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Therapeutic effect of *Sepia* ink extract against invasive pulmonary aspergillosis in mice

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Abstract Invasive pulmonary aspergillosis (IPA) is a life-threatening disease in immunocompromised patients that requires aggressive therapy. Because of the widespread use of antibiotics, corticosteroids, antitumor drugs, and immunosuppressive drugs, the morbidity of IPA is currently increasing. The ink secretion of molluscan species was identified as one of the novel sources of bioactive compounds. So the present study designed to investigate the antifungal and antioxidant effects of *Sepia officinalis* ink extract against IPA in mice. Eighty neutropenic infected mice were randomly assigned into four main groups (20 mice/group). The 1st group was treated with saline, neutropenic infected, the 2nd group was treated with ink (200 mg/kg) and the 3rd group was treated with amphotericin B (150 mg/kg) and the 4th group was treated with ink plus amphotericin B (Ink 200 mg/kg and AMB 150 mg/kg). Treatment was started at 24 h after fungal inoculation and was administered for 3 consecutive days. The present study demonstrated good *in vitro* and *in vivo* antifungal activity of IE against *Aspergillus fumigatus*. Compared with IPA group; IE-treated, AMB-treated, and AMB + IE-treated animals had a 67.80%, 83.41%, and 72.68% reduction in the pulmonary fungal burden, respectively. Treatment with IE and/or AMB for one and three days significantly decreased MDA and increased GSH and SOD levels in the lung tissues as compared with the infected untreated group. In conclusion, the results of our *in vivo* and *in vitro* studies demonstrate that IE has therapeutic effect against invasive pulmonary aspergillosis via reducing oxidative stress.

Introduction

Aspergillosis refers to the spectrum of diseases caused by *Aspergillus* species (Thompson and Patterson, 2008). *Aspergillus fumigates* (*Aspergillus fumigatus*) is the most common species among *Aspergillus*, accounting for 90% of all respiratory infections. *Aspergillus* species enter the host most commonly through the lungs by the inhalation of conidia. However, infection has also been reported by exposure and inhalation of

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water aerosols contaminated with *Aspergillus* conidia (Anaissie et al., 2002). Without effective host defenses following pulmonary exposure, the conidia resting in alveoli begin to enlarge and germinate. Hyphal transformation with vascular invasion and dissemination of infection are potential sequel (Thompson and Patterson, 2008). *A. fumigatus* is responsible for severe and often fatal infections in immunocompromised patients (Das Gupta et al., 2014). Moreover, *A. fumigatus* causes severe pulmonary infections in humans, such as invasive pulmonary aspergillosis (IPA) (Obar et al., 2013).

Invasive pulmonary aspergillosis (IPA) is a life-threatening disease of immunocompromised patients that requires aggressive therapy (Tsuji and Ogawa, 2011; Krel et al., 2014). Cancer chemotherapy and allogeneic bone marrow transplantation are associated with fungal disease, and up to 30% of patients with acute leukemia experience invasive fungal infections. Fungi are one of the most neglected pathogens, as demonstrated by the fact that the amphotericin B (AMB), a polyene antibiotic used for more than 50 years, is still used as a "gold standard" for antifungal therapy (Ostrosky-Zeichner et al., 2003; Gibbs et al., 2005; Chudzik et al., 2013). In neutropenic patients with IPA, treatment with amphotericin B is customary but often ineffective, with reported response rates of less than 55% in leukemia patients and bone marrow recipients (Becker et al., 2003). Antimicrobial resistance, and the resulting failure of antimicrobial therapies in humans, is a mounting public health problem of global significance (Marcos-Zambrano et al., 2014). Moreover, in humans, the most common and dose limiting side effect of amphotericin B is severe nephrotoxicosis (Geo vigila and Baskaran, 2011; Bes et al., 2014).

