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Allium cepa Inhibits *Aspergillus fumigatus* Growth and Virulence and Suppresses the Expression of *glip* Gene Involved in Fungal Pathogenesis

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ABSTRACT

Backgrounds: *Allium cepa L.* as a traditional medicine is a rich source of beneficial bioactive metabolites. In the present study, the effect of *A. cepa* ethanolic extract (EAC) was studied on *Aspergillus fumigatus* growth, ergosterol synthesis, gliotoxin production, and *gliP* gene expression.

Materials & Methods: The minimum inhibitory concentration (MIC) of EAC (125-4000 µg/mL) was determined against *A. fumigatus* isolates according to Clinical and Laboratory Standards Institute (CLSI) guidelines (M-38). Protease activity, gliotoxin production, cell membrane ergosterol content, ultrastructure, and *gliP* gene expression were evaluated in the fungus exposed to 0.5× MIC concentrations of EAC (1000 µg/mL) and fluconazole (FCZ: 64 µg/mL).

Findings: Ergosterol content was significantly reduced to 0.53 and 0.45 µg/mg in EAC- and FCZ-treated fungal cells, respectively ($p< .001$). The protease activity was significantly inhibited in both EAC- and FCZ-treated groups. The gliotoxin production was inhibited by 51.55 and 68.75% in the treated groups with FCZ and EAC, respectively. The expression of *gliP* in both EAC- and FCZ-treated *A. fumigatus* groups was significantly reduced by 0.40 and 0.53-fold, respectively ($p< .05$).

Conclusion: This study findings revealed that *A. cepa* ethanolic extract (EAC) effectively suppressed the growth and virulence factors of *A. fumigatus*, which could be attributed in part to its bioactive metabolites. Further studies are recommended to isolate and identify these metabolites as potential candidates for the development of antifungal drugs.

Keywords: *Aspergillus fumigatus*, *Allium cepa*, Antifungal activity, Ergosterol, Gliotoxin, *gliP*

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Introduction

Aspergillus fumigatus is the most important etiological agent of invasive aspergillosis (IA), especially in immunocompromised individuals [1]. The fungus could produce a wide array of secretory enzymes and secondary fungal metabolites with genotoxic and cytotoxic activities as potential virulence factors [2]. Gliotoxin as a member of the epipolythiodioxopiperazine (ETP) class of fungal toxins is produced by the non-ribosomal peptide synthetase (NRPS) enzyme encoded by *gliP* in *A. fumigatus* [2-4]. This toxin is able to suppress the host immune system by altering the function of neutrophils and leukotrienes via inhibiting migration, producing superoxide, and inducing apoptosis [5-8]. Different kinds of proteolytic enzymes such as elastase, serine protease, aspartic protease, and metalloproteases are also produced by *A. fumigatus* [9]. These enzymes have been shown to facilitate tissue invasion by the fungus [10].

The emergence of drug resistance to known antifungal drugs necessitates the need to find novel drugs from natural sources to combat fungal diseases [11]. Plant-derived compounds have received much attention in recent years due to their antifungal activities mediated via affecting fungal cell membrane, cell wall, and virulence factors. *Allium cepa* (onion) contains organosulfur compounds, flavonoids especially flavonols, quercetin, steroid saponins, sapogenins, tanins, alkaloids, kaempferol, and many other compounds with anti-inflammatory, anti-carcinogenic, antioxidant, antimicrobial, and antifungal properties [12-17]. Flavonoids and organosulfur compounds are the most important therapeutic phytochemicals which constitute the main parts of bioactive compounds in the *Allium* family (onions and garlic) [18, 19].

Objectives: This study aimed to investigate the effect of ethanolic extract of *A. cepa* L. on *A. fumigatus* growth and some major fungal virulence factors, such as gliotoxin production, protease activity, cell membrane ergosterol content, and *gliP* expression as an essential gene involved in gliotoxin production.

Material and Methods

Fungal strain and culture conditions*: A. fumigatus* clinical isolate PFCC 474 was obtained from Pathogenic Fungi Culture Collection of the Pasteur Institute of Iran and cultured on Sabouraud dextrose agar (SDA) (Merck, Germany) culture medium supplemented with chloramphenicol (0.005%) at 37°C for 48 hrs. Fungal spores were harvested by 3 mL of phosphate-buffered saline (FBS) containing 0.1% tween 80, adjusted to 1-2.5×10^5 CFU/mL, and kept at 4°C until use [20].

