Original Article

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.apjtb.org

doi: 10.4103/2221-1691.345516

Impact Factor: 1.55

Therapeutic potential of *Calotropis gigantea* extract against invasive pulmonary aspergillosis: *In vitro* and *in vivo* study

Enas M. Ali[1,2]✉, Manal A. Alfwuaires[1], Gehan M. Badr[1,3]

[1]*Department of Biological Sciences, Faculty of Science, King Faisal University, P.O. Box 380, Al-Ahsa 31982, Saudi Arabia*

[2]*Department of Botany and Microbiology, Faculty of Science, Cairo University, Cairo 12613, Egypt*

[3]*Department of Zoology, Faculty of Science, Ain Shams University, Cairo, 11566, Egypt*

ABSTRACT

Objective: To characterize the antifungal activity of methanolic leaf extract of *Calotropis gigantea* alone or in combination with amphotericin B against invasive pulmonary aspergillosis in mice.

Methods: GC/MS was used for analysis of active constituents of *Calotropis gigantea* extract. Spore germination assay and broth micro-dilution method were used to determine antifungal potential of *Calotropis gigantea*/amphotericin B against *Aspergillus fumigatus*. Neutropenic mice were randomly assigned into 5 groups: group 1 was neutropenic (control); group 2 was infected with *Aspergillus fumigatus*; group 3 was infected with *Aspergillus fumigatus*, and treated with *Calotropis gigantea* extract; group 4 was infected with *Aspergillus fumigatus* and treated with amphotericin B; group 5 was infected with *Aspergillus fumigatus* and treated with both *Calotropis gigantea* extract and amphotericin B. Fresh lung tissues were histopathologically examined. Fungal burden and gliotoxin concentration were evaluated in lung tissues. Catalase, superoxide dismutase, and malondialdehyde content were determined in lung tissues. Myeloperoxidase, tumor necrosis factor-alpha, interleukin-1, and interleukin-17 were also estimated by the sandwich enzyme-linked immuno-sorbent assay.

Results: *Calotropis gigantea*/amphotericin B had a minimum inhibitory concentration and minimum fungicidal concentration of 80 and 160 μg/mL, respectively, for *Aspergillus fumigatus*. Additionally, *Calotropis gigantea*/amphotericin B significantly reduced lung fungal burden by 72.95% and inhibited production of gliotoxin in lung tissues from 6320 to 1350 μg/g lung. *Calotropis gigantea*/amphotericin B reduced the oxidative stress of the lung *via* elevating the activity of antioxidant enzymes and decreasing the levels of lipid peroxidation. Myeloperoxidase activity and the production of pro-inflammatory cytokines were also significantly reduced. Scanning electron microscopy revealed deteriorations in the hyphae ultrastructure in *Calotropis gigantea*/amphotericin B treated *Aspergillus fumigatus* and leak of cellular components after damage of the cell wall. *In vivo* study revealed the suppression of lung tissue damage in mice of invasive pulmonary aspergillosis, which was improved with *Calotropis gigantea*/amphotericin B compared to the control group.

Conclusions: *Calotropis gigantea*/amphotericin B is a promising treatment to reduce lung fungal burden and to improve the drugs' therapeutic effect against invasive pulmonary aspergillosis.

Significance

Although *Aspergillus fumigatus* causes life-threatening infections, particularly in immunocompromised patients, only a few studies documented the antifungal potential of plant extracts in combination with commercially available antifungal drugs to overcome resistance and to reduce drug toxicities. So far, no studies claim the protective role of plant extracts on oxidative stress and its associated complications such as inflammation and lung tissue damage. This study shows the antifungal, antioxidant, and anti-inflammatory activities of *Calotropis gigantea*/amphotericin B.

✉To whom correspondence may be addressed. E-mail: Eabdelkader@kfu.edu.sa

How to cite this article: Ali EM, Alfwuaires MA, Badr GM. Therapeutic potential of *Calotropis gigantea* extract against invasive pulmonary aspergillosis: *In vitro* and *in vivo* study. Asian Pac J Trop Biomed 2022; 12(6): 243-252.

Article history: Received 27 December 2021; Revision 19 February 2022; Accepted 7 April 2022; Available online 30 May 2022

KEYWORDS: *Aspergillus fumigatus*; Antifungal; Invasive pulmonary aspergillosis; *Calotropis gigantea*; Amphotericin B; Mice

1. Introduction

Aspergillus fumigatus (*A. fumigatus*) is one of the most common fungi in the human environment. It is a saprophytic fungus responsible for invasive pulmonary aspergillosis (IPA), a life-threatening infection, especially among immunocompromised patients[1]. In addition, *A. fumigatus* can produce a diversity of secondary metabolites throughout growth[2]. The most commonly produced metabolite is the epipolythiodioxopiperazine metabolite gliotoxin, which has a wide spectrum of immunosuppressive action[3]. The mortality rate caused by IPA infection is about 80% due to the poor efficiency of the antifungal drugs that are currently available[4].

Few antifungal agents are existing in the market to fight fungal diseases. The target of these drugs is mostly fungal cell walls (echinocandins) and ergosterol through binding to ergosterol or suppressing enzymes responsible for ergosterol synthesis (azoles)[5]. These drugs have numerous side actions, for example, anaphylaxis, anxieties, malaise, nuisance, hepatotoxicity, vomiting, neurotoxicity, and reproductive problems. These drugs are also expensive[6]. Additionally, the incidence of drug resistance in *Aspergillus* is dramatically increasing worldwide[7]. Therefore, there is a crucial need for novel antifungal drugs to increase the treatment efficiency and minimize the adverse effects.

Numerous plant extracts display antibacterial and antifungal properties *in vitro*[8]. In this research, we choose *Calotropis gigantea* (*C. gigantea*) L. (Asclepiadaceae) for treatment of IPA because of its numerous described therapeutic actions, including anti-inflammatory, painkilling, anticonvulsant, anxiolytic, soothing, antidiarrheal, antipyretic, antifungal, and antibacterial activities[9]. Additionally, areal parts of the plant were described to have broad antimicrobial actions[9]. Several compounds have been extracted from *C. gigantea*, for example, cardenolides[10], flavonoids[11], and giganticine[12]. We have recently described the antifungal action of *C. gigantea* extract against growth of *Candida albicans* with a minimum inhibitory concentration (MIC) of 200 μg/mL[13]. In this research, we explored the antifungal action of a combination of *C. gigantea* plant extract with amphotericin B (AMB) (CG/AMB) against *A. fumigatus* for treatment of IPA.

2. Materials and methods

2.1. Plant material

Leaves of *C. gigantea* were collected from shudqum (Al Hassa-El Dammam Road), Eastern Providence, Saudi Arabia. The plant was classified at the Cairo University herbarium. Herbarium samples (voucher number S6E8) were kept at the Department of Botany and Microbiology, Cairo University[13].

2.2. Reagents and drugs

AMB was procured from North China Pharmaceutical Huasheng Co. Ltd. (Hebei, China). Methyl alcohol (purity 99.5%), chloroform (purity 99.5%), and dimethyl sulfoxide (purity 99.5%) were purchased from Samchun Pure Chemical (Pyeongtaek-si, Republic of Korea). MTT were obtained from Sigma-Aldrich (Saint Louis, MO, USA). All other chemicals and reagents were of analytical grade. Purified water of Milli-Q quality (Milli-Q Reference, Millipore®, Molshiem, France) was used throughout the study.

