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RESEARCH ARTICLE

STUDY OF ANTIGENOTOXIC EFFECT OF SEPIA INK EXTRACT AGAINST ASPERGILLOSIS

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ABSTRACT

One of the great challenges that face human is high mortality rate associated with invasive pulmonary aspergillosis (IPA). The sepia ink extract was studied for its potential antigenotoxic, antifungal and antioxidant effects against *Aspergillus fumigatus* in comparison to the common antifungal drug amphotericin B. Thirty-six mice were distributed randomly into four equal groups. All mice were immunosuppressed and infected with aspergillosis. Group I did not receive any treatment. The groups II, III and IV were treated after 1 day of infection with 200 mg ink extract, 150 mg amphotericin B and mixture of ink extract and amphotericin B, respectively. The current work showed that the ink extract has reduced the fungal load in the liver as well as the levels of fungal toxin gliotoxin in the serum. Throughout the 7 days' post-infection, after treatment with ink extract and/or amphotericin B, glutathione (GSH) content and superoxide dismutase (SOD) activities in the serum were markedly increased whereas the levels of malondialdehyde (MDA) were significantly reduced. The tail length (TL), %DNA in tail, tail moment (TM) and olive tail moment (OTM) in the hepatocytes were significantly alleviated after treatment with ink extract and/or amphotericin B. We can conclude that although ink extract and amphotericin B separately showed their antifungal, antioxidant and antigenotoxic properties, the combination between them can lead tothe antagonistic effect.

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INTRODUCTION

Pulmonary aspergillosis is a broad band of harmful pulmonary complication induced by fungus family Aspergillus (Knutsen *et al*., 2012). Angioinvasive aspergillosis is one of the most dangerous types being fatal and affects immunocompromised patients (Khan *et al*., 2003). *A. fumigatus* is a saprophytic filamentous fungus grows mainly on soil and decayed organisms (Latge, 1999). The heat and moisture are required for the development of this fungus (Chabi *et al*., 2015).It has two forms which are the reproductive form (conidiophores) and the mature spore (hyphae). Although asperigillus has low pathogenicity, pulmonary aspergillosis can be induced by pre-existing chronic lung disease, as tuberculosis, or impaired immunity (Khan *et al*., 2003).Commonly, corticosteroids were recommended as an effective therapy for controlling aspergillosis (Limper *et al*., 2011). However, the patient becomes dependent on steroids and cannot leave without them (Wark *et al*., 2003). For this reason, studies were needed to use alternatives such as antifungal drugs (Moss, 2014).Amphotericin B (AMB) is one of the most effective drugs against fungal infections (Khan *et al*., 2016). However, due to its adverse side effects such as induced fever, hypokalemia and in many cases nephrotoxicity, AMB is poorly applied in treatments (Saag *et al*., 2000 and Chappuis *et al*., 2007). Thus, trials have been made to decrease such undesirable effects as by encapsulation into liposomes (Wong-Beringer *et al*., 1998). However, a new trend was subjected to the natural extracts. In general, Molluscs contains bioactive constituents that has anti-microbial, anti-viral and anti-cancer activities (Rajaganapathy *et al*., 2000). Cuttlefish, *Sepia officinals* is a cephalopod that is characterized by ink production for self-defense. This ink is made of melanin granules suspended in viscous matrix (Palumbo, 2003). Previous studies were executed to study the antimicrobial effects of eggs and hemolymph extracts of mollusks (Haug *et al*., 2004). The current work aims to study the antimicrobial role of sepia ink extract against Aspergillosis on the molecular level.

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MATERIALS AND METHODS

Chemicals

Fungizone (Amphotericin B) drug is a trademark of E. R. Squibb and Sons, Princeton, N.J**.** was purchased. Agarose, 2,2-diphenyl-1-picrylhydrazyl (DPPH), absolute ethanol 99.5%, Tris-(hydroxymethyl)-amino methane (Tris–base), ethylenediaminetetracetic acid disodium salt (Na2EDTA), ethidium bromide(EtBr) were purchased from Sigma **(**St. Louis, MO, USA**)**. All other chemicals were of analytical grade.

Ink extract Preparation

Fresh samples of cuttlefish, *Sepia officinalias* were supplied by a fisherman in ice box direct to the laboratory to be dissected for the collection of ink. After draining of the ink, it was diluted with the same volume of distilled H_2O and then centrifuged at a speed of 15,000 g for 20 min at 4°C. The aliquot was collected and transformed into a black residue as described by Zhong *et al*. (2009), using LABCONCO lyophilizer, shell freeze system, USA.