Preparation of solvent-based extract of *A. cepa* L.: *A. cepa* L. (golden brown bulb onion harvested in autumn from northwestern Iran) (1000 g) was obtained from Herbarium of Forestry and Rangelands Research Institute (Karaj, Iran), homogenized using a blender, and dried. About 50 g of dried powder was separately mixed in 250 mL of methanol (99%), ethanol (99%), and sterilized distilled water and sonicated at 120 Hz for 1 hr. The mixtures were incubated at room temperature for 48 hrs while stirring at 120 rpm and filtered by Whatman No. 1. The aqueous extract of *A. cepa* (AAC) was dried by a freeze-dryer (Christ, Germany). Methanolic (MAC) and ethanolic (EAC) extracts were concentrated using a rotary evaporator (IKA, Germany) [21]. The yield percentage was calculated using the following formula:

Extract yield (%) =R (dry weight of the extracted plant) /S (raw plant sample) ×100

Screening of the inhibitory effect of solvent-based *A. cepa* L. extract on *A. fumigatus* growth: About 100 µL of

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fungal suspension (1-2.5×10^5 CFU/mL) was cultured on Sabouraud dextrose agar (SDA) culture medium, and AAC, MAC, and EAC extracts at a concentration of 500 µg/mL were inoculated into wells in agar plates. The plates were kept at 32°C for 4 days. The extract exhibiting the highest inhibitory activity against fungal growth was selected and stored at -20°C until used [18].

EAC minimum inhibitory concentration (MIC): EAC with the highest inhibitory effect was selected to determine its minimum inhibitory concentration (MIC). Briefly, 100 µL of fungal spore suspension (1-2.5×10^5 CFU/mL) in RPMI-1640 medium (Sigma-Aldrich, USA) was added to a 96-well U-shaped plate. Afterwards, 100 µL of two-fold serial concentrations of both EAC diluted with RPMI-1640 (125-4000 µg/mL) and fluconazole (FCZ) (0.5-256 µg/mL) as a drug control were separately added to each well and incubated at 32 ºC for 48-72 hours. The MIC was determined as the lowest concentration that could inhibit visible fungal growth, while the minimum fungicidal concentration (MFC) was determined as the lowest concentration resulting in no growth or turbidity compared to the control group. To determine the MFC of EAC, 50 µL of the contents of the wells with no visible fungal growth were cultured on SDA plates and incubated at 32°C for 48-72 hrs. The extract concentration in SDA plates in which no fungal colonies appeared was considered as MFC. Each experiment was repeated three times, and mean values were represented for MICs and MFCs [22].

Assessment of ergosterol content in EAC-treated *A. fumigatus*: Ergosterol content of fungal mycelia exposed to 0.5× MIC concentrations of EAC (1000 µg/mL) and FCZ (64 µg/mL) was determined as previously described and calculated by the following formulas [23].

(A): %Ergosterol+%24(28)dehydroergosterol (DHE)=[($Abs_{281.5}$/290)×F]/weightofpellet

(B): % 24(28) DHE = [(Abs_{230}/518) × F]/weight of pellet

% Ergosterol = A-B

Where F is the dilution factor, and 290 and 518 are the E values for crystalline ergosterol and 24(28) dehydroergosterol, respectively. The absorbance of the samples was read at two wavelengths of 281.5 ($A_{281.5}$) and 230 nm (A_{230}).

Production and activity of the secretory protease: Protease activity of *A. fumigatus* isolates treated with 0.5× MIC concentrations of EAC and FCZ was measured in a protein-based medium [24]. Briefly, 100 µL of fungal spore suspension (1-2.5×10^5 CFU/mL) in Czapek Dox medium (K_2HPO_4: 1 g/L, $MgSO_4 \cdot 7H_2O$: 0.5 g/L, KCl: 0.5 g/L, $FeSO_4 \cdot 7H_2O$: 0.01 g/L, and sucrose: 30 g/L) (HiMedia, India) containing 1% casein (Merck, Germany) was added to 0.5× MIC concentrations of EAC and FCZ and incubated at 32 ºC for 48-72 hours. Fungal cultures were centrifuged at 10,000×g for 15 min, and the supernatants were used for enzyme activity assay. Then 100 µL of each supernatant was mixed with 3 mL of reaction mixture containing 0.5%