2.3. Extract preparation

Collected plant leaves were air-dried and then crushed into a coarse powder using a laboratory grinder. The coarse powder was used for methanolic extract preparation. For the preparation of methanolic extract, 5 g dry plant powder was dissolved in 50 mL of methanol and extracted using soxhlet for 24 h at 50 ℃. The extract will be then filtered and stored at 4 ℃ in airtight bottles for further use.

2.4. GC-MS analysis

GC-MS analysis of the methanolic extract was carried out using Thermo Scientific Triple Quadrupole GC-MS (Trace 1300 GC, Tsq 8000 triple quadrupole MS) equipped with TG 5MS column. Helium was used as the carrier gas at a 1 mL/min, using an injection volume of 1.0 μL. Injector temperature was kept at 250 ℃ and ion source temperature was 230 ℃. The oven temperature was maintained at 50 ℃, isothermal at 280 ℃, Mass Spectra transfer line temperature.

2.5. Preparation of fungal spores and evaluation of spore germination

A strain of *A. fumigatus* was previously isolated by our group from an immunocompromised patient with IPA used in this study[14]. For the germination assay, fresh conidia were suspended in a peptone-yeast extract-glucose medium (10^6 conidia/mL) and incubated at 35 ℃ on a gyratory shaker. At various time intervals, 10 mL aliquots were removed and the numbers of germinated conidia were assessed by hemocytometer counting. Percent germination was calculated and graphed against the time of incubation[15]. Antifungal activity of different antifungal agents can be evaluated by spore germination assay using the slide technique[16].

Percentage spore germination was calculated according to the following formula: Percentage spore germination = Germinated spores (No.) /Total spores (No.)×100.

The effect of *C. gigantea* extract and AMB combination (1:1) was assayed with *A. fumigatus*, according to the checkerboard method. Synergistic, indifferent and antagonistic interactions were defined, respectively, by a fractional inhibitory concentration index of < 0.5, 0.5-4.0 or > 4.0[17].

2.6. Determination of MIC and minimum fungicidal concentration (MFC)

Determination of MIC was carried out according to the broth microdilution method (NCCLS, 2002). CG/AMB combination was diluted in a sterile solution of Tween 80 to final concentrations ranged from 10 to 1 280 μg/mL. At each different CG/AMB concentration, 100 μL of a suspension containing 4×10^5 CFU/mL of *A. fumigatus* were added to RPMI-1640 medium (0.1 mL). The microplates were incubated at 35 ℃ for 72 h. MIC was evaluated as the lowest concentration of CG/AMB that was able to inhibit the visual growth of *A. fumigatus*. The positive control was the medium containing only the conidial suspension. For MFC measurement of CG/AMB, samples were removed from the wells that did not display any visible fungal growth and re-inoculated in plates containing Sabourand Dextrose Agar. These plates were incubated at 25 ℃ for 72 h. The lowest concentration of CG/AMB that was able to inhibit the fungal growth was determined to be the MFC.

2.7. Determination of time killing activity using time–kill analysis assay

The time-kill curves of CG and/or AMB were evaluated using the broth microdilution assay and viable count performed at 0, 4, 8, 12, 16, and 24 h. The colony count of *A. fumigatus* for each plate was determined to obtain time-mortality curves.

2.8. Ultrastructural examination of A. fumigatus by scanning electron microscope (SEM)

The superior lobe of the right lung was cut into 5-mm cubes and fixed with 2.5% glutaraldehyde. The specimens were dehydrated in graded ethanol and finally freeze-dried in *t*-butyl alcohol. The specimens were coated with platinum-palladium and examined under examined under a Hitachi S-3000N SEM with an acceleration voltage of 20 kV[18].

2.9. In vivo experiment design

Thirty male Swiss albino mice [weighing (20±2) g] were obtained from a closed random-bred colony at the animal's house, National Research Center. Animals were housed in polycarbonate boxes with steel-wire tops (not more than 5 animals per cage) and bedded with wood shavings. Ambient temperature was controlled at (22±3) ℃ with a relative humidity of (50±15)% and a 12-h light/dark photoperiod. Food and water were provided *ad libitum*. Mice were treated with single intraperitoneal administration of 150 mg/kg of cyclophosphamide as a model of neutropenic mice[19]. Six neutropenic mice were assigned to the first group (neutropenic, control); the rest 24 neutopenic mice were infected by 5×10^4 CFU/mL of *A. fumigatus* conidia (three days after cyclophosphamide injection) to induce IPA. Then the IPA mice were divided into 4 groups: Group 2 was the IPA group; group 3 was treated with plant extract (50 mg/kg b.wt. single oral dose) (IPA+CG); group 4 was treated with AMB (150 mg/kg) (IPA+AMB) and group 5 was treated with both plant extract and AMB (IPA+CG+AMB). The dosages of AMB and plant extract were determined according to the results of our preliminary experiments (data not shown).

2.9.1. Histopathological study

Fresh lung tissues were fixed in 10% buffered formalin, dehydrated, and embedded in paraffin. The tissue sections were stained with hematoxylin and eosin dye and examined using a light microscope (Nikon Corporation, Tokyo, Japan).

2.9.2. Fungal burden quantification

The lungs were dissected out and homogenized with a mechanical grinder. A total of 100 μL of the homogenized sample and its serial dilutions were cultured in triplicate on plates containing Sabouraud-Dextrose Agar supplemented with chloramphenicol (20 mg/L) and gentamicin (10 mg/L). Plates were incubated at room temperature for 48 h. The results were described as the CFU average from 3 mice that were euthanized at different time points.

2.9.3. Assessment of gliotoxin in lung tissue

Lung sample macerated in a plastic bag as much as possible with a mallet then transferred with 5 mL of distilled water to Ten Broek tissue homogenizer. The sample was homogenized and 10 mL 6 mol/L HCl was added to homogenate on a shaker for 30 min. The mixture 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 ℃. A 10 mL syringe was attached to the silica column, then the column was rinsed with 4 mL hexane using vacuum and 8 mL hexane added to the reservoir. A total of 4 mL of chloroform and hexane added in column reservoir. Sample passed through the 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 a stream of

nitrogen at 30 ℃. The sample was quantitatively transferred to a 0.5-dram vial using ether: acetone (95:5, *v/v*) and the solution in the vial was dried. Samples were then analyzed by thin-layer chromatography[20].

2.10. Biochemical assays

Lung homogenates were centrifuged and the supernatants and sera were stored at –80 ℃ until analysis. Catalase (CAT), superoxide dismutase (SOD), and malondialdehyde (MDA) activities were measured according to the manufacturer's protocol (CAT, CAT. No.: MBS9718961; SOD, CAT. No.: MBS034842). MDA level was measured by the thiobarbituric acid test[21]. Myeloperoxidase (MPO), tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and interleukin-17 (IL-17) were estimated by the sandwich Enzyme-Linked Immuno-Sorbent Assay (ELISA) kits [MPO, CAT. No.: MBS700747; TNF-α, CAT. No.: MBS2502004; IL-1, CAT. No.: MBS264984 and IL-17, CAT. No.: MBS764076 (MyBioSource, Inc., San Diego, USA)].