In vitro study

Test organism

Aspergillus fumigatus strain was isolated from patient with Aspergillosis. The fungal colony was raised on a medium of peptone yeast glucose (PYG) agar at 35°Cfor 1 week. The PYG agar was prepared by the addition of 1g peptone, 1g yeast extract and 3g glucose to one litre of distilled water. The conidia were assembled according to the method described by Manavathu *et al*. (1999).

Spore germination

Conidia were dispersed in the PYG medium as 1 million conidia per 1 mL and were incubated on gyratory shaker with 160 rpm at 35°C. A 10 mL of the suspension was collected from the medium every 2 h, for counting the germinated conidia on a hemocytometer at 400x maginfication. A relationship was figured between the percentage of germination and the incubation period (Figure A).

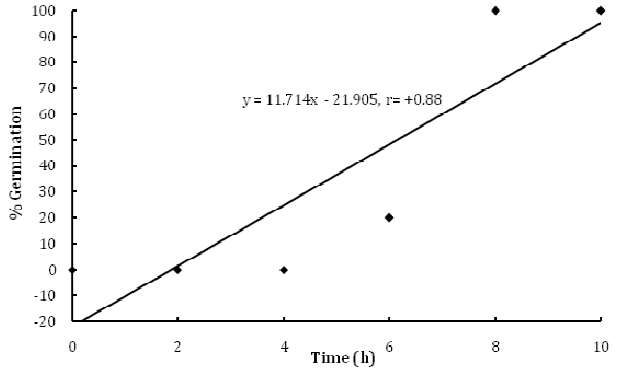


Figure A. Relationship of the % germination of fungal spores with the time of incubation on PYG medium

In order to study the antifungal properties of ink extract and/or amphotericin B, spore germination assay using the slide technique (Nair *et al*., 1991). The desired concentrations of ink extract and amphotericin B were spread as a film on dried slides.

After then, a 10^6conidia/ml from a series of concentrations of spore suspension (10-1280 µg/ml) of *Aspergillus* were spread over the films. In controls, dis. H_2O was spread instead of conidia suspension. Slides were incubated at 35°C and then were fixed in lactophenol-cotton blue and observed microscopically for spore germination. The calculations of % germination was according to the following formula:

$$\%\ Spore\ germination = \frac{Number\ of\ germinated\ spores}{Total\ number\ of\ spores} \times 100$$

In addition, the checkerboard method was applied to study the combined effect of ink extract and amphotericin B (1:1) mixture (Scott *et al*., 1995).

In vivo study

Experimental model

Healthy male mice, *Mus musculus* (weighing 20±2 g), were purchased from the animal's house, National Research Center (NRC). All mice were housed in polycarbonate cage bedded with wood shavings at temperature of 22±3 °C, relative humidity of 50±15% and a 12 h/ 12 h light/dark cycle. Feed and water were provided *ad libitum*.

Ethical Approval

All the procedures were approved by the Institutional Animal Care and Use Committee; (IACUC) at Faculty of Science, Cairo University, Egypt, under permit number of CUFS/F/ 10/13.

Toxicity study

In order to execute acute toxicity test of ink extract, the acute-toxic-class method guideline number 423 of the Organization for Economic Cooperation and Development was followed (OECD, 2001).Eighteen male albino miceweighing (20-25 g) were divided into control and ink administered groups each with six individuals. The normal groups received the saline whereas the others received 2 g of ink extract/kg body wt. The mice were observed during the first 4 h following administration and then at least one time every day till the 14th day to estimate the number of deaths. At the 15th day, mice were anesthetized, sacrificed, blood was collected analyses AST and ALT.

Immunosuppression

All the mice were immunosuppressed through intraperitoneal injection with 150 mg· of cyclophosphamide per kg body wt. per day as described by Sbaraglia *et al*. (1984) for three days prior to infection. Five mice were used as normal control for comparison. The mice were daily observed until the end of experiments. The blood was collected from the tail vein after anesthesia with sodium pentobarbital for analyses of total leukocyte count.

Infection (Inoculation)

The fungus of *Aspergillus fumigatus* was isolated from immunocompromised patients with IPA and then was cultured on agar to till harvesting the conidia. The conidia was

microscopically examined according to the method described by (Leenders *et al*., 1996) to ensure that they were free from hyphae. All the immunosuppressed mice were anesthetized using isoflurane and then inoculated with 0.025 ml of conidial suspension (about 10^6 conidia) through nasal instillation.

Experimental design

Randomly, thirty-six immunosuppressed infected male mice were divided into four main groups, with 9 mice per group.

- Group I did not receive any treatment.
- Group II received 200 mg of ink extract/kg body wt.
- Group III was treated with 150 mg of amphotericin B /kg body wt.
- Group IV was treated with a mixture of 200 mg of ink extract and 150 mg of amphotericin B per kg body wt.

