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# Therapeutic efficacy of chitosan against invasive candidiasis in mice

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**KEYWORDS**

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*In vivo*;
Antioxidant

**Abstract** The prevalence of antibiotic resistance has resulted in the need for new approaches to be developed to combat the previously easily treatable infections. This work aims to evaluate the anti-fungal and antioxidant effects of the chitosan, as a new alternative or complementary anti-fungal drug, alone or in combination with amphotericin B against a pathogenic *Candida albicans* 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 (NI group) (IPC group, invasive pulmonary candidiasis), the 2nd group was treated with chitosan ($ED_{50}$) (CE group), the 3rd group was treated with amphotericin B (150 mg/kg) (AMB group) and the 4th group was treated with chitosan plus amphotericin B (CE + AMB group). Treatment was started at 24 h after fungal inoculation and was administered for 3 consecutive days. All the previous treatments demonstrated notable growth inhibition against a *C. albicans* isolate as indicated by measuring the mean diameter of the inhibition zone. Compared with IPC group, CE, AMB, and AMB + CE-treated animals had 73%, 87%, and 90% reduction in fungal burden, respectively. Furthermore, treatment with CE and/or AMB for 24 and 72 h significantly decreased MDA, SOD, CAT and NO levels and increased GSH and in the lung tissues as compared with the infected untreated group. In conclusion, CE treatment, with the combination of antifungal therapy, can alleviate oxidative stress and lung injury associated with IPC in neutropenic mice.



## Introduction

*Candida albicans* (*C. albicans*) is an opportunistic pathogen that causes superficial and systemic infections (Selvaraj et al., 2014). *C. albicans* is a pathogenic yeast, which forms a range of polarized and expanded cell shapes (Canonico et al., 2014). It is the most common human fungal pathogen and causes significant morbidity and mortality worldwide (Noble and Johnson, 2005; Kaufman et al., 2014). It is a dimorphic yeast capable of producing alternate morphological forms (yeast or mycelium) in response to environmental changes (Manavathu et al., 1996). It exists as a commensal organism in healthy individuals by colonizing several niches of the human body which includes skin, mucosal surfaces, oral

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cavity, vagina, and gastrointestinal tract (Larriba et al., 2000). An altered balance between the host immunity and this opportunistic fungus, as in the case of immunocompromised patients, is one of the leading causes of candidiasis in humans (Bodey, 1993). After entering the blood stream, the yeast cells can infect all internal organs and may cause life-threatening septicemia (Karkowska-Kuleta et al., 2009). Candidiasis can develop as superficial candidiasis (skin and mucosa) which occurs in healthy individuals, or invasive candidiasis which is seen in cancer patients, AIDS patients, and immunocompromised individuals following transplantation (Larriba et al., 2000).

Invasive candidiasis remains a challenging complication, which frequently occurs in patients with one or more underlying diseases or surgical interventions. In recent point prevalence studies, a candidaemia incidence of 6.9 per 1000 ICU patients was reported, and 7.5% of ICU patients received antifungal therapy (Kett et al., 2011; Azoulay et al., 2012). Candidaemia increases mortality rates in the range of 20–49% (Gudlaugsson et al., 2003; Arendrup et al., 2011), but still there are many open management questions. Pulmonary candida infections may present as the manifestations of disseminated candidiasis spread by hematogenous route or as a primary bronchial or pulmonary process from the airways (Odds, 1988).

Highly reactive oxygen metabolites are one of the primary effector mechanisms used by the host immune system to control or clear microbial infections (Youseff et al., 2012). Reactive oxygen species (ROS) are essential components of the defensive mechanism against fungus infection (Ibrahim-Granet et al., 2003; Philippe et al., 2003). Initial host defenses against fungal invaders rely on the responses of innate immune cells, particularly macrophages, neutrophils and other phagocytic cells. These phagocytes generate potent reactive oxygen and nitrogen species (ROS and RNS), which are toxic to most fungal pathogens, causing damage to DNA, proteins and lipids (Bogdan et al., 2000; Youseff et al., 2012). To protect against damage, cells contain a number of defense mechanisms including endogenous well-characterized antioxidant enzymes, such as catalase, superoxide dismutase, nitric oxide and low molecular weight antioxidant, such as glutathione (GSH) (Mates et al., 1999). Indeed, ROS induce programmed cell death in *C. albicans* (Phillips et al., 2003).

Amphotericin B (AMB) is a polyene antifungal antibiotic by-product of the actinomycete bacterium *Streptomyces nodosus*. In spite of AMB's proven track record in the management of serious systemic fungal infections, its well-known side effects and toxicity will sometimes require discontinuation of therapy despite a life-threatening systemic fungal infection. The principal acute toxicity of AMB is nephrotoxicity (Geo vigila and Baskaran, 2011). Clinical manifestations of AMB nephrotoxicity include renal insufficient hypokalemia, hypomagnesaemia, metabolic academia, and polyuria due to nephrogenic diabetes insipidus (Laniado-Laborín and Cabrales-Vargas, 2009). There have been an increasing number of reports of clinically significant amphotericin B (AMB) resistance in fungal pathogens, including *C. albicans* (Sterling and Merz, 1998). Since many of the currently available drugs have undesirable side effects and are ineffective against *C. albicans* infection, there is now a greater interest in the next generation of antifungal agents. Many people worldwide, including those in developed countries, turn to complementary or alternative medicine. Products from freshwater and marine sources have recently become attractive as nutraceutical and functional foods and as a source material for the development of drugs (Koyama et al., 2006).