2.11. Statistical analysis

The results were represented as the mean of three independent replicates ± standard deviation. Statistical analysis of data was carried out by ANOVA and Duncan's test at $P<0.05$ using the Statistical Analysis Software package (SAS version 9.4; Cary, NC, USA).

2.12. Ethics approval

Animals were treated as stated by the national and institutional guidelines for the ethical and legal treatment of animals. Housing, handling, and treatment of animals were done in compliance with Egyptian national legislation on the care and use of laboratory animals. *In vivo* IPA experiment was approved by Cairo University, Egypt's Animal Ethics Committee (Approval No. 3006/442).

3. Results

3.1. Identification of bioactive compounds in methanolic extract of C. gigantea leaves

GC-MS analysis identified 24 compounds in the leaf extract of *C. gigantea* using methanol as solvent (Table 1, Figure 1A). The compounds were identified based on the peak area, molecular formula, and molecular weight. The GC-MS chromatogram displayed eleven prominent peaks. Compound cyclohexane was observed at retention time of 3.5 min with peak area 47.22%. It was the major compound present in leaf of *C. gigantea*.

Table 1. GC-MS analysis of methanolic extract of *Calotropis gigantea* (CG) leaves.

No.	Compounds	RT (min)	Peak area (%)
1	1-Octanol-3,7-dimethyl	22.7	5.98
2	2-Methoxy 4-vinyl phenol ethanone	53.2	2.88
3	4-Methyl-2-phenylindole	4.3	5.11
4	5-Nonadecen-1-ol	33.2	7.60
5	9,12,15-Octadecatricenoic acid, methyl ester	52.8	2.33
6	Azulene	11.5	3.11
7	Benalaxy	22.7	5.98
8	Beryllium sulfate tetrahydrate	50.1	1.59
9	Biphenyl	3.4	45.11
10	Butane-2,2-dimethyl	6.6	5.90
11	Campesterol	49.8	2.33
12	Cholest-5-en-3 ol, 24, Propylidene	41.7	3.11
13	*Cis*-vaccenic acid	22.7	5.98
14	Cyclohexane	3.5	47.22
15	Decane	46.9	2.15
16	*D*-Mannose-1-phosphate sodium salt	6.6	5.90
17	Eicosane	46.9	2.15
18	Ethion	53.2	1.55
19	Guanidine nitriate	51.7	2.33
20	Levomenol	49.8	2.89
21	Pentacosane	33.4	7.55
22	Profenofos	22.7	5.98
23	β-Tocopherol	22.7	5.98
24	β-Sitosterol	46.9	2.15

RT= retention time.

Table 2. Antimicrobial activities of CG and/or amphotericin B (AMB) against *Aspergillus fumigatus*.

Concentration (μg/mL)	Spore germination (%)			Effect
	CG	AMB	CG/AMB	
0	100.00 ± 0.50^{Aa}	100.00 ± 0.22^{Aa}	100.00 ± 0.20^{Aa}	-
10	100.00 ± 0.30^{Ac}	92.00 ± 0.19^{Bb}	75.00 ± 0.17^{Ba}	Synergetic
20	96.00 ± 0.11^{Bc}	83.00 ± 0.30^{Cb}	59.00 ± 0.50^{Ca}	Synergetic
40	89.00 ± 0.22^{Bc}	64.00 ± 0.40^{Db}	28.00 ± 0.44^{Da}	Synergetic
80	78.00 ± 0.30^{Cc}	45.00 ± 0.11^{Eb}	15.00 ± 0.12^{Ea}	Synergetic
160	65.00 ± 0.16^{Dc}	36.00 ± 0.17^{Fb}	0.00 ± 0.00^{Fa}	Synergetic
320	59.00 ± 0.11^{Eb}	0.00 ± 0.00^{Ga}	0.00 ± 0.00^{Fa}	Synergetic
640	48.00 ± 0.40^{Fb}	0.00 ± 0.00^{Ga}	0.00 ± 0.00^{Fa}	Synergetic
1 280	0.00 ± 0.00^{Ga}	0.00 ± 0.00^{Ga}	0.00 ± 0.00^{Fa}	Indifferent

Data are presented as mean ± SD (n=3 in each group). The same capital letters in the same raw and lowercase letters in the same column mean no significant difference according to ANOVA and Duncan's multiple range tests at $P<0.05$.

3.2. Synergetic antifungal activity of CG/AMB

A. fumigatus was cultivated on Sabaroud dextrose agar showing blue-green pigmentation and the surface containing dense conidiophores (Figure 1B). Microscopic observation of *A. fumigatus* displayed a single series of phialides giving origin to the rounded conidia disposed in long and parallel chains (Figure 1C). During the first four hours of incubation, the conidia displayed a significant increase in volume until becoming fully germinated after 8 h (Figure 1D). We studied the antifungal potential of plant extract, AMB, and CG/AMB using spore germination method. In this assay, the AMB showed a higher antifungal action with a germination percentage of 36%, while plant extract (CG) showed

moderate antifungal potential with a germination percentage of 48% (Table 2). The MIC of AMB and CG were 160 and 640 μg/mL, respectively (Table 2). CG/AMB displayed synergistic antifungal potential against *A. fumigatus*, with a germination percentage of 15% and MIC was 80 μg/mL. The results obtained from the MIC test were confirmed with further MFC tests, where the inhibitory activity of CG/AMB upon *A. fumigatus* occurred at a concentration of 160 μg/mL. In addition, the time-kill curves displayed the fungistatic activity of both AMB and leaf extract at 160 μg/mL and 640 μg/mL, respectively, on the growth of *Aspergillus* cells (Figure 2A). After 4 h of incubation, CG/AMB completely inhibited *A. fumigatus* growth to zero colonies while AMB and CG still had living colonies (Figure 2A).

3.3. CG/AMB reduced colonization of A. fumigatus in lung tissues

A mouse model of IPA was used to determine the therapeutic activity of CG/AMB for the treatment of IPA *in vivo*. To determine *A. fumigatus* colonization in pulmonary structures of infected mice, the mean load of *Aspergillus* cells in the autopsied lung following 24 h of inoculation was recorded (Figure 2B). Lung cultures had a negligible fungal burden in the CG/AMB group. CG, AMB, and CG/AMB-treated animals showed 26.22%, 46.72%, and 72.95% decrease in fungal load, respectively, as compared with the IPA group.

3.4. CG/AMB inhibited gliotoxin production in IPA mice

Gliotoxin was detected in lung samples of all mice (Figure 2C). The results revealed that the levels of gliotoxin in tissues varied significantly along with treatment type. Gliotoxin levels were significantly decreased in mice lungs treated with plant extract and/or AMB during the infection, from a mean of 6320 μg/g lung in the IPA group to a mean of 1350 μg/g in the lung of CG/AMB treated group.