The onset of all treatments was 24 h post-inoculation of the fungus.

Sampling

The mice were immediately dissected and the liver was removed and stored at –80°C for further investigations. The liver was homogenized (10% w/v) in ice-cold 0.1 M phosphate buffer (pH 7.6) and then centrifuged for 15 min at 3000 rpm at 4^oC in ice-cool centrifuge. The aliquot was used for assessment of MDA, GSH and SOD whereas the precipitate was cultured on potato dextrose agar plates. Animals were euthanized by exsanguinations under overdose of sodium pentobarbital after 1, 3 and 7 days post-infection. The mice were humanely sacrificed, serum was collected by cardiac puncture, and the livers were removed quickly and stored at –80°C for further investigations. The liver was homogenized (10% w/v) in ice-cold 0.1 M phosphate buffer (pH 7.6) and then centrifuged for 15 min at 3000 rpm at $4^{\circ}C$ in ice-cooled centrifuge. The aliquot was used for detection of hepatic DNA damage using comet assay whereas the precipitate was cultured on potato dextrose agar plates for the determination of hepatic fungal burden of *A. fumigatus*. While serum was used for assessment of MDA, GSH and SOD.

Gliotoxin assay

According to the method described by Richard and DeBey (1995), 10 ml 6N HCl was added to serum and shaken 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 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 were added to reservoir. The round bottom was rinsed, four times with 1ml each of CHCl3 and hexane was added in column reservoir. Sample was drown 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 25 ml beaker and evaporated under stream of nitrogen at 30 °C. Sample was transferred to 0.5-gram vial using ether-acetone (95:5, v/v) and the solution in the vial was dried. Samples were then analyzed by thin-layer chromatography (TLC).

Fungal load assay

In order to measure its fungal burden, the liver was homogenized in 5 ml phosphate buffer saline (PBS). The aliquot of homogenate was cultured on potato dextrose agar using serial 10-fold colony countdilutions. The cultures were incubated at 37°C for 48 h. The number of colonies was reported and expressed as CFU/organ.

Oxidative stress assay

The serum levels of MDA, GSH and activities of SOD were measured in the liver homogenate, using Biodiagnostic kits according to the methods described by Ohkawa *et al*. (1979), Beutler *et al.* (1963) and Nishikimi *et al*. (1972), respectively.

Comet assay

Alkaline comet assay was executed to follow up damage in the genomic DNA of hepatocytes. Small pieces (50 mg) of liver were homogenized gently into mincing solution. About 10,000 cells were mixed with 75 μL of 0.5% low melting point agarose (Sigma) and spread on a fully frosted slide predipped in normal melting agarose (1%) as described by Tice *et al*. (2000). After solidification, cells were lysed in cold lysis buffer (2.5M NaCl, 100mM EDTA, and 10mM Tris, pH 10) with freshly added 10% DMSO and 1% Triton X-100 for 24 h at 4∘C in dark. Subsequently, the slides were incubated in fresh alkaline buffer (300mM NaOH and 1mM EDTA, pH 13) for 20 min. The unwinding DNA was electrophoresed for 20min at 300mA and 25V (0.90 V/cm) and neutralized in 0.4M Trizma base (pH7.5) and, finally, fixed in 100% cold ethanol, air-dried, and stored at room temperature until cells were scored. The extent of DNA migration for each sample was determined by simultaneous image capture and scoring of 100 cells stained with ethidium bromide at ×400 magnification using Komet 5 image analysis software developed by Kinetic Imaging, Ltd. (Liverpool, UK). The extent of DNA damage was evaluated using tail length, % tail DNA, and tail moment as DNA damage endpoints.

Statistical analysis

Data were analyzed using Statistical Package for the Social Sciences (SPSS) software version 22. Two-ways analysis of variance (ANOVA) was used to study the effect of treatment, post-infection time and their interaction on the studied parameters. Duncan's test for homogeneity was applied to test the similarity among the studied groups. Data was expressed as means ± standard error of mean (SEM).

RESULTS

Immunosuppression was confirmed by the marked reduction ($p<0.05$) of WBCs count of the immunosuppressed mice ($0.60 \pm 0.09 \times 10^9$/L) in comparison with the normal uninfected mice ($2.3 \pm 1.2 \times 10^9$/L). According to toxicity test, the sepia ink extract did not induce any mortality at 2000 mg/kg body weight of the mice throughout the 14 days of experiment. This was confirmed by insignificant change ($P>0.05$) in the activities of AST (92.0 + 4.0) and ALT (68.11 + 2.9) of mice treated with ink extract as compared to those of control group (87.02 + 8.0) and (65.50 + 3.5), respectively. Thus, the median lethal dose (LD_{50}) of ink extract was considered beyond the 2000 mg/kg. The effective dose was considered as the tenth of the LD_{50} which is 200 mg/kg. According to two-ways ANOVA, all the studied parameters were significantly affected by the treatment but not the post-infection duration except for the serum levels of MDA and gliotoxicn were influenced with the time (Table 1).