Chitosan is a linear polysaccharide composed of randomly distributed β-(1–4) linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). It is made by deacetylation of chitin, the primary polysaccharide component of crustacean shells with the alkali sodium hydroxide (Shahidi and Synowiecki, 1991). Chitosan can be used to produce value-added products because it is rich in protein, carotenoids and chitin (Lertsutthiwong et al., 2002). This polysaccharide was found to be non-toxic, biocompatible and biodegradable (Arvanitoyannis et al., 1998). Chitosan has several applications being employed either alone or in blends with other natural polymers (starch, gelatin and alginates) in the food and pharmaceutical industries mainly due to its high biodegradability and antimicrobial properties (Hague et al., 2005). Microbiological activity of chitosan has been detected for many bacteria, filamentous fungi and yeasts (Hirano and Nagao, 1989). Data in the literature have the tendency to characterize chitosan as bacteriostatic rather than bactericidal (Coma et al., 2002), although the exact mechanism is not fully understood and several other factors may contribute to the antibacterial action (Raafat et al., 2008). Three models have been proposed, the most acceptable being the interaction between positively charged chitin/chitosan molecules and negatively charged microbial cell membranes. In this model the interaction is mediated by the electrostatic forces between the protonated $NH^{+3}$ groups and the negative residues (Tsai and Su, 1999), presumably by competing with $Ca^{+2}$ for electronegative sites on the membrane surface (Young and Kauss, 1983). Since such mechanism is based on electrostatic interaction, it suggests that the greater the number of cationized amines, the higher will be the antimicrobial activity (Yalpani et al., 2002; Másson et al., 2008). This suggests that chitosan has higher activity than that found for chitin and this has been confirmed experimentally (Tsai and Su, 1999; Másson et al., 2008).

To improve the suboptimal therapy for many fungal infections, the efficacy of some drug combinations has been examined. Several studies involving combinations of amphotericin B with other antimicrobial agents have been reported. Such combinations were expected to be synergistic because amphotericin B facilitated the entry of the second agent into the fungal cell (Jit Sud and Feingold, 1983).

Therefore, this study aims to evaluate the antifungal and antioxidant effects of the chitosan, as a new alternative or complementary anti-fungal drug, alone or in combination with amphotericin B against a pathogenic *C. albicans* in mice.

## Materials and methods

### *Chemicals, media, and drugs*

Chitosan (CAT No. 50494) and Sabouraud Dextrose Agar (Product No. S 3181) were purchased from Sigma–Aldrich (St Louis, MO, USA). Cyclophosphamide (Endoxan), and Amphotericin B (supplied as Fungizone; E.R. Squibb & Sons, Princeton, NJ) were purchased. All other chemicals were purchased from local standard companies and were of reagent grade or better.

*Preparation of C. albicans and growth condition*

A registered isolate of lyophilized *C. albicans* (ATCC No. 10321) was obtained from ATCC (American Type Culture Collection, National Research Center, Cairo, Egypt). The growth is indicated by growth of white, soft, cream-colored colonies with yeasty odor which were confirmed by gram staining reaction and germ tube test (Doughari and Peter, 2009).

*Preparation of serial dilution*

The yeast suspension was harvested by washing the organism culture with sterile physiological saline. A sample was serially diluted and plated on Sabouraud dextrose agar (SDA) to determine the numbers of colony forming unit CFU/1 ml. The plates were inverted and incubated at 37 °C for 18–24 h till the required growth was obtained (Doughari and Peter, 2009) and then the CFU was counted.

*Standard in vitro agar diffusion growth inhibition*

A standard *in vitro* agar diffusion growth inhibition assay was used to evaluate the anti-candidal activity of chitosan, amphotericin B and their combination. *C. albicans* isolates were cultured on SDA plates. Each plate was inoculated with 50 µl of candida isolate suspension and swabbed evenly to generate a 'lawn' of yeast growth. Following inoculation and swabbing, a cork borer of 5 mm diameters was used to create wells in each plate with a concentration of *C. albicans* suspension $1 \times 10^7$ CFU/ml. Thereafter, 15 µl chitosan, amphotericin and their combination (ratio 1:1) were pipetted into the wells. Plates were placed in an incubator at 37 °C for 18–24 h until colonies were formed (Doughari and Peter, 2009). After the incubation period, zones of growth inhibition (clear zone appeared around each well) were measured using an image analysis software program (Quantimet 500, Windows version; Leica). The diameter of each inhibition zone around a well (including the diameter of the well itself) was measured and the software automatically calculated the arithmetic mean of 4 measurements at different angles (Sitheeque et al., 2009).