3.5. Ultrastructural analysis of the interaction between CG/AMB and A. fumigatus by SEM

The results of Figure 2D-a & b display distinctive morphology of *A. fumigatus* hypha in control mice. Hypha elongated, infrequently branched in several directions. The thickness of the hypha was nearly unvarying and the exteriors of the hypha were mostly flat; though, some hypha showed a wrinkly shape (b). In contrast, hypha

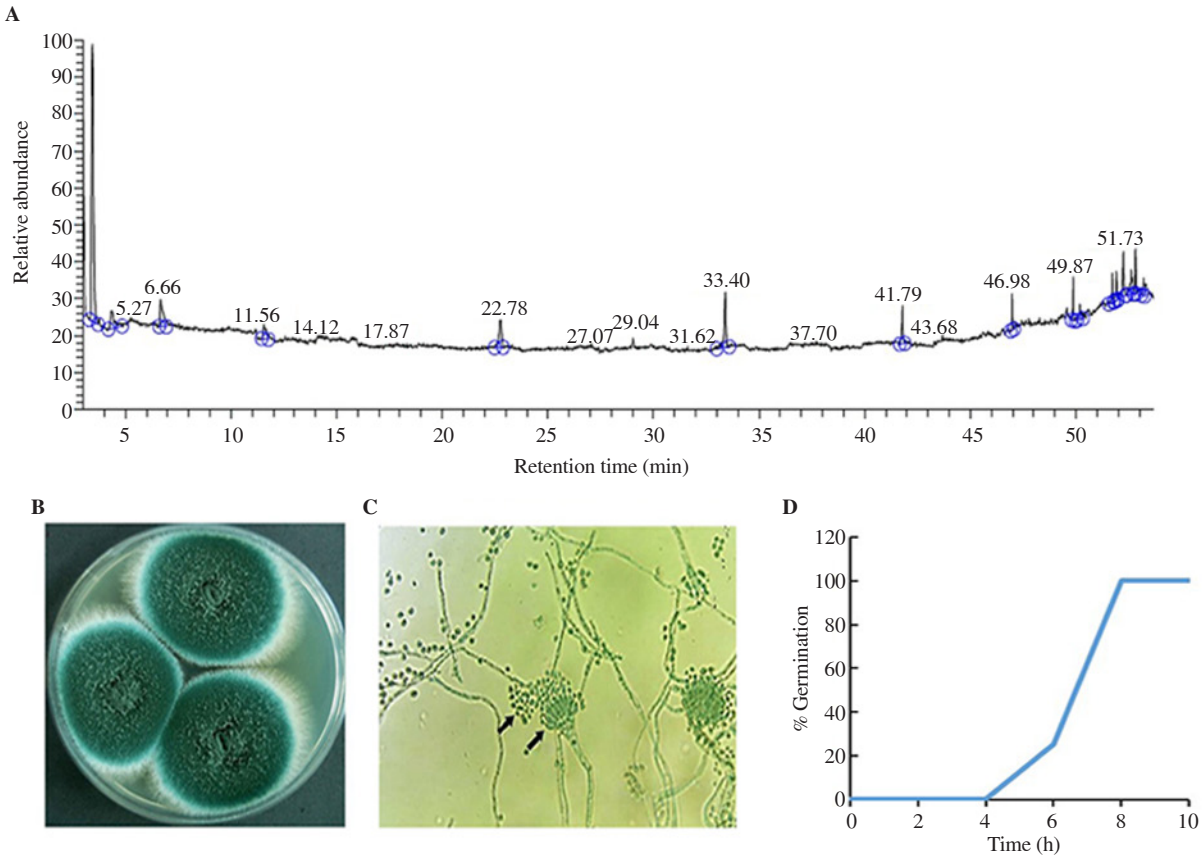

Figure 1. (A) GC-MS chromatogram of CG; (B) *Aspergillus fumigatus* (*A. fumigatus*) on Sabaroud dextrose agar after 4 days of incubation at 30℃ colonies showing blue-green pigmentation and the surface containing dense conidiophores; (C) *A. fumigatus* microscopic features. A single series of phialides (right arrow) can be observed, giving origin to the rounded conidia disposed in long and parallel chains (left arrow) (Lacto phenol cotton blue ×400); (D) Kinetics of conidial germination of *A. fumigatus* in PYG broth at 35℃. Magnification 400×. Scale bar 100 μm.

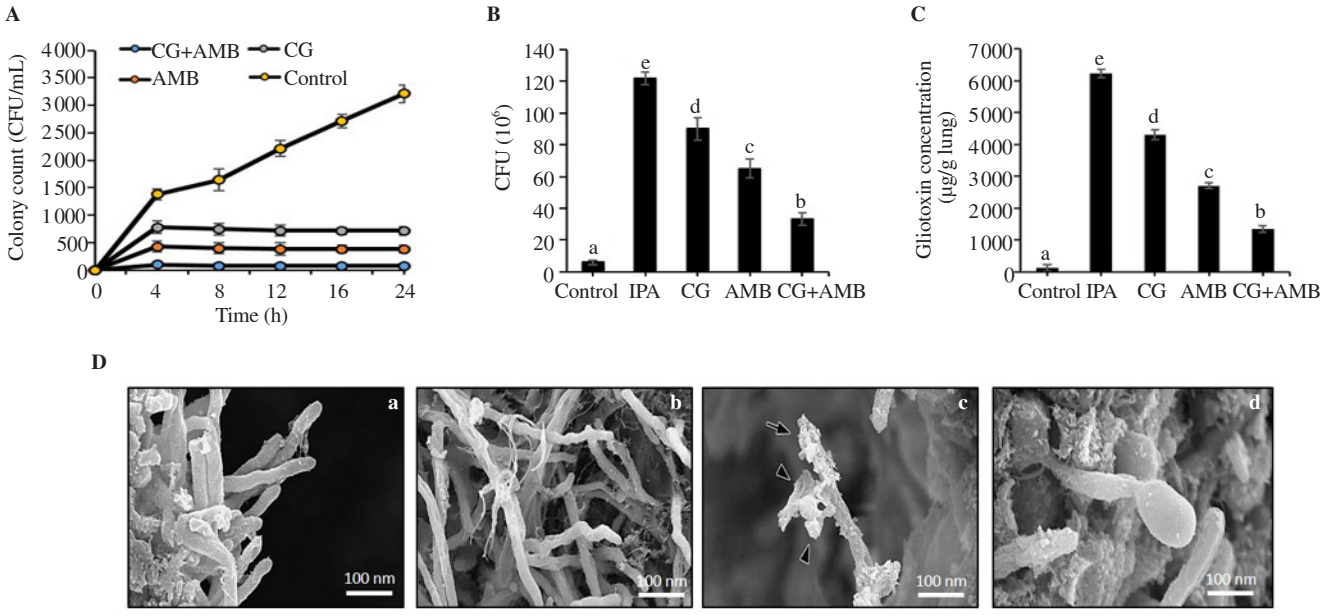

Figure 2. (A) Time kill curves of *A. fumigatus* after treatment with AMB, CG, and CG/AMB; (B) Effect of CG and/or AMB on fungal load in lung homogenate; (C) Effect of CG and/or AMB on lung gliotoxin concentration. IPA: invasive pulmonary aspergillosis. Data are expressed as means ± SD. Bars with different letters show statistically significant differences between the groups ($P<0.05$); (D) SEM of *A. fumigatus* treated with saline (a and b) or CG/AMB (c, d). Scale ba: 100 nm. (a,b) Hyphae are elongated and infrequently branched. The surfaces of the hyphae are smooth. (c) An atrophied hyphal tip is displayed separately. The branched hypha (arrowheads) has been destroyed beyond recognition. The hyphal surface is rough. Clear bumps seen at the tip (arrow) cannot be specified and are either abnormally shaped cell walls or attachments of some unknown material. (d) Typical swollen cell visible at the hyphal tip. The neck of the swollen cell has become thin and the hyphal surface is smooth. Magnification 100×. Scale bar: 100 nm.