Table 1. Two-ways ANOVA to test the effect of the treatment (Tr: Untreated, Ink-treated, Amphotericin-treated, Ink+ Amphotericin-treated), experimental periods (Ti:1, 3, 7 days) and their interaction on the MDA, SOD, GSH, %DNA, OTM, TL, TM, glitoxin and CFU. r_g and r_f: correlation coefficients in relation to the serum level of gliotoxin and fungal load in the liver of mice, respectively

Parameter	Source	SS	df	MS	$F_{calculated}$	P-value	r_g	r_f
MDA	Treatment	2343.53	3	781.18	936.09	<0.0001	+0.95	+0.92
	Time	16.50	2	8.25	9.89	<0.0001		
	Tr * Ti	66.09	6	11.02	13.20	<0.0001		
	Error	20.03	24	0.84				
SOD	Treatment	3254.72	3	1084.91	124.54	<0.0001	-0.80	-0.75
	Time	7.22	2	3.61	0.41	>0.05		
	Tr * Ti	225.49	6	37.58	4.31	<0.0001		
	Error	209.08	24	8.71				
GSH	Treatment	1892.94	3	630.98	240.80	<0.0001	-0.94	-0.86
	Time	9.83	2	4.92	1.88	>0.05		
	Tr * Ti	263.31	6	43.89	16.75	<0.0001		
	Error	62.89	24	2.62				
%DNA	Treatment	2.01	3	0.67	58.77	<0.0001	+0.84	+0.82
	Time	0.06	2	0.03	2.48	>0.05		
	Tr * Ti	0.17	6	0.03	2.46	>0.05		
	Error	0.27	24	0.01				
OTM	Treatment	0.084	3	0.028	56.404	<0.0001	+0.89	+0.93
	Time	0.001	2	0.001	1.365	>0.05		
	Tr * Ti	0.024	6	0.004	8.062	<0.0001		
	Error	0.012	24	0.001				
TL	Treatment	10.18	3	3.39	12.40	<0.0001	+0.78	+0.68
	Time	0.83	2	0.41	1.51	>0.05		
	Tr * Ti	0.49	6	0.08	0.30	>0.05		
	Error	6.56	24	0.27				
TM	Treatment	0.010	3	0.003	8.469	<0.01	+0.68	+0.66
	Time	0.000	2	0.000	0.275	>0.05		
	Tr * Ti	0.007	6	0.001	2.998	<0.05		
	Error	0.009	24	0.000				
Gliotoxin	Treatment	125348783.19	3	41782927.73	5894.26	<0.0001	---	+0.95
	Time	2522343.50	2	1261171.75	177.91	<0.0001		
	Tr * Ti	12380566.06	6	2063427.68	291.09	<0.0001		
	Error	170130.00	24	7088.75				
CFU	Treatment	86238.44	3	28746.15	345.70	<0.0001	+0.95	---
	Time	234.31	2	117.16	1.41	>0.05		
	Tr * Ti	21015.39	6	3502.57	42.12	<0.0001		
	Error	1995.68	24	83.15				

P > 0.05: insignificant effect; P<0.05, P<0.01, P<0.0001: significant effect at α= 0.05, 0.01 and 0.0001, respectively. **SS**: Sum of squares, **df**: degree of freedom, **MS**: mean of squares. **MS**= SS/df; $\mathbf{F_{calculated}}$=MS_{factor}/MS_{error}.

The *in vitro* data revealed that in group A, B and C, the percentage of germination of *Aspergillus fumigatus* was inversely correlated to the applied concentrations of ink extract, Amphotricin B and their mixture together, respectively (Figure 1).