*Animals*

Specific pathogen-free, 8–10 weeks old male Swiss mice, and weighing 20–25 g obtained from a closed random-bred colony at the animal house, National Research Center. Animals were housed in polycarbonate boxes with steel-wire tops (not more than five animals per cage) and bedded with wood shavings. Ambient temperature was controlled at 22 ± 3 °C with a relative humidity of 50 ± 15% and a 12-h light/dark photoperiod. Food and water were provided *ad libitum*.

*Ethical consideration*

Experimental protocols and procedures used in this study were approved by the Cairo University, Faculty of Science, and Institutional Animal Care and Use Committee (IACUC) (Egypt) (CUFS/S/08/13). All the experimental procedures were carried out in accordance with international guidelines for care and use of laboratory animals.

*Toxicity study (OECD 420)*

Acute oral toxicity test was done according to the organization for economic cooperation and development (OECD) based on acute oral toxicity up and down procedure 425 guideline (OECD, 2001). Two groups, each of five healthy male mice, were selected randomly and fasted overnight. The first group was administered, via gavage, chitosan extract (CE) powder suspension at a limit dose of 5000 mg/kg body weight. The second group (control group) was given an equal volume of distilled water. All animals were observed at 0, 30 min, 1, 2, 4, 6 h and thereafter every day for 14 days to check the mortality and abnormal clinical manifestation. The rats were sacrificed after 14 days and their liver and lung were excised and fixed in 10% formalin for 24 h. They were processed and stained with hematoxylin and eosin dyes for histopathological examination. The median effective dose ($ED_{50}$) of CE was selected based on $LD_{50}$ obtained from acute toxicity study.

*Immunosuppression*

Neutropenia was induced by intraperitoneal (i.p) 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 on a daily basis until the end of the study. At this time point mice were at an immunocompromised state, as determined by the decrease in the number of white blood cells (WBC) and reduction in the body weight (unpublished data).

*Murine model of candidiasis*

The standard inoculation of *Candida albicans* was cultured on Sabouraud agar and washed three times in sterile normal saline and adjusted to a concentration of $1 \times 10^7$ viable cells/ml on a hemocytometer. On day 0, all neutropenic mice were infected intravenously with *C. albicans* via the lateral tail vein (Jothy et al., 2012). Each inoculum consisted of 0.1 ml of fungal suspension.

*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) (IPC group, invasive pulmonary candidiasis), the 2nd group was treated with chitosan ($ED_{50}$) (CE group), the 3rd group was treated orally with Amphotericin B (150 mg/kg) (AMB group) and 4th group was treated with chitosan plus amphotericin B (CE + AMB group). Treatment was started at 24 h after fungal inoculation and was administered for 3 consecutive days.

*Sample collection for analysis*

Animals were euthanized after being anesthetized with sodium pentobarbital and sacrificed after 24 h and 72 h of treatment after being fasted over night; blood was collected in EDTA and centrifuge tubes for haematological and biochemical parameters. The superior lobe of the right lung was removed and immediately blotted using a filter paper to remove traces

of blood and stored at −80 °C for biochemical studies. However, the inferior lobe of the right lung was suspended in 10% formal saline for fixation preparatory to histological processing.

*Sample preparation*

*Lung tissue homogenate preparation*

The superior lobe of the right lung was homogenized (10% w/v) in ice-cold 0.1 M phosphate buffer (pH 7.6). The homogenate was centrifuged at 3000 rpm for 15 min at 4 °C and the resultant supernatant was used for different oxidative stress markers.

*Histopathological preparation*

The fixed inferior lobe of the right lung was sectioned (5-micron thickness); sections were firstly stained with basic dyes, hematoxylin and Eosin (H&E) according to the method described by Conn (1946).

*Assessment of fungal load in lung tissue*

For tissue burdens, aliquots of tissue were semi-quantitatively cultured on Sabouraud dextrose agar that was prepared as previously described using serial 10-fold colony count dilutions. Plates were placed in an incubator at 37 °C until colonies could be counted (Doughari and Peter, 2009). Count was expressed as CFU/organ.

*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), glutathione reduced (GSH) (Aykac et al., 1985), superoxide dismutase (SOD) (Nishikimi et al., 1972), Catalase (Aebi, 1984) and Nitric oxide (NO) (Montgomery and Dymock, 1961).

*Statistical method*

All data are expressed as means ± SEM. In general, data were analyzed by two-way ANOVA followed by the Bonferroni test 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 5.01v (La Jolla, CA, USA).