Because of the widespread use of antibiotics, corticosteroids, antitumor drugs, and immunosuppressive drugs, the morbidity of IPA is currently increasing. Emerging evidence suggests that marine natural products continue to be a major source of nutraceutical and functional foods and as a source material for the development of drugs (Koyama et al., 2006). The cuttlefish *Sepia officinalis* relies for defense on the ejection of a dark ink in order to create a dark, diffuse cloud which can obscure the predator's view, allowing the cephalopod to make a rapid retreat by jetting away. *Sepia* ink consists of a suspension of melanin granules in a viscous colorless medium (Palumbo, 2003). Most of the studies concerning antimicrobial activity include specific compartments like egg masses, hemolymph or whole body extracts of mollusk (Haug et al., 2004). Mollusks not only exhibit the anti-microbial activity, but also constitute many classes of bioactive compounds which include antitumor, antileukemic and antiviral activities (Premanand et al., 1997; Rajaganapathy et al., 2000). It has been reported that, *Sepia* ink extract at the suitable concentration is able to alleviate the *in vivo* immunosuppression induced by cyclophosphamide in mice (Guang et al., 2009). Also, Zhong et al. (2009) studied the protective effects of squid ink extract toward hemopoietic injuries induced by cyclophosphamine.

Reactive oxygen species (ROS) are essential components of the defensive mechanism against fungus infection (Ibrahim-Granet et al., 2003; Noubade et al., 2014). Following inhalation, *A. fumigates* conidia are either phagocytosed by alveolar macrophages or germinate to form hyphae and are then phagocytosed by the second line of defense, the neutrophils (Dagenais and Keller, 2009). Both macrophages and neutrophils mediate powerful fungicidal effects on *A. fumigates* by producing reactive oxygen species (ROS) (Chauhan et al., 2006). Oxidative stress in the lung is induced by the oxidant-antioxidant imbalance; high levels of ROS in macrophages can result in DNA damage, lipid peroxidation, and protein inactivation. The present investigation aims to evaluate the antifungal and antioxidant effects of *S. officinalis* ink extract against IPA in mice.

Materials and methods

Chemicals

Amphotericin B was purchased as Fungizone (E.R. Squibb & Sons, Princeton, NJ). All other chemicals were purchased from local standard companies and were of reagent grade or better.

Preparation of cuttlefish ink extract (IE)

The cuttlefish ink extract (IE) was prepared according to our previous study, Fahmy et al. (2014). In brief, fresh cuttlefish (*S. officinalis*) were purchased directly from a fishmonger and rapidly transferred to the laboratory where they were dissected and the ink was collected and diluted immediately with an equal volume of dist. water and ground sufficiently. The admixture was collected immediately, concentrated and lyophilized to a black residue using LABCONCO lyophilizer, shell freeze system, USA.

Free radical scavenging activity

The free radical scavenging activities of the extract and vitamin C were analyzed by the DPPH assay (Sanchez-Moreno et al., 1998). A 1.0 ml of the test extract, at gradient final concentrations of 10–80 mg/ml, was mixed with 2 ml of 0.3 mM DPPH solution in MeOH in a cuvette. The absorbance was taken at 517 nm after 20 min of incubation in the dark at room temperature. The experiment was done in triplicates. The percentage antioxidant activity was calculated as follows:

$$\%\text{Antioxidant Activity [AA]} = 100 - \left[\{(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}) \times 100\} / \text{Abs}_{\text{control}}\right].$$

where $\text{Abs}_{\text{sample}}$ was the absorbance of sample solution (2.0 ml) + DPPH solution (1.0 ml, 0.3 mM), $\text{Abs}_{\text{blank}}$ was the absorbance of methanol (1.0 ml) + sample solution (2.0 ml), $\text{Abs}_{\text{control}}$ was the absorbance of DPPH solution (1.0 ml, 0.3 mM) + methanol (2.0 ml).

Test organism and growth conditions

A strain of *A. fumigates* isolated from an immunocompromised patient with IPA served as the parental strain in this investigation. A working culture of this strain was maintained on peptone yeast extract glucose (PYG: peptone 1 g; yeast extract 1 g; glucose 3 g; per liter of distilled water) agar slants at room temperature. For the preparation of conidial suspension, a culture of *A. fumigatus* was grown on PYG agar for 6 days at 35 °C, and the conidia were collected as described previously (Manavathu et al., 1999).

Ethical consideration

Experimental protocols and procedures used in this study were approved by the Cairo University, Faculty of Science Institutional Animal Care and Use Committee (IACUC) (Egypt), (CUFS/F/10/13). All the experimental procedures were carried out in accordance with international guidelines for care and use of laboratory animals.

