effect of gamma and UV irradiation on the survival of *Cladosporium* sp. and *Oidiodendron cerealis*. Mikrobiol. Zh. 35: 449-452.