Table 1) In vitro antifungal susceptibility of *A. fumigatus* to *A. cepa* ethanolic extract (EAC) and fluconazole (µg/mL)

Antifungal Compound	MIC Range (µg/mL)	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)	MFC (µg/mL)
EAC	125-4000	1000	2000	4000
Fluconazole	0.5-256	64	128	256

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casein in 2.95 mL of Tris-HCl buffer (0.1 M, pH 8.0) and incubated at 40°C for 30 min. The reaction was stopped by adding 3 mL of cold trichloroacetic acid (10%), and the mixture was kept at room temperature for 1 hr. The supernatants were removed by centrifugation at 8,000×g for 5 min, and their absorbance was read at 540 nm using a UV/Vis-1601 PC spectrophotometer (Shimadzu, Japan). The enzyme unit (µg/mL/min) was defined as the amount of enzyme able to oxidize one µmoL of substrate per min under assay conditions [24].

Measurement of gliotoxin production: *A. fumigatus* spores (1-2.5×10^5 CFU/mL) were cultured in 100 mL of Czapek Dox broth (HiMedia, India) supplemented with yeast extract (0.5%) in culture flasks and treated with 0.5× MIC concentrations of EAC and FCZ. The flasks were kept in a shaking incubator at 32°C for 72 hrs. The culture media were centrifuged at 4000 rpm for 15 min and extracted by methanol (1:1 v/v). Methanolic extracts were concentrated using a rotary evaporator (IKA, Germany). The residue was dissolved in acetonitrile/water/acetic acid (99:99:2), and 50 µL the extract was injected into the HPLC column (TS TSK-GEL ODS-80 4.6×150 mm). Methanol/water (70:30) as the mobile phase was pumped at a rate of 1.0 mL/min during 6 min. Standard curves were constructed using gliotoxin (Sigma-Aldrich, USA). The levels of gliotoxin in EAC- and FCZ-treated samples were measured and compared to the standard level [25].

Expression of gliP gene in EAC-treated *A. fumigatus*: *A. fumigatus* spore suspension (1-2.5×10^5 CFU/mL) was cultured in 100 mL of Czapek Dox broth (HiMedia, India) in culture flasks and treated with 0.5× MIC concentrations of EAC and FCZ. The flasks were kept in a shaking incubator at 32 °C for 72 hrs. The mycelial mass was harvested and ground in a porcelain mortar. Total RNA was purified from the homogenized fungal mycelia using RNX-Plus kit (Sinaclone, Iran) and treated with RNase-free DNase (Fermentas, USA). First-strand cDNA synthesis was carried out using the related kit (Fermentas, USA). Real-time PCR reaction was carried out in a final volume of 20 µL consisting of 10 µL of 2× prime Q-Master SYBR mix (Genet Bio, Korea), 1 µL of each primer solution (10 mM) containing *gliP* gene (forward: 5'- AGCACCACAAAGGTCATTCC-3'; reverse: 5'-GTAGAGGCTATGCCGTGAGC-3') and *β-acti* gene (forward: 5'-TGCTCTCGTTATCGACAATGGT-3'; reverse: 5'-CATCGTCACCGGCGAAA-3'), 1 µL of total cDNA sample, and distilled water using an ABI PRISM 7500 thermal cycler (Applied Biosystems) [20, 26]. PCR thermal cycling conditions were as follows: a pre-denaturation step at 94°C for 10 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30s, and extension at 72°C for 30 s. Differences in Ct values between the target (*gliP*) and reference (*β-actin*) genes in treated and non-treated samples were evaluated using the comparative threshold (CT) method ($2^{-\Delta\Delta Ct}$) and analyzed by REST© software (2009, Version 2.0.13).

Statistical analysis: All data were analyzed and compared using ANOVA in GraphPad Prism software Ver. 9.2.0 (San Diego, CA). *P* values lower than 0.05 were considered as statistically significant.