Animals

The experiment was conducted on healthy Swiss male albino mice weighing 20 ± 2 g, obtained from the animal house, National Research Center. The animals were housed in controlled environmental conditions (temperature 25 ± 2 °C and 12 h dark/light cycle) with standard diet and water *ad libitum*.

Toxicity study (OECD 420)

The acute oral toxicity of *S. officinalis* ink extract (IE) was evaluated in mice according to the procedures outlined by the Organization for Economic Co-operation and Development (OECD, 2001). Mice were administered orally with cuttlefish ink extract (IE) at dose levels of 5 g/kg (high dose) and 2 g/kg (low dose). Normal control mice received the same amount of vehicle (distilled water) only. Food was provided to the mice approximately an hour after treatment. The mice were observed in detail for any indications of toxicity effect within the first six hours after the treatment period, and daily further for a period of 14 days. Surviving animals were weighed and visual observations for mortality, behavioral pattern, changes in physical appearance, injury, pain and signs of illness were conducted daily during the period.

Fungal strain

A strain of *A. fumigates* was isolated from an immunocompromised patient with IPA. The fungus was cultured on Sabouraud agar and conidia were harvested in sterile saline. Preparation of a microscopically confirmed, hyphae-free conidial suspension was performed as previously described (Leenders et al., 1996).

Murine model of immunosuppression and invasive pulmonary aspergillosis (IPA)

Neutropenia was induced by intraperitoneal (ip) administration of 150 mg kg^{-1} d^{-1} cyclophosphamide (Sbaraglia et al., 1984) 3 days before fungal inoculation. Animals of control (20 mice) (injected intraperitoneally by saline, for 3 days) and neutropenic groups (80 mice) were kept under strict hygienic conditions and were observed daily until the end of the study. On day 0, all neutropenic mice were infected with conidia of *A. fumigatus*, a suspension of 1×10^9 conidia/ml was then prepared prior to inoculation. Each inoculum consisted of 0.025 ml of suspension (10^6 conidia) administered by intranasal instillation, using a 1 cc insulin Syringe, under light chloroform anesthesia.

Experiment design

Eighty neutropenic infected mice were randomly assigned into four main groups (20 mice/group). The 1st group was treated with saline, neutropenic infected (NI group), the 2nd group was treated with ink (200 mg/kg) (Ink group) and the 3rd group was treated with amphotericin B (150 mg/kg) (AMB group) and the 4th group was treated with ink plus amphotericin B (Ink 200 mg/kg and AMB 150 mg/kg) (I + AMB group). Treatment was started at 24 h after fungal inoculation and was administered for 3 consecutive days.

Collection of samples for analysis

Animals were euthanized under chloroform vapor and sacrificed after 24 h and 72 h of treatment after being fasted overnight; blood was collected in EDTA tubes for haematological and biochemical parameters. The superior lobe of the right lung was removed and immediately blotted using filter paper to remove traces of blood stored at −80 °C for biochemical studies. The inferior lobe of the right lung was suspended in 10% formal saline for fixation preparatory to histological processing. Part of the frozen liver tissues was shipped to micro analytical center, Faculty of science, Cairo University in frozen state. Upon receipt, the samples were kept frozen (−20 °C) until used for analysis of gliotoxin.

Assessment of fungal load in lung tissue

For tissue burdens, aliquots of tissue were semi-quantitatively cultured on potato dextrose agar plates using serial 10-fold colony count dilutions. Plates were placed in an incubator at 37 °C until colonies could be counted. The undiluted homogenate was saved at 4 °C until initial cultures were counted. When the undiluted homogenate had no counts, the entire organ homogenate was cultured. Counts were expressed as cfu/organ.