## Results

*Toxicity study (OECD 420)*

The oral administration of CE powder (5000 mg/kg BW) caused neither mortality nor signs of clinical abnormality. At necropsy, no gross pathological observation was found in the target organs like liver and lung (Figure 1). Liver sections of control and chitosan groups show normal histological

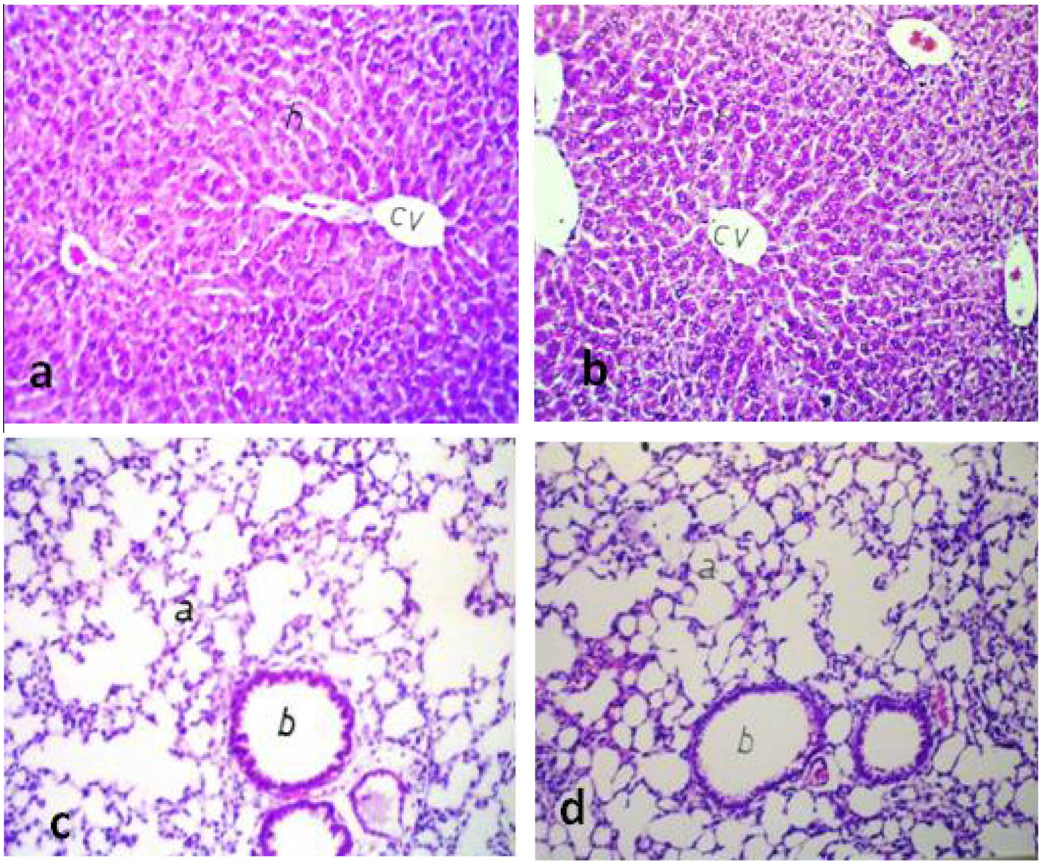


**Figure 1** Photomicrographs of hematoxylin-eosin stained mice liver and lung sections of control group (a and c), and chitosan group (5000 mg/kg body weight) (b and d) (40×).

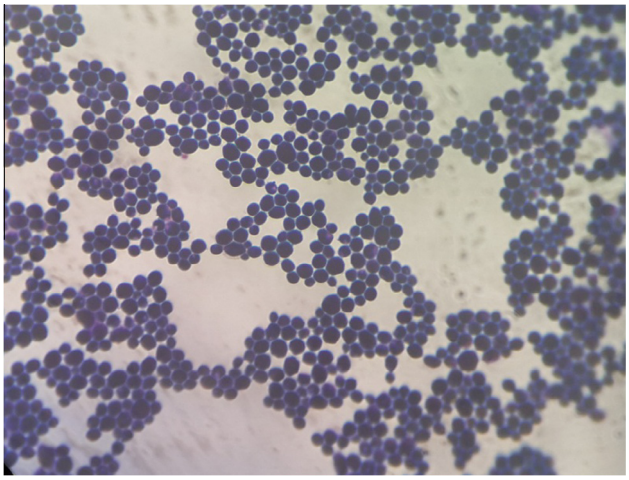

**Figure 2** *Candida albicans* was examined under oil immersion at 100× magnification Gram positive oval shaped large purple cells were identified as *Candida albicans*.

structure of the central vein (cv) and surrounding hepatocytes (h) (Figs. 1a and b). Meanwhile, the lung sections of control and treated mice show-normal histological structure of the bronchioles and surrounding air alveoli (a) (Figs. 1 c and d). The $LD_{50}$ of CE powder was found to be more than 5000 mg/kg BW. 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 $LD_{50}$ (500 mg/kg body weight, P.O).

*Identification of C. albicans by gram stain reaction and germ tube*

All the isolates showed Gram positive pseudohyphae or oval shaped large purple cells after Gram staining (Fig. 2) and small filaments projecting from the cell surface after the respective germ tube tests (Fig. 3).