Findings

Screening of the inhibitory effect of solvent-based *A. cepa* L. extract against *A. fumigatus* growth: Screening of the inhibitory effects of aqueous (AAC), methanolic (MAC), and ethanolic (EAC) extracts of *A. cepa* on *A. fumigatus* fungal growth showed that the highest zone of inhibition was related to EAC (2.6±0.1 cm)

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Table 2) Effect of *A. cepa* ethanolic extract (EAC) on protease activity, cell membrane ergosterol content, and gliotoxin production in *A. fumigatus* isolates

Groups	Protease Activity (pmol/min) Mean±SD	Inhibition of Protease Activity (%)	Ergosterol (µg) Mean±SD	Inhibition of Ergosterol (%)	Gliotoxin Production (µM) Mean±SD	Inhibition of Gliotoxin Production (%)
EAC-treated	45.081.06±	64.23	0.4520.067±	56	2.30±0.09	68.75
Fluconazole-treated	60.514.01±	46.21	0.5370.012±	48	3.56±0.03	51.55
Non-treated control	115.87±9.0	0.0	1.0450.010±	0	6.23±0.6	0.0

in comparison with AAC (0.0±0.0 cm) and MAC (2.1±0.1 cm) (Fig. 1).

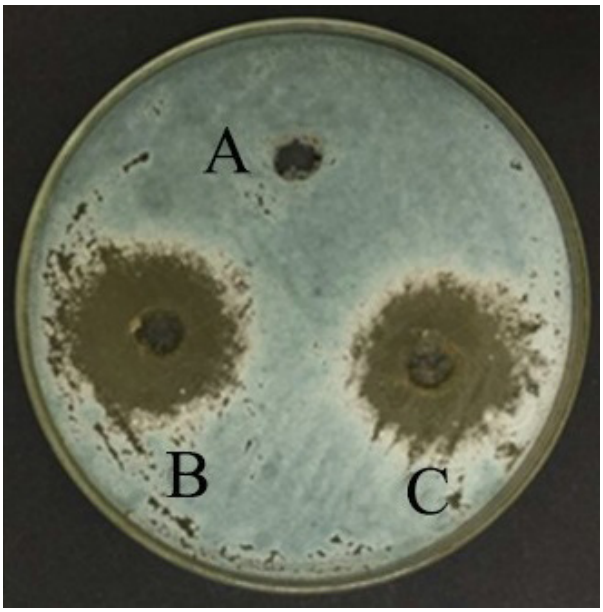

Figure 1) Screening of antifungal effects of aqueous (A), ethanolic (B), and methanolic (C) extracts of *A. cepa* on *A. fumigatus*

Determination of minimum inhibitory concentration: MIC and MFC values of EAC against the standard clinical isolate of *A. fumigatus* were determined and compared with those of FCZ (Table 1). The results indicated that MIC_{90} and MFC values of EAC against *A. fumigatus* growth were 2000 and 4000 µg/mL, respectively. For FCZ, these values were reported to be 128 and 256 µg/mL, respectively.

Evaluation of ergosterol content in EAC-treated *A. fumigatus*: The mean ergosterol production in *A. fumigatus* isolates treated with EAC and FCZ at concentrations of 1000 and 64 µg/mL was 0.452 and 0.537 µg/mg, respectively. While this value in non-treated group was 1.045 µg/mg (Table 2). Therefore, ergosterol production was inhibited by 48 and 56% in the FCZ- and EAC-treated groups, respectively. The analysis of the results was performed by one-way ANOVA, and significant differences were shown between the EAC/FCZ-treated and non-treated groups (p< .001).

Evaluation of protease activity in EAC-treated *A. fumigatus:* Total activity of protease enzyme was reduced in *A. fumigatus* isolates treated with EAC and FCZ at concentrations of 1000 and 64 µg/mL, respectively. The mean protease production was determined to be 45.08, 60.51, and 115.87 pmol/min/mg in the EAC and FCZ-treated and non-treated groups, respectively (Table 2). Therefore, protease production was inhibited by 64.23 and 46.21% in the EAC and FCZ-treated groups, respectively. The analysis of the results showed significant differences between the EAC/FCZ-treated and non-treated groups (ANOVA, p< .001)

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(Fig. 2).

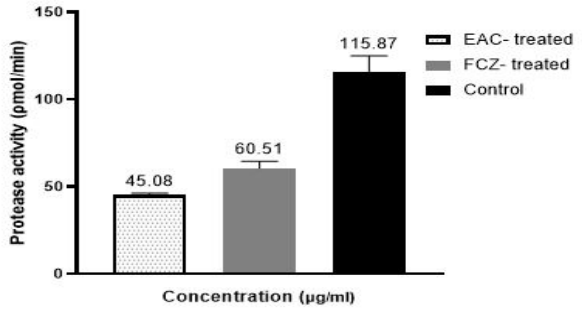

Figure 2) Evaluation of protease activity in *A. fumigatus* isolates treated with EAC and FCZ (MIC_{50} concentration; µg/mL). The results showed significant differences between the treated and non-treated groups (p<.001).