Assessment of gliotoxin in lung tissue

Extraction of gliotoxin from infected lung tissue was performed according to the method described by Richarad and DeBey (1995). Briefly, lung sample was macerated in a plastic bag as much as possible with mallet then was transferred with 5 ml of distilled water to Ten Broek tissue homogenizer. The sample was homogenized and 10 ml 6 N HCl was added to homogenate on a shaker for 30 min. The mixture was extracted with 200 ml of chloroform passed through 5 g sodium sulfate and filtered through No. 580 glass filter. The eluate was transferred to a round bottom flask and dried on rotavap at 30 °C. A 10 ml syringe was attached to silica column then column was rinsed with 4 ml hexane using vacuum and 8 ml hexane was added to reservoir. Round bottom was rinsed, from above, 4× with 1 ml each of $CHCl_3$ and hexane was added in column reservoir. Sample was drowning through column and rinsed with 12 ml hexane and 12 ml hexane: diethyl ether (1:1, v/v). Gliotoxin was eluted with 15 ml ether: acetone (95:5, v/v) in a 25 ml beaker and evaporated under stream of nitrogen at 30 °C. Sample was quantitatively transferred to 0.5 dram vial using ether: acetone (95:5, v/v) and

the solution in the vial was dried. Samples were then analyzed by TLC.

Oxidative stress marker assessment

Oxidative stress markers were detected in the resultant supernatant of lung homogenate. The appropriate kits (Biodiagnostic kits, Biodiagnostic Dokki, Giza, Egypt) were used for the determination of malondialdehyde (MDA) (Ohkawa et al., 1979), reduced glutathione (GSH) (Aykaç et al., 1985) and superoxide dismutase (SOD) (Nishikimi et al., 1972).

Statistical analysis

All data are expressed as means ± SEM. In general, data were analyzed by two-way ANOVA followed by the Bonferroni test and Duncan's multiple range test. Student's *t* test was used when only two data groups were compared with each other. *P*-value of <0.05 was considered as statistically significant. All calculations were performed using GraphPad Prism software 5.01 (La Jolla, CA, USA).

Results

Free radical scavenging activity

The results of DPPH scavenging activity of IE and vitamin C are shown in Fig. 1. The radical scavenging activities were estimated by comparing the percentage of inhibition of DPPH radical by the tested extract and the vitamin C. Data were displayed with mean ± SEM of three replications. The present results showed that IE produced dose dependent inhibition of DPPH radical ranging from 86.3% to 94.8% as compared to ascorbic acid.

Antimicrobial activities of ink extract (IE) and/or amphotericin B (AMB) against A. fumigates

Fig. 2 summarizes the minimum inhibitory concentrations (MICs) of ink extract, amphotericin B, and their combination. Data revealed that *A. fumigatus* isolate used in this study was susceptible to antifungal drugs. The antifungal potency of all

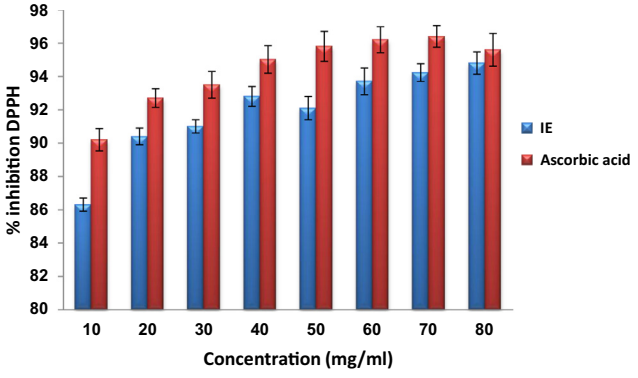

Fig. 1 Antioxidant activity of *Sepia officinalis* ink extract (IE) and ascorbic acid. Each vertical column represents the mean ± SEM change of three replications.

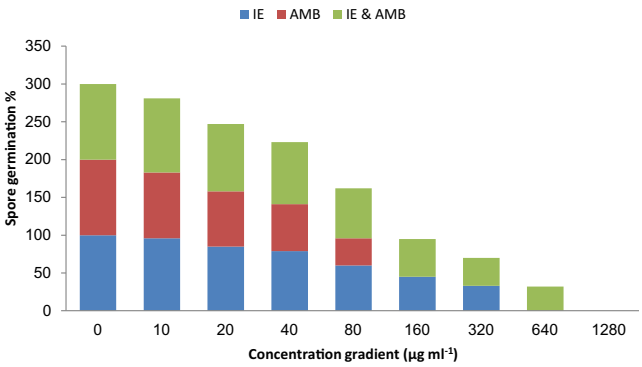

Fig. 2 Antimicrobial activities (determined as inhibition of spore germination) of *Sepia officinalis* ink extract (IE) and its antagonistic effect on amphotericin B (AMB) of germinated conidia of *Aspergillus fumigates*.

treatments varies significantly. Amphotericin B seemed to be the most effective antifungal drug with the lowest MIC value of 80 µg ml^{-1} followed by ink extract (MIC 320 µg ml^{-1}) whereas their combination was relatively a weak antifungal drug to *A. fumigatus* growth (MIC 1280 µg ml^{-1}). The interaction between amphotericin B and ink extract was antagonistic for *A. fumigatus* isolate. For the antagonistic interactions the concentrations of ink extract and amphotericin B in the combination were 1:1.