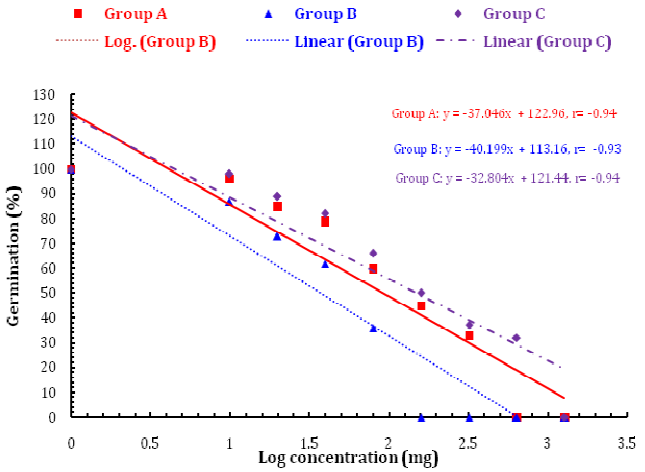


Figure 1. The percentage of germination of *Aspergillus fumigatus* after treatment with ink extract and/or amphotericin B

According to regression analysis, the concentrations of either ink extract (93.2 mg) or Amphotricin B (37.25 mg) required to cause 50% reduction of germination of *Aspergillus Fumigates* was smaller than the concentration (150.6 mg) required from their mixture together (Figure 1).

The levels of gliotoxin and fungal load in the liver of the experimental groups, at all the experimental periods were in the following order: Group I > Group IV > Group II > Group I (Figure 2). In addition, with increasing the time post-infection, the serum levels of gliotoxin and fungal load in the liver of group I were significantly increased whereas those of group II, III and IV were markedly reduced (Figure 2). The concentrations of MDA in the serum of group I were significantly higher than those of groups II, III and IV, at any experimental period (Figure 3). The serum levels of MDA of group II and III were similar throughout the experiments but significantly lower than those of group IV (Figure 3). Among all the experimental periods, the levels of MDA in the serum of groups II, III and IV were homogenous and did not show any significant differences (Figure 3).

The levels of GSH and activities of SOD in the serum of group I were significantly depleted in comparison with those of group II, III and IV, during the course of experiments (Figure 3). Upon increasing the post-infectiontime, the activities of SOD and GSH contents in the serum of group I and IV were markedly decreased whereas those of group II and III were significantly increased (Figure 3). The TL, %DNA in tail, TM and OTM in the hepatocytes of group I were significantly higher than those of the treated groups throughout the

experiments and were markedly elevated at the end of experiments than those at the first day (Figure 4). However, the values of all comet parameters of group II, III and IV were significantly reduced with increasing the post-infection period (Figure 4).

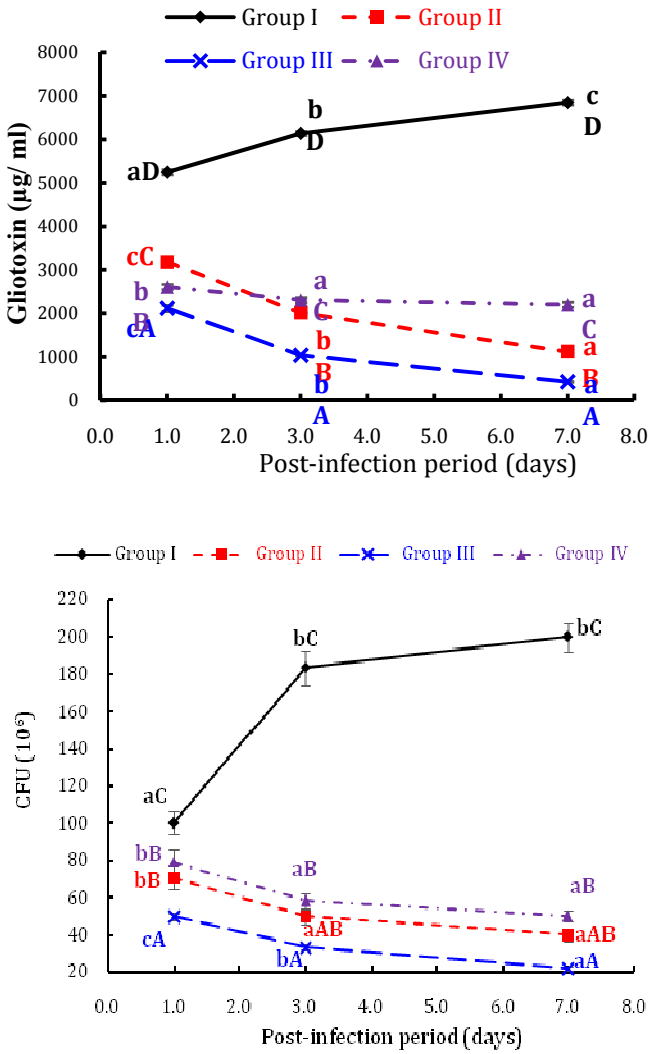
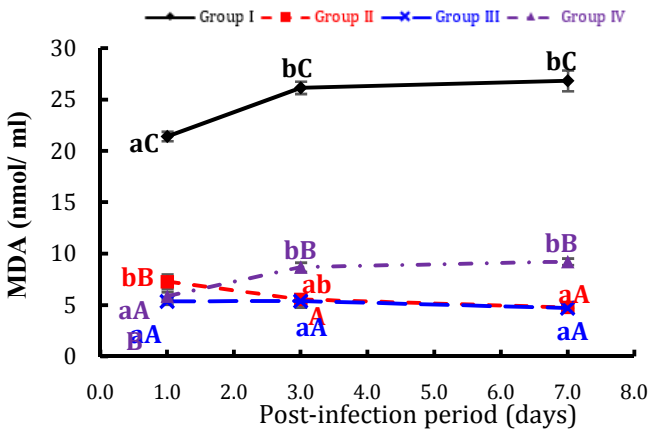