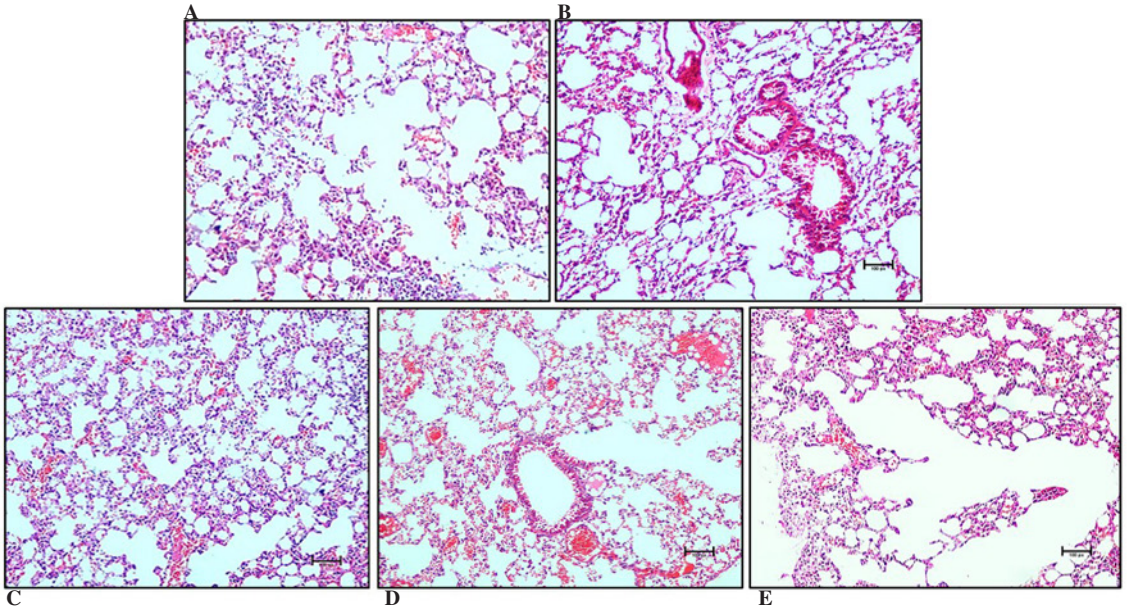

Figure 3. Histopathological light micrographs. Representative micrographs of lungs stained with hematoxylin and eosin. (A) The control group where alveolar space and bronchioles were normal. (B) The IPA mice group showed an increased number of inflammatory cells in alveolar septum and around bronchi and alveolar septum was widened and lung tissue damage also appeared. (C) The IPA/CG group showed histological improvement somehow when being treated with the extract, some inflammatory cells still appeared and the tissue damage had been repaired to some extent. (D) The IPA/AMB group showed inflammatory cells and the tissue injury was still clear. (E) The IPA+CG+AMB group showed obvious lung tissue repair nearest to control. Scale bar= 100 μm. Magnification 40×.

in CG/AMB treated mice were often branched at different sites of the trunk hypha and the branched hypha was dumpy (Figure 2D-c & d). The surfaces of the hyphal tips were rough and displayed a fibril-like shape. The hypha was severely deformed where they looked to have atrophied, and some other structures were not identified (Figure 2D-c). An enlarged hyphal tip was also detected (Figure 2D-d).

3.6. Histopathological examination result

Histopathological examination of lungs harvested from mice showed that IPA mice had several large abscesses of *A. fumigatus* hyphae, the number of inflammatory cells increased in alveolar septum, and the lung tissue damage appeared (Figure 3B). In contrast, mice treated with CG had fewer inflammatory cells and the tissue damage was repaired (Figure 3C). Interestingly, mice treated with CG/AMB displayed no fungal abscesses and regular tissue architecture (Figure 3E). These results confirmed the efficacy of CG/AMB in this IPA murine model.

3.7. CG/AMB reduced the oxidative stress in IPA mice

In the IPA group, the antioxidant enzyme activities of CAT (Figure 4A), and SOD (Figure 4B) were significantly reduced in the lung, while MDA level, an indicator of non-enzymatic lipid peroxidation (Figure 4C) was significantly increased compared to the control. CAT and SOD activities showed significant increases in the IPA+CG, IPA+AMB and IPA+CG+AMB compared to the IPA group. SOD showed no significant change between the IPA+CG and IPA+AMB groups. Lung MDA levels in the IPA+AMB, IPA +CG, and IPA+CG+AMB groups were significantly (P<0.05) decreased as compared to the IPA group. Additionally, there was also a significant difference in MDA level between the IPA+CG and IPA+AMB. The results showed that the synergetic treatment with extract and drug (IPA+CG+AMB) showed significant increases in SOD and CAT and a decrease in MDA compared to all treatments.

3.8. CG/AMB relieved inflammation in IPA mice

The pro-inflammatory cytokines such as MPO activity (Figure 4D), TNF-α (Figure 4E), IL-1 (Figure 4F), and IL-17 (Figure 4G) in the lung tissue were significantly increased in the IPA group compared to the control group. The levels of these inflammatory mediators were modulated in the group IPA+CG, IPA+AMB, and IPA+CG+AMB when compared to the IPA group. Moreover, the group IPA+CG+AMB showed significant alleviation compared to other treatments.

4. Discussion

A. fumigatus is the major cause of IPA in immunocompromised patients. In this research, we investigated the antifungal activity of *C. gigantea* against *A. fumigatus* in the IPA animal model[22,23].

A total of 24 compounds were identified from the methanolic extract of *C. gigantea* leaves with cyclohexane and biphenyl as major compounds. Our results are in agreement with Sharma *et al.*[24], who analyzed the active constituents found in *C. gigantea*. They identified 46 compounds, 22 of which were from latex and 24 from leaves of the sample with cyclohexene as the main compound present in the leaf. Similarly, Pattnaik *et al.*[25] identified palmitic