*Standard in vitro agar diffusion growth inhibition*

Fig. 4 shows the anti-candidal activity of chitosan extract (CE) (500 mg/1 ml) and Amphotericin B (AMB) (150 mg/1 ml) and their combination (CE + AMB) (1:1) against *C. albicans*. All the previous treatments demonstrated notable growth inhibition against *C. albicans* isolate as indicated by measuring the mean diameter of the inhibition zone (Fig. 4).

*Estimation of fungal load in lung tissue*

Fig. 5 shows the microbial burden in the autopsied lungs. Lung cultures had minimal fungal load in the CE + AMB group. Mean burden of fungal organisms in the lung (CFU/g lung tissue) after 72 h of inoculation was $344 \times 10^7$ CFU/g lung tissue (IPC group), $93 \times 10^7$ CFU/g lung tissue (CE group), $46 \times 10^7$ CFU/g lung tissue (AMB group), and $35 \times 10^7$ (CE + AMB). Compared with IPC group, the CE-treated, AMB-treated, and AMB + CE-treated animals had 73%, 87%, and 90% reduction in fungal burden, respectively.

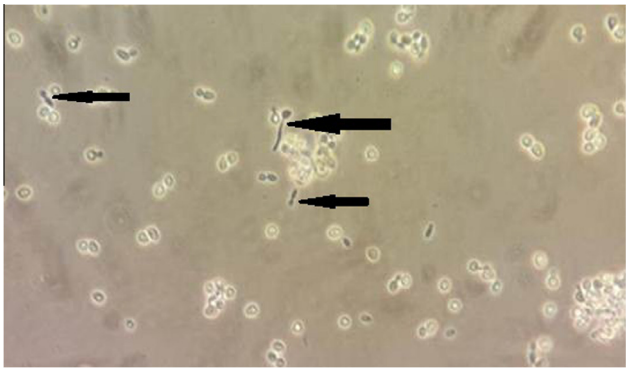

**Figure 3** *Candida albicans* examined microscopically, using the 10× and 40× objective lenses. The appearance of small filaments projecting from the cell surface confirmed formation of germ tubes.

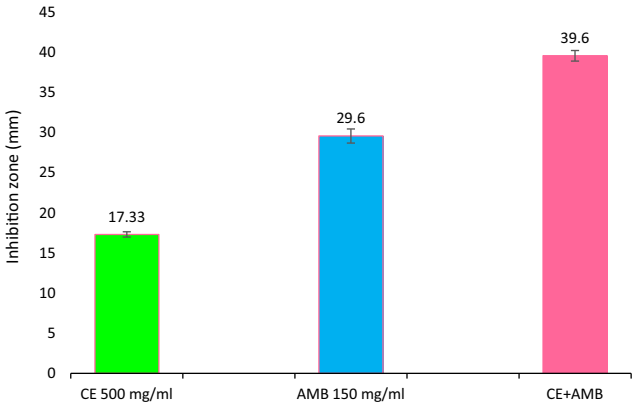


**Figure 4** Growth inhibitory activity (mean diameter of inhibition zone in mm) of different treatments for candidiasis : chitosan extract (CE; 500 mg/1 ml), amphotericin B (AMB; 150 mg/ml), and their combination (CE + AMB) (1:1) ratio, respectively.

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

*Lung malondialdehyde (MDA)*

There was a significant increase ($P < 0.05$) in the lung MDA level of the neutropenic infected mice after 24 and 72 h period, as compared to control mice. Lung MDA levels of CE, AMB, and CE + AMB groups were significantly ($P < 0.05$) decreased after 24 and 72 h of treatment, as compared with the infected untreated groups (Table 1). Meanwhile, a significant increase ($P < 0.05$) was noticed in the level of lung MDA after 24 and 72 h of CE administration as well as after 24 h of combined treatment, as compared to the corresponding AMB groups.

*Lung glutathione reduced (GSH)*

Table 1 shows that Candida infection to neutropenic mice caused a significant decrease ($P < 0.05$) in the lung GSH level, as compared to control mice. On the other hand, treatment with CE, AMB, and CE + AMB for 24 and 72 h significantly ($P < 0.05$) increased the lung GSH levels, as compared with the infected untreated groups. Additionally, a significant

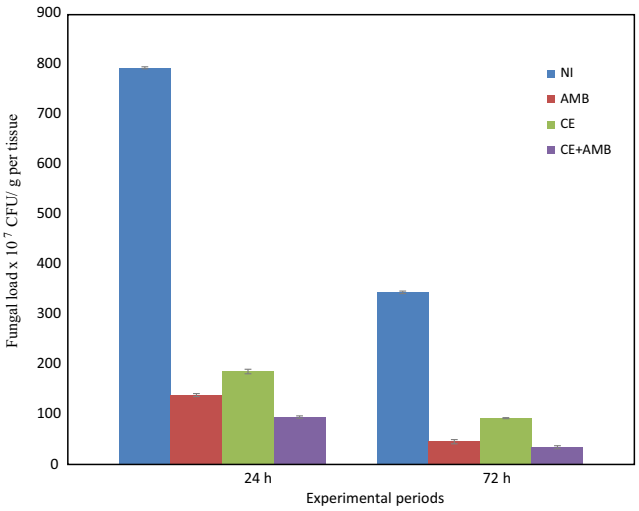


**Figure 5** Screening of fungal load in lung homogenate of mice infected with *Candida albicans*. IN: infected and neutropenic group; CE: chitosan extract (neutropenic, infected with *C. albicans*, treated with chitosan extract); AMB: amphotericin B (neutropenic, infected with *C. albicans* and treated with amphotericin B); CE + AMB: amphotericin B and chitosan extract (neutropenic, infected with *C. albicans* and treated with chitosan extract and amphotericin B).