Figure 2. The serum level of gliotoxin and the fungal load in the liver of infected mice with pulmonary aspergillosis and those after treatment with ink extract and/ or AMB throughout seven days post-infection. In each group, the points marked with the same small letters are significantly indifferent (P>0.05) whereas those with different ones are significantly different (P<0.05). At any corresponding time, the points with the same capital letters are significantly indifferent (P>0.05) whereas those with different ones are significantly different (P<0.05)



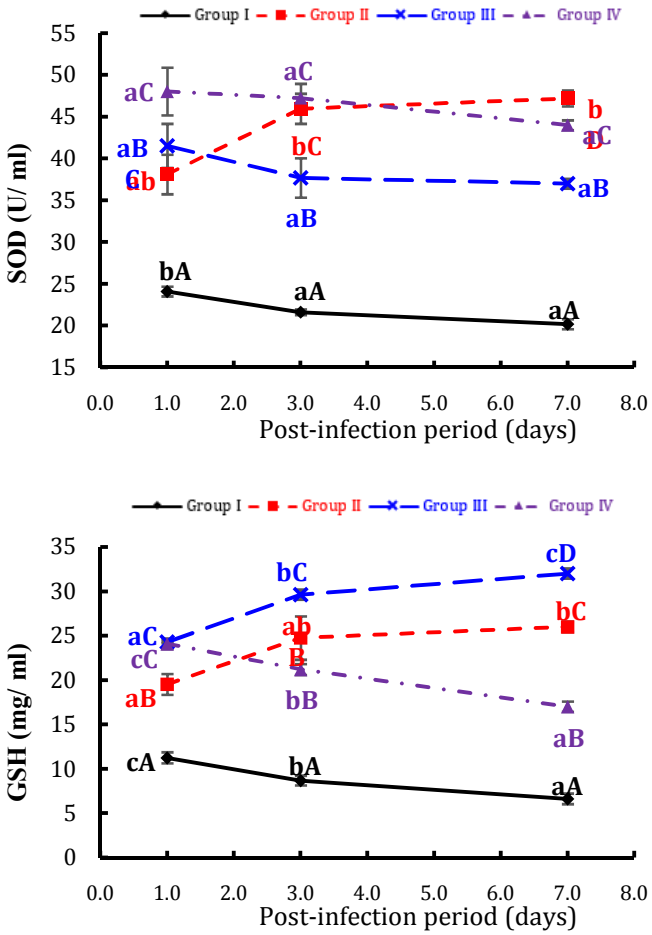


Figure 3. The levels of MDA, GSH and activities of SOD in the serum of infected mice with aspergillosis and those after treatment with ink and/ or AMB throughout seven days post-infection. In each group, the points marked with the same small letters are significantly indifferent (P>0.05) whereas those with different ones are significantly different (P<0.05). At any corresponding time, the points with the same capital letters are significantly indifferent (P>0.05) whereas those with different ones are significantly different (P<0.05)

In the present study, the serum level of gliotoxin and fungal load in the liver were correlated positively with each other as well as with all the studied parameters except for negative correlations with the activities of SOD and GSH content (Table 1).

DISCUSSION

Invasive pulmonary aspergillosis (IPA) is considered one of the most dangerous diseases that affect the immunocompromised patients (Chabi *et al*., 2015). The IPA occurs after inhalation of an air contaminated with the saprophytic *Aspergillus fumigatus* fungus (Jolink *et al*., 2017). Accordingly, in the present study, the intranasal route of administration was chosen to introduce the fungus in the murine model in order to induce infection such as in natural conditions. The hepatic tissue is one of the vital organs that is affected by the *Aspergillus* fungus (Khan *et al*., 2003).Nowadays, the oxidative damage is considered a reasonable mechanism to explain many of the consequences behind the occurrence of many diseases including aspergillosis (Xu *et al*., 2009). Oxidative stress conditions were reported associated with the majority of respiratory disorders (Morcillo *et al*., 1999).