Acute toxicity

From the experiment performed as per the OECD Guidelines 420, the results reveal that the cuttlefish ink extract (IE), has been found toxic at 5000 mg/kg body weight of experimental animals as in the first 4 h of observation 2/3 morbidity was observed. None of the 6 rats died or showed any sign of toxicity at the limit dose of 2000 mg/kg p.o. for IE in the first 48 h. No evidence of toxicity was noted during the period of observation. The LD_{50} was therefore taken as above 2000 mg/kg p.o. The median effective dose (ED_{50}) was selected based on the proposed LD_{50} obtained from the acute toxicity study. This dose was considered one tenth of the proposed LD_{50}, that is, 200 mg/kg body weight.

Induction of IPA in neutropenic mice

A neutropenic mice model was successfully established, as evidenced by a median white blood cell count of $6.02 \pm 0.92 \times 10^8$/L, as compared with $2.3 \pm 1.2 \times 10^9$ in normal mice. Lung of control mice showed healthy pulmonary tissue (Fig. 3A), while that infected with *A. fumigatus* for one day showed peribronchitis which was represented by peribronchial congestion and mononuclear cell infiltration (Fig. 3B). Lung infected with *A. fumigatus* for three days showed diffuse interstitial mononuclear cell infiltration (Fig. 3C).

Estimation of fungal load in lung tissue

Fig. 4 shows the microbial burden in the autopsied lungs. Lung cultures had minimal fungal load in the AMB-group. Mean burden of fungal organisms in the lungs (cfu/g liver tissue) after 72 h. of inoculation was 205×10^6 (IPA group), 66×10^6

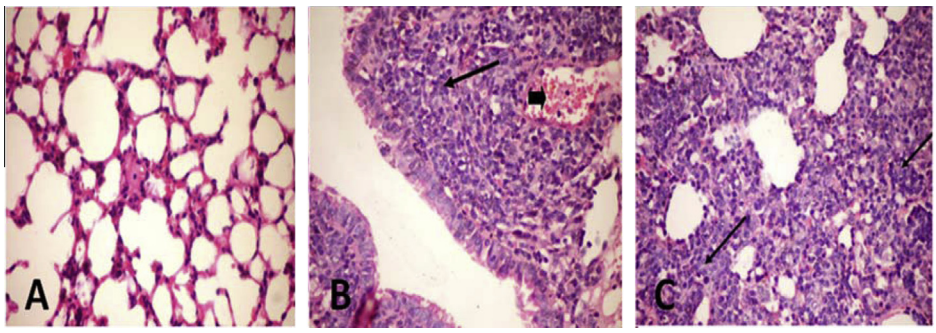

Fig. 3 Histological study of hematoxylin & eosin stained lung sections (400×) of control (A), infected with *A. fumigatus* for one day (B) and infected with *A. fumigatus* for three days (C). Lung of control mice showing healthy pulmonary tissue. Lung infected with *A. fumigatus* for one day showing peribronchitis which represented by peribronchial congestion (arrow head) and mononuclear cells infiltration (arrow). Lung infected with *A. fumigatus* for three day showing diffuse interstitial mononuclear cells infiltration (arrows).

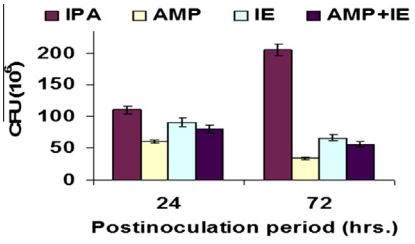

Fig. 4 Screening of fungal load in the lung homogenate of mice infected with *Aspergillus fumigatus*. IPA: invasive pulmonary aspergillosis (neutropenic, infected with *A. fumigates* conidia); AMP: amphotericin B (neutropenic, infected with *A. fumigates* conidia, and injected with amphotericin B); IE: ink extract (neutropenic, infected with *A. fumigates* conidia, and injected with ink extract); AMP + IE: amphotericin B and ink extract (neutropenic, infected with *A. fumigates* and injected with amphotericin B and ink extract).