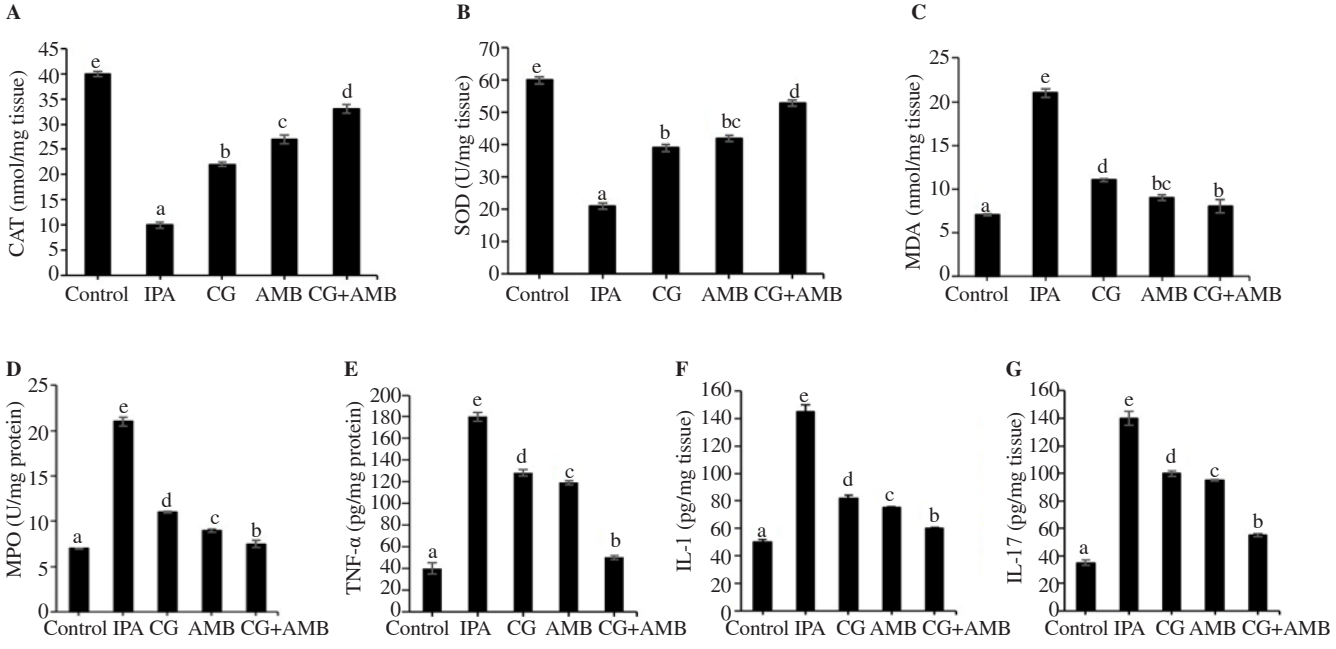

Figure 4. Antioxidant and inflammatory effect of CG and/or AMB in IPA mice. (A): Catalase (CAT), (B): superoxide dismutase (SOD), (C): malondialdehyde (MDA); (D): myeloperoxidase (MPO); (E): tumor necrosis factor-alpha (TNF-α); (F): interleukin (IL)-1; (G): IL-17. Data are expressed as means ± SD, n=6 mice/group. Bars with different letters show statistically significant differences among the groups (P<0.05).

acid, diterpene, triterpene, and linoleic acid as the major active constituents in leaf extract of *C. gigantea*.

Our data determined the MIC of *C. gigantea* to be 640 μg/mL with a moderate antifungal activity as compared to AMB. This might be attributed to the presence of cardiac glycosides, saponins, flavonoids, steroids, and terpenoids in the extract[26]. In this context, we have recently reported the antifungal activity of *C. gigantea* extract against *Candida albicans* with a MIC of 100 μg/mL[13]. Several studies investigated the anti-*Aspergillus* activity of *C. gigantea*, for example, acetone extracts of *C. gigantea* displayed inhibitory action against *Aspergillus* with inhibition zone diameter (IZD) of 7.1 mm at a concentration of 10% *v/v*[27]. Similarly, Pattnaik *et al.* showed that the methanolic extract of *C. gigantea* displayed a higher inhibition with IZD of 15 mm against *Aspergillus niger* with a MIC of 7.5 mg/mL[25]. Ethyl acetate extract of *C. gigantea* resulted in IZD between 7 to 15 mm against *Aspergillus flavus* and *A. fumigatus*, respectively[28]. Therefore, our results identified *C. gigantea* extract as a novel alternative source of biological components that could be used in treatment of IPA.

A promising approach to reduce the toxicity of AMB is to decrease its dose by combination therapy with other antifungals, showing synergistic interactions. In this study, we examined the efficiency of the antifungal activity of the CG/AMB to provide a different approach for the effective treatment of IPA. AMB is an ergosterol-binding polyene that disrupts the fungal membrane and is considered the standard therapy for IPA[29]. *A. fumigatus* is well susceptible to AMB therapy, but higher doses must be applied which might result in severe side effects[30]. Previously, the antifungal activity of different medicinal plants against *Aspergillus* was improved by a combination with commercially available antifungal drugs such as fluconazole or AMB[31]. In this context, Sim and Shin[32] reported that the combination of AMB with the essential oil of *Ligusticum chuanxiong* exhibited a synergistic effect against *Aspergillus*, leading to fractional inhibiting concentration indices between 0.12 to 2. Our results confirmed that the antifungal activity of CG plant extract could be enhanced through the combination therapy of plant extract and AMB to provide a pioneering approach for the effective treatment of IPA.

Our data displayed the inhibitory action of CG/AMB on the production of gliotoxin in lung tissues. The production of gliotoxin by *A. fumigatus* during growth in tissues could support the invasion by the fungus of innate immune cells. Similarly, Lewis *et al.*[33] reported that gliotoxin in mice with IPA was decreased with antifungal treatment. Gliotoxin that is involved in the pathogenesis of IPA could be an indicator of infection with *A. fumigatus*[33] and is reported to inhibit the activation of the transcription factor NF-κB[34], neutrophil and macrophage oxidative killing[35]. Higher gliotoxin concentrations could prompt apoptosis in macrophages and lymphocytes *via* a mechanism different from its anti-phagocytic effects[3].

Our data confirmed that the treatment of *A. fumigatus* with CG/AMB resulted in significant alterations in the cell wall when observed by SEM. Short, greatly branched, or swollen hyphae were detected in mice treated with CG/AMB. Additionally, CG/AMB induced severe fungal destruction such as partially distorted trunk hyphae, atrophied hyphae, and the leak of cytoplasmic components after damage of the cell wall, which finally led to fungal cell death. These morphological changes were also reported by other antifungals in rabbit models of IPA[36], indicating that they are mechanism-based distinctive modifications of the glucan synthesis repressors.

IPA causes an imbalance between oxidants and antioxidants leading to oxidative stress in the lung tissue, which may directly damage tissues and induce inflammation[37]. IPA mice treated with *C. gigantea* extract and/or AMB showed elevation in SOD, and CAT while MDA was significantly decreased. Our data are consistent with Rathod *et al.*[38], who suggest that *C. gigantea* extract has antioxidant activity, which might have an advantageous action against pathological changes resulting from reactive oxygen species. Our results also proposed that the combination of *C. gigantea* extract and AMB could alleviate the oxidative stress, increase the antioxidant ability of IPA mice, ameliorate tissue injury resulting from free radicals during IPA progress, and improve the drugs' therapeutic effect.