Effect of EAC on gliotoxin production in *A. fumigatus*: Gliotoxin production was affected by EAC and FCZ and reduced in *A. fumigatus* isolates treated with EAC and FCZ at concentrations of 1000 and 64 µg/mL, respectively. The mean gliotoxin production was determined to be 2.30±0.15, 3.56±0.9, and 6.23±0.14 µM in the EAC and FCZ-treated and non-treated groups, respectively (Table 2). The results showed significant differences between the treated and non-treated groups (ANOVA, p< .01) (Fig. 3).

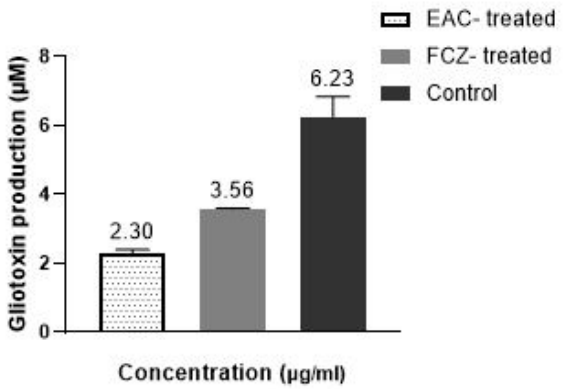

Figure 3) Gliotoxin production in *A. fumigatus* isolates treated with EAC and FCZ (MIC_{50} concentration; µg/mL). The results showed significant differences between the treated and non-treated groups (p< .01)

Expression of gliP gene in *A. fumigatus*: Real-time PCR was performed using specific primers to evaluate the expression of the *gliP* gene, as a mediator of gliotoxin biosynthesis, in *A. fumigatus* isolates treated with 0.5× MIC concentrations of EAC (1000 µg/mL) and FCZ (64 µg/mL). For relative quantitative measurements, the expression of the *gliP* gene was measured according to the $2^{-\Delta\Delta CT}$ formula. As shown in Fig. 4, the *gliP* gene expression in the EAC- and FCZ-treated groups was reduced by around 2 fold at mRNA level, which was significant in comparison with non-treated control group (p< .05). Both EAC and FCZ were able to inhibit *gliP* expression similarly. Thus, there was no significant difference in inhibition of *gliP* expression between the EAC- and FCZ-treated groups (p> .05).

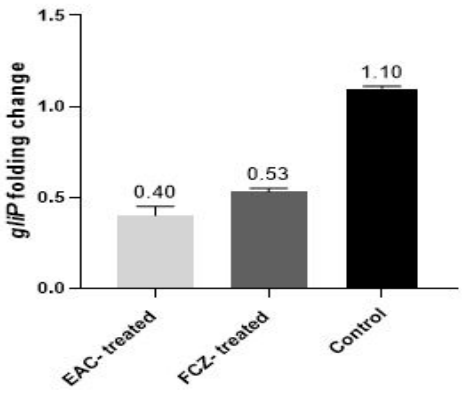

Figure 4) Comparison of *gliP* gene expression at mRNA level in *A. fumigatus* between the control and treated groups with EAC (1000 µg/mL) and fluconazole (64 µg/mL) (p< .05).

Discussion

In the present study, the effect of ethanolic extract of *A. cepa* L. was investigated on *A. fumigatus* growth and some major fungal virulence factors, such as gliotoxin and protease production, cell membrane ergosterol content, and *gliP* gene expression. Although the growth inhibitory activity of *A. cepa* has been proven against various fungi including dermatophytes [14] and saprobes such as *Aspergillus* species [21, 27-30], little is known about this plant mechanism of action at cellular and molecular levels.