(IE group), 34×10^6 (AMB group), and 56×10^6 (AMB + IE). Compared with IPA group; IE-treated, AMB-treated, and AMB + IE-treated animals had a 67.80%, 83.41%, and 72.68% reduction in fungal burden, respectively.

Detection of gliotoxin in lung tissue

Gliotoxin was found in lung tissue samples from all mice inoculated with *A. fumigatus* (Table 1). It is obvious from the results that concentration of gliotoxin in lung tissues varies significantly in all samples according to treatment type and days after inoculation. The highest concentration of gliotoxin was recorded in sample No. 3 in which mice were infected with *A. fumigatus* conidia and were not subjected to any treatment while the lowest concentration was observed in sample No. 11 where mice were treated with amphotericin B for 7 days. It is also clear from the results that in sample No. 14 where combination treatment was used the concentration of gliotoxin significantly decreased as compared with the infected group but the reduction is less than that when amphotericin B was used separately.

Effect of the IE and/or AMB on lung oxidative stress markers

Oxidative stress markers, malondialdehyde (MDA), reduced glutathione (GSH) levels superoxide dismutase (SOD) activities in control, *A. fumigatus* infection, IE and/or AMB treated mice are shown in Table 2.

MDA levels were assessed as an indicator of lipid peroxidation, *A. fumigatus* infection caused a significant increase ($P < 0.05$) in MDA level as compared to control. Lung MDA levels of IE, AMB, and IE + AMB groups were significantly ($P < 0.05$) decreased after one and three days of treatment, as compared to the infected untreated group (Table 2). Meanwhile, no significant change ($P < 0.05$) was noticed in the level of lung MDA following all treatments.

As shown in Table 2, *A. fumigatus* infection challenge significantly decreased GSH level ($P < 0.05$) as compared to the control group. Treatment with IE, AMB, and IE + AMB for one and three days significantly ($P < 0.05$) increased the lung GSH levels, as compared with the infected untreated group (Table 2). Additionally, the highest GSH level was noticed following three days of IE treatment.

Lung SOD activity of the *A. fumigates* infected group decreased significantly ($P < 0.05$) as compared to the infected untreated group (Table 2). All experimental groups at the two selected time periods were significantly ($P < 0.05$) higher than those of the infected untreated group (Table 2).

Discussion

The source of the respiratory infection is the fungal spores present in air and enter the lung which is the first line of defense which can localize and kill the spores after cellular infiltration as the lung can be an important defense line against *Aspergillus* (Luther et al., 2007). Aspergillus infections are among the most feared opportunistic infections in humans due to their ability to cause several distinct pulmonary diseases. Invasive pulmonary aspergillosis (IPA) is a rapidly progressing, often fatal disease that commonly affects immunocompromised patients and is characterized by necrotic tissue containing abundant hyphae (Krel et al., 2014). Invasive pulmonary aspergillosis (IPA) pathogenesis in the present study was induced by the intratranasal instillation which best

Table 1 Lung gliotoxin concentration in mice with invasive pulmonary aspergillosis (IPA) treated with *Sepia officinalis* ink (IE) extract and/or amphotericin B.

Sample No.	Neutropenia	Infection	Treatment	Post inoculation period (h)	Gliotoxin conc. (μg/g lung)
1	No	No	No	72	$0^{a} \pm 0.0$
2	Yes	No	No	24	$0^{a} \pm 0.0$
3	Yes	Yes	No	24	$5200^{h} \pm 7.6$
4	Yes	Yes	No	72	$6320^{i} \pm 8.1$
6	Yes	Yes	IE	24	$3320^{g} \pm 3.3$
7	Yes	Yes	IE	72	$2535^{d} \pm 3.8$
9	Yes	Yes	AMB	24	$2555^{e} \pm 2.5$
10	Yes	Yes	AMB	72	$1133^{b} \pm 2.6$
12	Yes	Yes	IE & AMB	24	$2910^{f} \pm 5.9$
13	Yes	Yes	IE & AMB	72	$1760^{c} \pm 2.0$

Values are mean (n = 3) ± SE. The means followed by different letters in the same column are significantly different according to ANOVA and Duncan's multiple range tests.