decrease was noticed in the level subsequent to administration of CE (500 mg/kg) for 24 and 72 h. However, lung the GSH level increased significantly ($P < 0.05$) after 72 h of CE + AMB administration, as compared to the AMB group (Table 1).

*Lung superoxide dismutase (SOD)*

A significant increase ($P < 0.05$) was noticed in the lung SOD activity of infected untreated mice at the two selected time periods, as compared to the control group. However, lung SOD activities of all experimental groups at the two selected time periods were significantly ($P < 0.05$) lower than those of the infected untreated group (Table 1). However, by comparing with the AMB group, the lung SOD activity of mice treated with CE for 24 h only and CE + AMB for 24 and 72 h were significantly decreased ($P < 0.05$) (Table 1).

*Lung catalase (CAT)*

Table 2 shows that lung CAT activity significantly increased ($P < 0.05$) after 24 and 72 h of infection, as compared to control mice. A significant decrease ($P < 0.05$) was noticed in the lung catalase activity of mice receiving chitosan extract (500 mg/kg) or Amphotericin B (150 mg/kg) and their combination (CE + AMB) for 24 and 72 h, as compared to the infected untreated group (Table 2). However, lung catalase activity of mice significantly decreased ($P < 0.05$) after the administration of CE (500 mg/kg) and also increased significantly ($P < 0.05$) (CE + AMB) for 24 and 72 h, as compared to the corresponding AMB group.

*Lung nitric oxide (NO)*

Lung nitric oxide level increased significantly ($P < 0.05$) after 24 and 72 h of infection, as compared to control mice. A significant decrease ($P < 0.05$) in lung nitric oxide (NO) concentration was recorded after the administration of chitosan extract (500 mg/kg body weight) and Amphotericin B (150 mg/kg body weight) and their combination for 24 and 72 h as compared to the infected group (Table 2). On the other hand, a significant increase ($P < 0.05$) was noticed in the NO level subsequent to chitosan administration (500 g/kg body weight) for 24 h. However, treatment with CE + AMB for 24 and 72 h caused a significant decrease ($P < 0.05$) in the level of lung nitric oxide, as compared to the AMB group (Table 2).

## Discussion

Infection with pathogenic fungi increased dramatically over the past two decades (Kupfahl et al., 2007). Although bacteria are the causative organisms of most of the infectious episodes, fungi, particularly *C. albicans* (which causes >80% of all fungal infections) and account for >20% of the fatal infections in patients with leukemia and for 13% of those in patients with lymphoma (Lopez-Berestein et al., 1983). *C. albicans* an important aerobic eukaryotic pathogen causes the majority of human fungal infections. These infections range from thrush in immunocompetent colonized hosts to life-threatening systemic infections in immunocompromised individuals such as patients with cancer (Martchenko et al., 2004).

The search for new antimicrobial agents is of great concern today, because of the increasing development of drug

**Table 1** Effect of chitosan extract (CE); amphotericin B (AMB) and their combination on some lung oxidative stress markers in mice model of invasive pulmonary candidiasis (IPC).

| Groups | MDA (nmole/g tissue) | | GSH (mg/g tissue) | | SOD (U/g tissue) | |
|---|---|---|---|---|---|---|
| | 24 h | 72 h | 24 h | 72 h | 24 h | 72 h |
| Control | 3.69 ± 0.14 | 90 ± 2.80 | 36.46 ± 5.44 | | | |
| Infected untreated | 29.51 ± 1.00[a] | 19.92 ± 0.55[a] | 55.52 ± 1.06[a] | 74.51 ± 1.05[a] | 133.44 ± 4.54[a] | 206.04 ± 2.00[a] |
| AMB | 6.64 ± 0.14 [b] | 3.88 ± 0.15 [b] | 96.60 ± 1.320[b] | 115.48 ± 1.04[b] | 52.08 ± 6.80[b] | 30.34 ± 1.28[b] |
| CE | 7.32 ± 0.19[bc] | 4.96 ± 0.08[bc] | 88.80 ± 0.680[bc] | 113.80 ± 0.86[bc] | 35.14 ± 2.47[bc] | 31.08 ± 4.33[b] |
| CE + AMB | 8.00 ± 0.29[bc] | 3.76 ± 0.09[b] | 97 ± 0.70[b] | 125.96 ± 0.89[bc] | 23.29 ± 3.34[bc] | 17.85 ± 2.63[bc] |

Data are presented as mean ± SEM ($n = 5$ in each group).
[a] Significantly different from control group at $P < 0.05$.
[b] Significantly different from infected untreated group at $P < 0.05$.
[c] Significantly different from AMB treated group at $P < 0.05$.