Pro-inflammatory cytokines, IL-1 and IL-17, were significantly increased in IPA mice as compared to all other groups. The levels of these inflammatory interleukins showed significant reduction in IPA mice treated with either CG or AMB groups, as compared to the IPA group. In addition, the CG/AMB-treated group showed significant improvement compared to all other groups. Some studies reported that invasive fungal growth and cellular damage triggered the inflammatory response in lung tissue of IPA mice; releasing the lung IL-1 and causing the lung tissue injury, which is interceded by the neutrophils[39]. The eosinophils migration in response to infection with *A. fumigatus* in lung tissue generates a high level of IL-17, which is produced during an inflammatory response by innate immune cells[40]. Polymorphonuclear neutrophils are essential in the innate host defense against *A. fumigatus* by killing pathogens and results in excessive release of oxidants and proteases which may be responsible for organ injury as well as fungal sepsis[41]. Accordingly, CG/AMB treatment improves the lung tissue by reducing IL-1 and IL-17 levels in IPA mice which in turn reduces migration and infiltration of neutrophils and eosinophils into the lungs.

Our data displayed the inhibition of TNF-α and MPO activities in the lung tissues in all treated groups compared to the IPA group. MPO initiates the inflammatory response therefore any decrease in level of MPO indicated neutrophil recruitment into the lungs during inflammation[42]. TNF-α is a pro-inflammatory Th1 cytokine that is important for the host response to fungal pathogens and has a major role in amplification of inflammatory response by activating the NF-κB pathway[43]. Additionally, TNF-α has a role in induction

of leukocytes which results in neutrophil recruitment in the lungs. TNF-α may be released from various recruited neutrophils and alveolar macrophages in lungs infected with *A. fumigatus* both *in vitro* and *in vivo*[44].

Our *in vivo* results demonstrated the therapeutic potential of CG/AMB for lung tissue repairing in the IPA mouse model. This effect is mediated through improving the inflammatory cell recruitment in lung tissue and decreasing the concentrations of pro-inflammatory cytokines. Sumathy *et al*.[45], reported lung tissue reparation and recovery from IPA infection in mice treated with *Cassia surattensis* flower extract. Additionally, we previously reported the therapeutic potential of sepia ink extract in treatment of IPA and repairing lung tissue damage *via* reducing oxidative stress[14].

In conclusion, CG/AMB protected the lung from oxidative damage by its antioxidant property. Our results confirmed the greater antifungal action of CG/AMB *in vitro* and *in vivo* over AMB alone in inhibiting *A. fumigatus* growth. In addition, CG/AMB displayed anti-inflammatory activity. Therefore, it could be a better therapeutic agent for several oxidative stress-related pathologies and fungal *Aspergillus* infections. However, additional studies especially in the clinical setting are required to confirm this finding. Furthermore, the purification and characterization of major active compounds from the CG/AMB might provide an enhanced vision into understanding the exact molecular mechanism of this combination.

Conflict of interest statement

The authors declare no conflict of interest.

Funding

This work was supported by the Deanship of Scientific Research, Vice Presidency for Graduate Studies and Scientific Research, King Faisal University, Saudi Arabia [Project No. GRANT93 (170061)].

Authors' contributions

EMA conceived, designed the project, and drafted the manuscript. MAA and GMB performed experiments, data analysis, and revised the article, gave final approval of the version, and agreed to all aspects of the work.

References

[1] Sabino R, Veríssimo C. Novel clinical and laboratorial challenges in aspergillosis. *Microorganisms* 2022; **10**(2): 1-7.

[2] Tomee JF, Kauffman HF. Putative virulence factors of *Aspergillus fumigatus*. Clinnical experimental allergy. *J Allergy Clin Immunol* 2000; **30**(4): 476-484.

[3] Waring P, Eichner RD, Müllbacher A. The chemistry and biology of the immunomodulating agent gliotoxin and related epipolythiodioxopiperazines. *Med Res Rev* 1988; **8**(4): 499-524.

[4] Reichenberger F, Habicht JM, Gratwohl A, Tamm M. Diagnosis and treatment of invasive pulmonary aspergillosis in neutropenic patients. *Eur Clin Respir J* 2002; **19**(4): 743-755.

[5] Lavergne RA, Albassier M, Hardouin JB, Alvarez-Moreno C, Pagniez F, Morio F, et al. Impact of TR(34)/L98H, TR(46)/Y121F/T289A and TR(53) alterations in azole-resistant *Aspergillus fumigatus* on sterol composition and modifications after *in vitro* exposure to itraconazole and voriconazole. *Microorganisms* 2022; **10**(1): 1-13.

[6] Mazu TK, Bricker BA, Flores-Rozas H, Ablordeppey SY. The mechanistic targets of antifungal agents: An overview. *Mini Rev Med Chem* 2016; **16**(7): 555-578.

[7] Nywening AV, Rybak JM, Rogers PD, Fortwendel JR. Mechanisms of triazole resistance in *Aspergillus fumigatus*. *Environ Microbiol* 2020; **22**(12): 4934-4952.

[8] Schoss K, Kočevar Glavač N, Dolenc Koce J, Anžlovar S. Supercritical CO(2) plant extracts show antifungal activities against crop-borne fungi. *Molecules* 2022; **27**(3): 1-15.

[9] Kumar G, Loganathan K, Rao B. Antibacterial activity of aqueous extract of *Calotropis gigantea* leaves - An *in vitro* study. *Int J Pharm Sci Rev Res* 2010; **3**: 141-144.

[10]Lhinhatrakool T, Sutthivaiyakit S. 19-Nor- and 18,20-epoxy-cardenolides from the leaves of *Calotropis gigantea*. *J Nat Prod* 2006; **69**(8): 1249-1251.

[11]Tenpe CR, Upaganlawar AB, Dongre PA, Yeole PG. Screening of methanolic extract of *Calotropis gigantea* leaves for hepatoprotective activity. *Int J Res Pure Appl Microbiol* 2007; **44**: 874-875.

[12]Gupta J, Sanjrani M. Rare chemical constituents from *Calotropis gigantea* roots. *Indian J Pharm Sci* 2000; **136**: 29-32.

[13]Ali EM, Abdallah BM. Effective inhibition of candidiasis using an eco-friendly leaf extract of *Calotropis gigantean*-mediated silver nanoparticles. *Nanomaterials* 2020; **10**(3): 1-16.

[14]Fahmy SR, Ali EM, Ahmed NS. Therapeutic effect of sepia ink extract against invasive pulmonary aspergillosis in mice. *J Basic Appl Zool* 2014; **67**(5): 196-204.

[15]Manavathu EK, Cutright J, Chandrasekar PH. Comparative study of susceptibilities of germinated and ungerminated conidia of *Aspergillus fumigatus* to various antifungal agents. *J Clin Microbiol* 1999; **37**(3): 858-861.

[16]Nair MG, Safir GR, Siqueira JO. Isolation and identification of vesicular-arbuscular mycorrhiza-stimulatory compounds from clover (*Trifolium repens*) roots. *Appl Environ Microbiol* 1991; **57**(2): 434-439.

[17]Petersen PJ, Labthavikul P, Jones CH, Bradford PA. *In vitro* antibacterial activities of tigecycline in combination with other antimicrobial agents determined by chequerboard and time-kill kinetic

analysis. *J Antimicrob Chemother* 2006; **57**(3): 573-576.

[18]Nakai T, Hatano K, Ikeda F, Shibuya K. Electron microscopic findings for micafungin-treated experimental pulmonary aspergillosis in mice. *Med Mycol* 2005; **43**(5): 439-445.