Table 2 Effect of *Sepia officinalis* ink extract (IE) and amphotericin B (AMB) on some pulmonary oxidative stress markers in neutropenic mice with invasive pulmonary aspergillosis (IPA).

Parameter	Duration of infection	Control	Infected			
			Vehicle	IE	AMB	IE + AMB
MDA	One days	$4.83 \pm .19^{a}$	8.59 ± 0.26^{d}	4.80 ± 0.45^{a}	5.05 ± 0.34^{a}	5.81 ± 0.45^{ab}
	Three days	$4.73 \pm .02^{a}$	15.07 ± 0.76^{e}	9.13 ± 1.99^{bc}	6.49 ± 0.73^{bc}	6.86 ± 0.35^{bc}
GSH	One days	122.45 ± 4.7^{f}	74.75 ± 7.44^{bc}	86.26 ± 5.12^{cd}	82.48 ± 3.96^{bc}	68.156 ± 8.14^{ab}
	Three days	121.05 ± 4.5^{f}	55.508 ± 4.78^{a}	106.63 ± 4.55^{e}	83.90 ± 4.83^{bc}	84.914 ± 3.25^{cd}
SOD	One days	37.80 ± 0.28^{d}	24.28 ± 1.25^{a}	41.52 ± 2.64^{cd}	38.1 ± 2.37^{bc}	48.02 ± 2.85^{cde}
	Three days	38.30 ± 0.30^{d}	21.92 ± 1.189^{ab}	37.68 ± 2.36^{de}	45.96 ± 1.80^{e}	47.24 ± 1.72^{e}

Values are given as mean ± SE for 5 mice in each group.
Values with different superscript letters are significantly different ($P < 0.05$).

mimics the natural route of infection in humans, in whom the lung is the primary target organ (Pasqualotto, 2008).

Murine models of aspergillosis have been used extensively in the study of antifungal drug efficacy (Xu et al., 2009). However, the development of multidrug resistance to fungal therapeutic drugs is a main obstacle for the successful treatment of many microbial diseases (Vadlapudi, 2011). Marine environment comprises of many organisms which are known to possess bioactive components as a common means of self-defense or for the protection of eggs and embryos (Peruru et al., 2012). The ink secretion of molluscan species was identified as one of the novel sources of bioactive compounds. The ink extract of *Sepia pharaonis* showed inhibitory effect against *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, and *Klebsiella pneumonia* (Nithya et al., 2011). Vennila et al. (2011) showed the strongest antifungal activity of octopus ink with the inhibition zone of 20 mm against *Fusarium* sp. and *A. fumigatus*, respectively (Vennila et al., 2011). So, the present study extended the previously reported finding of Fahmy and Soliman (2013) and Fahmy et al. (2014) who showed the antioxidant and the antifungal efficacy of *S. officinalis* ink extract (IE) to explore its efficacy against oxidative stress induced in the lung following IPA in mice. The present study demonstrated good antifungal potential of IE of *S. officinalis* as compared to amphotericin B against *A. fumigatus in vitro*, as observed by a reduction in percentage of spore germination and a reduction in pulmonary fungal burden. In conjunction with our results Peruru et al. (2012) and Shrivastava et al. (2006), reported that the eumelanin extracted from *S. officinalis* ink showed strong antimicrobial activity. In addition, Nithya et al. (2011), showed that the ink extract of *S. pharaonis* exhibited inhibitory effect against *P. aeruginosa*, *S. epidermidis*, and *K. pneumonia*. *A. fumigatus* produces a number of toxins, the most abundant mycotoxin produced by *A. fumigatus* is gliotoxin (GT) (Waring and Beaver, 1996). Gliotoxin is the only toxin isolated from the sera of rodents and of patients suffering from invasive aspergillosis (Lewis et al., 2005). Gliotoxin showed immunosuppressive activities including an inhibition of macrophage phagocytosis (Dagenais and Keller, 2009). The results from the present work revealed that, ink extract and AMB significantly reduced the concentration of gliotoxin secreted by *A. fumigatus* in the lung tissues. In consonance with the results of Lewis et al. (2005), data from the present study showed that gliotoxin levels in mice with IPA reduced with antifungal therapy.