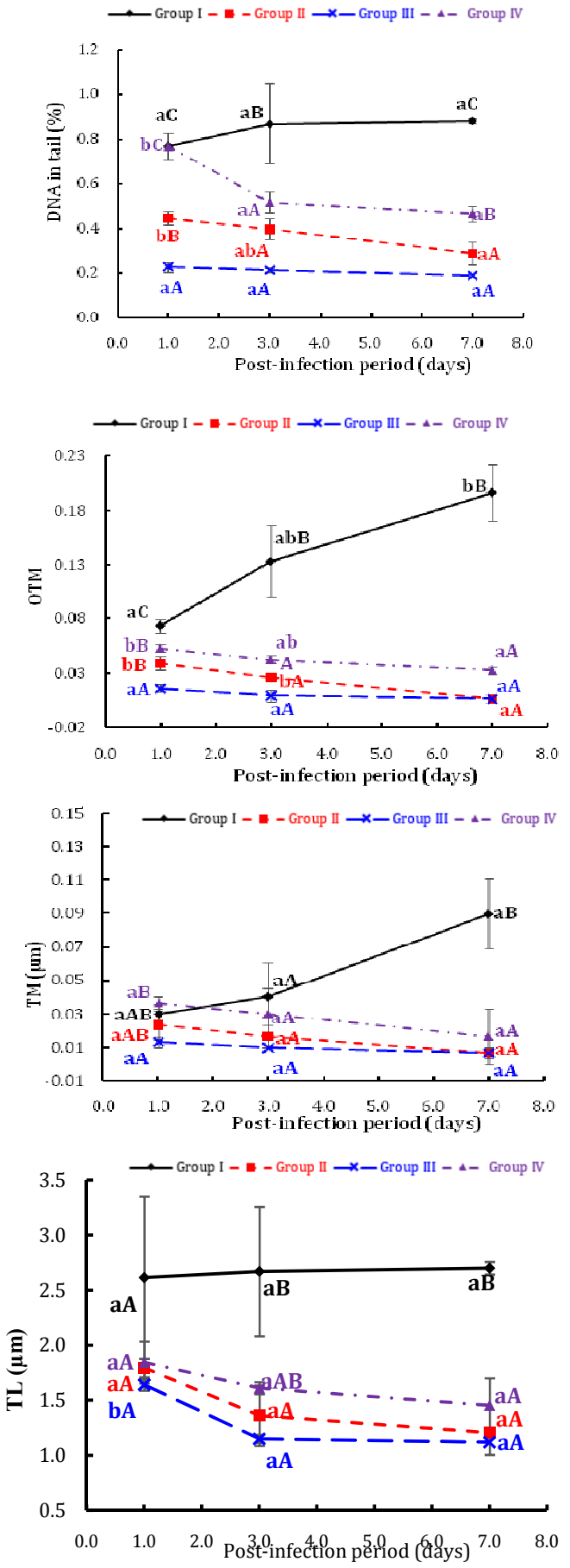


Figure 4. %DNA in tail, TL, TM and OTM in the liver of infected mice with aspriglosesis and those after treatment with ink and/ or AM throughout seven days post-infection. In each group, the points marked with the same small letters are significantly indifferent (P>0.05) whereas those with different ones are significantly different (P<0.05). At any corresponding time, the points with the same capital letters are significantly indifferent (P>0.05) whereas those with different ones are significantly different (P<0.05)

Although, Amphotericin B (AMB) is considered a standard therapy for aspergillosis, its successful outcome was only achieved in 34% of the patients (Denning, 1996). Thus, there was a necessity to discover a new effective antifungal drug. Sepia ink extract was a suitable candidate as being composed of eumelanin which has free radical scavenging properties (Vennila *et al*., 2011). In the present study, *in vitro* data revealed that the germination of *Aspergillus* was inhibited greatly in the mice after administration of either ink extract or AMB separately. However, the combination of both treatments together led to an antagonistic effect. This was confirmed by comparing the required dose of each of treatment alone with their combination for inhibiting germination by 50%, which showed that the dose of mixture was greater than each of them alone. Similarly, Becker *et al.* (2002) recorded that the amphotericin B had antifungal action against *Aspergillus fumigatus*. Peruru *et al*. (2012) attributed the antifungal effect of sepia ink to its content of eumelanin. The reported antagonism between the ink extract and amphotericin B, in the current study, may be attributed to the ability of ink to accumulate in the cell membrane leading to inhibition of the binding of amphotericin B to ergosterol in the fungal membranes (Gill *et al*., 1999 and Milhaud *et al*., 2002). Gliotoxin is considered the most common mycotoxin produced by *Aspergillus fumigatus* (Dolan *et al*., 2014). The present results showed that ink extract and amphotericin B significantly reduced the levels of gliotoxin in the liver. This may be attributed to the ability of both ink and amphotericin B to decrease the fungal load in the liver as shown by our results. The present data is parallel to that reported by Lewis *et al*. (2005) in IPA-infected mice after treatment with antifungal drug. In addition, the reduced levels of gliotoixn and fungal load after treatment with either amphotericin B or ink extract indicates a good therapeutic response (Dagenais and Keller, 2009).