**Table 2** Effect of chitosan extract (CE); amphotericin B (AMB) and their combination on some lung oxidative stress markers in mice model of invasive pulmonary candidiasis (IPC).

| Groups | CAT (U/min) | | NO (μmol/g tissue) | |
|---|---|---|---|---|
| | 24 h | 72 h | 24 h | 72 h |
| Control | 5.16 ± 0.42 | 20.42 ± 0.7 | | |
| Infected untreated | 12.77 ± 0.70[a] | 14.01 ± 0.50[a] | 162.72 ± 7.89[a] | 70.9 ± 3.90[a] |
| AMB | 6.80 ± 0.66[b] | 7.70 ± 0.45[b] | 62.18 ± 1.132[b] | 41.82 ± 0.52[b] |
| CE | 4.2 ± 0.21[bc] | 5.5 ± 0.36[bc] | 68.48 ± 0.687[bc] | 44.02 ± 3.37[b] |
| CE + AMB | 8.6 ± 0.40[bc] | 9.1 ± 0.38[bc] | 33.36 ± 0.591[bc] | 19.46 ± 0.41[bc] |

Data are presented as mean ± SEM ($n = 5$ in each group).
[a] Significantly different from control group at $P < 0.05$.
[b] Significantly different from infected untreated group at $P < 0.05$.
[c] Significantly different from AMB treated group at $P < 0.05$.

resistance to human pathogens and the appearance of undesirable effects of certain antifungal agents (Phongpaichit et al., 2005). A multidisciplinary approach to drug discovery, involving the generation of truly novel molecular diversity from natural product sources, providing the best solution to the current productivity problems in the scientific society involved in drug discovery and development (Newman and Cragg, 2007).

Chitosan (CE) exhibits various potential biological activities, such as antitumor, immunostimulatory, antibacterial and antifungal properties (Chung et al., 2011). To improve the suboptimal therapy for many fungal infections, the efficacy of some drug combinations has been examined. Several studies involving combinations of amphotericin B with other antimicrobial agents have been reported. Such combinations were expected to be synergistic because amphotericin B facilitated the entry of the second agent into the fungal cell (Graybill et al., 1980; Sud and Feingold, 1983). The current study revealed that the synergism of amphotericin B with the CE has shown in the standard *in vitro* agar diffusion growth inhibition test. The mean inhibition zone was the largest in the CE + AMB group. In addition, the estimation of fungal burden in lung tissue revealed that the administration of CE + AMB has the highest percentage in the reduction of fungal load in the infected lung. These aforementioned effects may be due to the chitosan's immuno-enhancing effect which has a satisfactory stimulatory effect on macrophages and its internalization was mediated by a specific receptor on macrophages (Feng et al., 2004).

The virulence of *C. albicans* seems to be multifactorial (Chauhan et al., 2003), but the ability of this fungus to mount stress responses is an important aspect, as this promotes survival in the host during systemic infections (d'Enfert and Hube, 2007). It was demonstrated that a large proportion of *C. albicans* cell surface antigens related to acute candidemia are involved in oxidative stress (Mochon et al., 2010). Lung is remarkably vulnerable to injury induced by candidiasis as a result of the reactive oxygen species (ROS).

The lipid peroxidation was assessed on the basis of malondialdehyde (MDA) estimation. Lung MDA level of neutropenic infected mice with *C. albicans* increased significantly with a peak at 24 h post infection. The results of Zgai and Chhibber (2010) and Mahmoud et al. (2011) are consistent with the present finding. It has been reported that the increase in the MDA level enhances the lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanism to prevent formation of excessive free radical (Park et al., 2010). The decreased level of lung MDA of treated mice with CE alone or in combination with AMB may be due to the scavenging effect of CE on hydroxyl radicals which inhibits lipid peroxidation of phosphatidyl choline and linoleate liposomes (Ozcelik et al., 2014).

Glutathione reduced (GSH) is the most abundant non-protein thiol compound present in mammalian cells and serves many physiological roles, particularly as cellular antiperoxidation in peripheral tissue (Liu and Gaston Pravia, 2010). It acts as an electron donor in the glutathione peroxidase catalyzed reactions of organic and hydrogen peroxide. The decreased GSH contents indicate increased oxidative stress. The sustained oxidative challenge to the lung results in depletion of lung GSH (El-Sayed and Rizk, 2009). Furthermore, the lung GSH levels of all treated mice in the present study increased with specific improvement in the combination groups. Such combinations were expected to be synergistic because amphotericin B facilitated the entry of the second agent into the fungal cell (Jit Sud and Feingold, 1983).