[19]Perito S, Vecchiarelli L, D'Errico P, Serafini S, Sbaraglia G. Pathogenicity of various species of *Candida* in an experimental murine system. *Boll Soc Ital Biol Sper* 1984; **60**(7): 1415-1419.

[20]Richard JL, DeBey MC. Production of gliotoxin during the pathogenic state in turkey poults by *Aspergillus fumigatus* Fresenius. *Mycopathologia* 1995; **129**(2): 111-115.

[21]Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979; **95**(2): 351-358.

[22]Clemons KV, Espiritu M, Parmar R, Stevens DA. Comparative efficacies of conventional amphotericin b, liposomal amphotericin B (AmBisome), caspofungin, micafungin, and voriconazole alone and in combination against experimental murine central nervous system aspergillosis. *Antimicrob Agents Chemother* 2005; **49**(12): 4867-4875.

[23]Johnson MD, MacDougall C, Ostrosky-Zeichner L, Perfect JR, Rex JH. Combination antifungal therapy. *Antimicrob Agents Chemother* 2004; **48**(3): 693-715.

[24]Sharma S, Kumari A, Sharma M. Comparative GC-MS analysis of bioactive compounds in methanolic extract of *Calotropis gigantea* (L) W.T. Aiton leaf and latex. *Int J Pharmacogn Phytochem Res* 2016; **8**: 1823-1827.

[25]Pattnaik PK, Kar D, Chhatoi H, Shahbazi S, Ghosh G, Kuanar A. Chemometric profile & antimicrobial activities of leaf extract of *Calotropis procera* and *Calotropis gigantea*. *Nat Prod Res* 2017; **31**(16): 1954-1957.

[26]Kumar JS, Chezhian A, Senthilraja P. Computational selections of terpenes present in the plant *Calotropis gigantea* as mosquito larvicide's by blocking the sterol carrying protein, AeSCP-2. *Bangladesh J Pharmacol* 2012; **7**(1): 1-5.

[27]Viji R, Palanichamy A, Mani P, Velavan. Biological control of *Calotropis gigantea* leaf extracts against pathogenic fungus, infecting *Oryza sativa*. *Int J Res Pure Appl Microbiol* 2013; **3**: 107-112.

[28]Habib MR, Karim MR. Antimicrobial and cytotoxic activity of di-(2-ethylhexyl) phthalate and anhydrosophoradiol-3-acetate isolated from *Calotropis gigantea* (Linn.) Flower. *Mycobiology* 2009; **37**(1): 31-36.

[29]Delhom R, Nelson A, Laux V, Haertlein M, Knecht W, Fragneto G, et al. The antifungal mechanism of amphotericin B elucidated in ergosterol and cholesterol-containing membranes using neutron reflectometry. *Nanomaterials* 2020; **10**(12): 1-17.

[30]Rudramurthy SM, Paul RA, Chakrabarti A, Mouton JW, Meis JF. Invasive aspergillosis by *Aspergillus flavus*: Epidemiology, diagnosis, antifungal resistance, and management. *J Fungi* 2019; **5**(3): 1-23.

[31]Ullmann AJ, Aguado JM, Arikan-Akdagli S, Denning DW, Groll AH, Lagrou K, et al. Diagnosis and management of *Aspergillus* diseases. executive summary of the 2017 ESCMID-ECMM-ERS guideline. *Clin Microbiol Infect* 2018; **24**(Suppl 1): e1-e38.

[32]Sim Y, Shin S. Anti-*Aspergillus* activities of the *Ligusticum chuanxiong* essential oil alone and in combination with antibiotics. *Nat Prod Sci* 2010; **16**: 175-179.

[33]Lewis Russell E, Wiederhold Nathan P, Chi J, Han Xiang Y, Komanduri Krishna V, Kontoyiannis Dimitrios P, et al. Detection of gliotoxin in experimental and human aspergillosis. *Infect Immun* 2005; **73**(1): 635-637.

[34]Pahl HL, Krauss B, Schulze-Osthoff K, Decker T, Traenckner EB, Vogt M, et al. The immunosuppressive fungal metabolite gliotoxin specifically inhibits transcription factor NF-kappaB. *J Exp Med* 1996; **183**(4): 1829-1840.

[35]Tsunawaki S, Yoshida LS, Nishida S, Kobayashi T, Shimoyama T. *Fungal metabolite* gliotoxin inhibits assembly of the human respiratory burst NADPH oxidase. *Infect Immun* 2004; **72**(6): 3373-3382.

[36]Petraitis V, Petraitiene R, Groll AH, Roussillon K, Hemmings M, Lyman CA, et al. Comparative antifungal activities and plasma pharmacokinetics of micafungin (FK463) against disseminated candidiasis and invasive pulmonary aspergillosis in persistently neutropenic rabbits. *Antimicrob Agents Chemother* 2002; **46**(6): 1857-1869.

[37]Xu P, Qu JM, Xu JF, Zhang J, Jiang HN, Zhang HJ. NAC is associated with additional alleviation of lung injury induced by invasive pulmonary aspergillosis in a neutropenic model. *Acta Pharmacol Sin* 2009; **30**(7): 980-986.

[38]Rathod NR, Raghuveer I, Chitme HR, Chandra R. Free radical scavenging activity of *Calotropis gigantea* on streptozotocin-induced diabetic rats. *Indian J Pharm Sci* 2009; **71**(6): 615-621.

[39]Teschner D, Cholaszczyńska A, Ries F, Beckert H, Theobald M, Grabbe S, et al. CD11b regulates fungal outgrowth but not neutrophil recruitment in a mouse model of invasive pulmonary aspergillosis. *Front Immunol* 2019; **10**: 123.

[40]Guerra ES, Lee CK, Specht CA, Yadav B, Huang H, Akalin A, et al. Central role of IL-23 and IL-17 producing eosinophils as immunomodulatory effector cells in acute pulmonary aspergillosis and allergic asthma. *PLOS Pathog* 2017; **13**(1): e1006175.

[41]Hohl TM, Feldmesser M. *Aspergillus fumigatus*: Principles of pathogenesis and host defense. *Eukaryot Cell* 2007; **6**(11): 1953-1963.

[42]Tayman C, Cekmez F, Kafa IM, Canpolat FE, Cetinkaya M, Tonbul A, et al. Protective effects of *Nigella sativa* oil in hyperoxia-induced lung injury. *Arch Bronconeumol* 2013; **49**(1): 15-21.

[43]Miyata K, Yotsumoto F, Nam SO, Kuroki M, Miyamoto S. Regulatory mechanisms of the HB-EGF autocrine loop in inflammation, homeostasis, development and cancer. *Anticancer Res* 2012; **32**(6): 2347-2352.

[44]Mehrad B, Strieter RM, Standiford TJ. Role of TNF-alpha in pulmonary host defense in murine invasive aspergillosis. *J Immunol* 1999; **162**(3): 1633-1640.

[45]Sumathy V, Zakaria Z, Jothy SL, Gothai S, Vijayarathna S, Yoga Latha L, et al. *In vitro* and *in vivo* antifungal activity of *Cassia surattensis* flower against *Aspergillus niger*. *Microb Pathog* 2014; **77**: 7-12.