Reactive oxygen intermediates (ROS) are involved in many of the complex interactions between the invading microorganisms and its host (Miller and Britigan, 1997). *A. fumigatus* conidias are phagocytosed and killed by alveolar macrophages, using ROS (Peng et al., 2009). In addition NADPH-oxidase mediated production of ROS by alveolar macrophages and neutrophils is a critical mechanism for immune defense against *A. fumigates* (Fréalle et al., 2013). In accord with the results of Peng et al. (2009) and Mahmoud et al. (2011) the intensity of oxidative stress was measured as enhancement in the levels of lipid peroxidation end product, malondialdehyde (MDA) in

the lung of IPA mice following all experimental periods. It has been reported that increased MDA level suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals (Park et al., 2010; Vinodini et al., 2010).

Glutathione (GSH) is the main nonprotein thiol responsible for cellular homeostasis and maintenance of the cellular redox balance (Forman et al., 2009). Existing in two forms, oxidized (GSSG) and reduced/free form (GSH), only GSH exhibits antioxidant activity. In accordance with the report of Hagens et al. (2006) and Fahmy et al. (2014) our results also support the notion that depletion of tissue GSH, as observed in the IPA, is one of the major factors that permit lipid peroxidation and subsequent tissue damage. Mycotoxins such as gliotoxin generally do not cause health problems as they are bound to glutathione via interaction with the enzyme glutathione S-transferases (GST), which facilitates the excretion of mycotoxin (Roebuck et al., 2009). Additional suggestion that the interaction between glutathione and mycotoxin plays an important role in immune cell function comes from studies of the mycotoxins gliotoxin and patulin which show that in murine dendritic cells (antigen-presenting macrophage cells), the mycotoxins depleted glutathione in a concentration-dependent fashion (Wichmann et al., 2002). Insufficiency in non-enzymatic antioxidant GSH following fungal intoxication in the present study could be the consequence of increased utilization for detoxification of gliotoxin.

Antioxidant enzymes crucial for protection of the airway against oxidant stress include superoxide dismutases (Comhair et al., 2005). Superoxide dismutase (SOD), one of the important intracellular antioxidant enzymes, is present in all aerobic cells and has an antitoxic effect against superoxide anion. SOD activity is reduced in the oxidant-rich environment of the asthmatic airway (Comhair et al., 2005) and during asthma exacerbation further loss of SOD activity occurs with enhanced production of oxygen radicals by inflammatory cells (Comhair et al., 2000). The present investigation showed a significant decrease in SOD in the lung of the IPA-untreated group as compared to the control group. In accord with our results Escobar et al. (1996) and Sanzgiri et al. (1997), have reported that the enhanced free radical concentration resulting from the oxidative stress conditions can cause loss of enzymatic activity. The present study extended the previously reported finding that SOD activity significantly decreased following *A. fumigates* infection (Peng et al., 2009; Xu et al., 2009; Fahmy et al., 2014).

The present study revealed that, treatment with IE and/or AMB significantly elevated SOD activity and GSH level and decreased MDA level in comparison with the IPA group, suggesting that the mechanism of IE may be due to its antioxidant effect. Chen et al. (2007) showed that melanin of squid ink, like superoxide dismutase, can catalyze O^{2-} to H_2O_2, and thus avoid the free radical chain reaction triggered by O^{2-}. In conjunction with the reports of Zhang et al. (2003), Zhong et al. (2009) and Fahmy et al. (2014), data from the present investigation showed that squid ink elevated SOD activity in the liver and kidney of mice in a dose-dependent manner.

In conclusion, the present study serves to extend the growing number of earlier investigations on fungal therapeutic drugs from marine sources and confirms that IE decreased lipid peroxidation, improved antioxidant status, and thereby prevented the damage to the lung during IPA. In combination with antifungal therapy (AMB), IE treatment can reduce pulmonary fungal burden and alleviate oxidative stress associated with IPA in neutropenic mice. However, further studies must be carried out to elucidate the mechanisms of the antifungal efficacy of IE.

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