Aerobic eukaryotic pathogens as *C. albicans* can encounter superoxide radicals ($O_2^-$) generated from several sources. These sources can be internal or external. An important internal source is the mitochondrial respiratory chain (Casteilla et al., 2001; Lenaz, 2001), and thus the rate of respiration can have a significant impact on the reactive oxygen species (ROS) production. A key external source of ROS encountered by pathogens is from phagocytes. The superoxide radical is the first intermediate in the oxidative burst generated in the phagosome, and this burst is thought to be involved in pathogen killing (Reeves et al., 2002). The superoxide radicals are known to inactivate [4Fe-4S] cluster-containing enzymes by oxidizing one iron and releasing it from the cluster (Liochev and Fridovich, 1994; Fridovich, 1995). Free iron can react with hydrogen peroxide to generate toxic hydroxyl radicals ($OH^-$) by Fenton chemistry (Meneghini, 1997). The hydroxyl and superoxide radicals react with cellular components, resulting in oxidation of proteins and nucleic acids as well as lipid peroxidation. These effects can lead to inactivation of enzymes, disruption of membranes, mutations, and ultimately cell death (Halliwell and Gutteridge, 1990, 1999). To reduce the harmful effects of superoxide radicals, cells express detoxifying enzymes. Superoxide dismutase (SOD) is an antioxidant enzyme involved in the elimination of superoxide anions; it catalyzes the reaction: $O2^- + O2^- + 2H^+ \rightarrow H_2O_2 + O_2$.

Normally, $H_2O_2$ is still toxic to the cell; therefore, another enzyme, catalase, converts it to water (Martchenko et al., 2004). So, this describes why the lung SOD activity significantly increased in the infected untreated mice and returned toward normality in the treated mice groups of the present study. This increase of SOD activity may be a response of the antioxidant system to high superoxide radicals produced via infection.

Catalase plays a key role as an antioxidant, protecting aerobic organisms from the toxic effects of hydrogen peroxide (Wysong et al., 1998). This shows the primary importance of oxidative fungicidal mechanisms by human neutrophils (polymorphonuclear leukocytes [PMNs]) and monocytes. These mechanisms largely depend on the ability of PMNs to synthesize potent oxidants primarily derived from hydrogen peroxide, including $H_2O_2$ itself, as well as hydroxyl radical, hypochlorous acid, and chloramines (Diamond et al., 1980). Thus, treatment with CE, AMB and (CE + AMB) has a significant decrease in the lung catalase level as compared to the infected group. This crucial role of oxidant-mediated fungicidal effects dictates a need to define fungal antioxidant defenses because exogenous antioxidants, including catalase, impair killing of *C. albicans* hyphae by PMNs (Diamond et al., 1980; Wagner et al., 1986) and the production of $H_2O_2$ by PMNs correlates directly with fungicidal catalase activity presumed to be an important antioxidant defense in *C. albicans*. Therefore, the role of catalase in resistance of the fungus to leukocyte-mediated killing was investigated. Since-$H_2O_2$ is highly diffusible across cell walls, catalases provide essential intracellular antioxidant activity for many organisms, including *Saccharomyces cerevisiae* and other fungi (Cohen et al., 1988; Ueda et al., 1990; Kawasaki et al., 1997). Again, the present data confirmed the result of Qiao et al. (2011).

One of the other cytokines involved in the pathogenesis of Candidiasis is the endogenous nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS). NO is primarily produced in the lungs by the epithelial cells in the airways, endothelial cells of vessels and neurons (Dweik, 2001). Once produced, it freely diffuses and enters target cells where it activates guanylate cyclase to produce cyclic guanosine monophosphate, which promotes smooth muscle relaxation (Ozkan and Dweik, 2001). NO plays a central role in regulating airway blood flow in lungs. The role of altered NO homeostasis in asthma has been extensively studied. NO strongly promotes chemotaxis of inflammatory cells in the lung. In asthmatics, NO production is greatly enhanced due to the induction of iNOS by pro-inflammatory cytokines leading to elevated levels of exhaled NO (Pendharkar and Mehta, 2008). In Candidiasis, the production of NO is greatly increased due to the induction of iNOS by pro-inflammatory cytokines leading to elevated levels of exhaled NO (Karaman et al., 2011). NO is an antimicrobial factor generated by NO synthase in activated macrophage and plays a role in the killing of bacteria, protozoa and fungi (Alspaugh and Granger, 1991; Chan et al., 1992). In our study, we could investigate the increased lung NO levels in the infected neutropenic mice and the recovery effect of CE and their combination with AMB by decreasing NO levels. It may be hypothesized that alterations of the NO levels may lie behind the antiasthmatic effect of chitosan (Chung et al., 2012).

In conclusion, CE has a significant anticandidal activity *in vitro* and *in vivo*. The combination of both CE + AMB can be used to dampen the toxic effect of AMB. Such combinations were expected to be synergistic because amphotericin B facilitated the entry of CE into the fungal cell as manifested by the results of both the *in vitro* and *in vivo*.

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