Lipid peroxide (LPO) formation can be a good indicator of the pathological conditions of the cells and degenerated tissues (Morsy *et al*., 2016b). Malondialdehyde (MDA) is the end product of lipid peroxidation (Peng *et al*., 2009). In the present study, the treatment with ink extract as well as AMB caused significant depletion of MDA in the serum as compared to those of infected mice. This may be attributed to their ability to scavenge the ROS that is overproduced during infection (Maitraie *et al*., 2009). Sepia ink also contains considerable amount of taurine, a sulphur containing amino acid that act as antioxidant (Derby *et al*., 2007). Glutathione (GSH) is a non-enzymatic endogenous antioxidant in the mammalian cells (Toklu *et al*., 2013). The present data showed marked increase in the serum levels of GSH of mice treated with ink and/or amphotericin B as compared to the infected untreated group. This increase in GSH content was accompanied by decrease in the levels of gliotixin as manifested by correlation coefficients, in the present study. This may be attributed to the ability of GSH to bind to the gliotoxin to facilitate its exertion (Roebuck *et al*., 2009). On the other hand, the reduction in GSH of infected group may be considered a trigger for the occurrence of LPO (Thomas *et al*., 2009). Superoxide dismutase (SOD) is an enzymatic antioxidant that is involved in the detoxification of super oxide anion into hydrogen peroxide (Morsy *et al*., 2016b). The amount of SOD is abundant in the liver (Singh and Ahluwalia, 2002). The present results showed that the activities of serum SOD of the mice administered ink extract and /or amphotericin B were markedly increased as compared to those of the infected untreated group. The reduced activity

of SOD in the infected group may be attributed to the overproduction of free radicals by inflammatory responses (Comhair *et al*., 2000). In addition, the infection can lead to damage of mitochondria, decreased mitochondrial antioxidant manganese superoxide dismutase (MnSOD) activity and consequently the total SOD activity (Sun *et al*., 1989). Similarly, Peng *et al.* (2009) reported that SOD activity significantly decreased following *A. fumigates* infection. However, the increased activity of SOD in the serum after treatment with ink extract may be attributed to the ability of eumelanin in sepia ink to act like SOD, leading to keeping the normal activity of SOD (Chen *et al*., 2007). In the same way, ink extract was reported to increase the SOD activities and reduce the levels of MDA in the kidney (Liu *et al.,* 2009),lung (Wang *et al.,* 2009), heart (Wang *et al.,* 2010)and spleen of mice (Zhong *et al.,* 2009).

Single cell electrophoresis (or comet assay) is an efficient technique to study the DNA damage in the mammalian cells (Morsy *et al*., 2016a). The current results of comet assay showed that all the studied comet parameters were markedly reduced after treatment with ink and /or AMB as compared to the infected untreated group. The reduction in the comet parameters was in a time-dependent manner as indicated by significant differences between the experimental periods. This indicates that the DNA breaks caused by the infection were alleviated by the ink as well as AMB. The interaction of *Aspergillus fumigatus* with the host immune cells can lead to DNA fragmentation, and cell death as a result of gliotoxin production **(**Morton *et al*., 2012**)**. This may be attributed to the ability of gliotoxin to suppress the immune responses against fungal growth causing its invasiveness and consequently interaction with macromolecules including DNA (Tsunawaki *et al.,* 2004). In the present study, the increased levels of GSH after treatment with ink extract and/or AMB can lead to protection of cells from the oxidative stress and assist to capture free radicals that can injure RNA and DNAthrough thiol-disulphide exchange (Pastore *et al.*, 2003).

Eid *et al*. (2014) studied the effect of aspergillosis infection on DNA damage qualitatively using agarose gel electrophoresis, and observed that IPA infection caused high degree of random DNA degradation in pulmonary tissues, they attributed the observation to the fact that the inflammatory action result from aspergillosis infection leads to oxidative DNA damage and also due to the necrotic cell death which accompanied in the inflammatory process. In conclusion, the both ink extract and amphotericin B showed antimicrobial, antioxidant and antigenotoxic properties in a time-dependent manner. On the other hand, the treatment with combination of ink extract and amphotericin B can lead to antagonistic effect.

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