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Three ceposides were extracted from white onion in a study and shown to strongly inhibit the growth of some filamentous fungi at 200 ppm concentration [28]. Kocic´-Tanackov et al. (2017) identified 21 compounds in onion essential oil, and the major components were dimethyl-trisulfide (16.64%) and methyl-propyl-trisulfide (14.21%). They showed that onion essential oil caused macro-morphological and micro-morphological changes and delayed or prevented fungal growth at different concentrations (3.5, 7.0, 14.0, and 28.0 µL/100 mL). At a concentration of 28.0 µL/100 mL, the growth of *A. carbonarius*, *A. wentii*, *A. versicolor*, *Penicillium brevicompactum*, *P. glabrum*, *P. chrysogenum*, and *Fusarium* spp. was completely inhibited, and the growth of *A. niger* and *P. aurantiogriseum* was reduced [15]. In another study, organic ethanolic extract of onion (*A. cepa*) (MFC: 275 mg/mL) was shown to inhibit the growth of *A. flavus*, *A. niger*, and *Cladosporium herbarum*, and this inhibitory activity was highly significant against *A. flavus* and *A. niger* ($p< .0032$ and $p< .0041$, respectively) [21]. Significant antifungal activity of onion-based synthesized nanoparticles against various *Aspergillus* spp. was revealed by Balamanikandan et al. (2015) [29]. Antifungal activities of aqueous and alcoholic extracts of *A. cepa* against *Candida albicans*, *A. niger*, and *Trichophyton rubrum* were reported by Ikegbunam et al. (2016) [30]. In another study, ultrastructural cell damage was reported in *T. rubrum* and *T. mentagrophytes* after exposure to fresh *A. cepa* aqueous extract at a concentration of 200 mg/mL. In addition, breakage and rupture in the cell wall, loosening and reducing of cell membrane, excessive vacuolization, deposition of lipid globules, and disintegrated cytoplasm were observed in *A. cepa*-treated *T. rubrum* and *T. mentagrophytes* [14]. The present study findings showed that EAC at a concentration of 1000 µg/mL reduced the growth rate of *A. fumigatus* by 50%, and the fungal growth was completely inhibited at a concentration of 4000 µg/mL.

Ergosterol, as the main sterol in fungal membranes, is essential for the developmental growth of the fungus [31, 32]. Studies have proven that *Thymus pulegioides* essential oil has high levels of carvacrol and thymol, which have antifungal activity against *Candida*, *Aspergillus*, and dermatophytes and reduce ergosterol content [33]. The present study results showed that EAC and FCZ at 0.5× MIC concentrations (1000 and 64 µg/mL, respectively) decreased the cell membrane ergosterol content to 0.45 and 0.53 µg/mg of fungal dry weight, respectively. These results indicate that EAC has a high potential activity to damage *A. fumigatus* cell membrane permeability.

Studies have reported the inhibitory activity of *Carum copticum* and *T. vulgaris* oils against elastase and keratinase activities in *A. fumigatus* and *T. rubrum* [34, 35]. This study results showed that EAC reduced protease activity (38.84 ρmol/min/mg) in treated *A. fumigatus* isolates more than fluconazole (58.14 ρmol/min/mg).

Heidary et al. (2022) showed that *A. cepa* extract at 0.5× MIC concentration (1.1 mg/mL) potentially inhibited *C. albicans* growth and reduced proteinase and phospholipase activity and cell membrane integrity, hydrophobicity, and ergosterol content as the major fungal virulence factors [36]. Bruns et al. (2010) reported an increase in gliotoxin production in *A. fumigatus*, which might be a putative fungal factor for persistence in chronic lung infections [37]. Various studies have shown that cinnamon, clove, oregano, palmarosa, and lemongrass-derived essential oils inhibit the production of mycotoxin and decrease the expression of related genes [20, 23, 38]. In this study, gliotoxin production was reduced to 2.30 and 3.56 µm

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in EAC and FCZ-treated *A. fumigatus* isolates compared with the control (6.23 µM), respectively. *GliP* gene has been proved to be as one of the crucial mediators of gliotoxin biosynthesis in *A. fumigatus*. This study results showed that *gliP* expression was significantly down-regulated by 0.40 and 0.53-fold in *A. fumigatus* isolates exposed to 0.5× MIC concentrations of EAC and FCZ, respectively. The importance of biologically active organosulfur compounds such as sulfide derivatives, thiopropanal-S-oxide, fistulosin, and alicepin in *Allium* species could be attributed to their antifungal activities [15, 19, 28]. Further studies are needed to purify and characterize *A. cepa* active components and their mode of antifungal action at cellular and molecular levels.

Conclusion

The present study showed that ethanolic extract of *A. cepa* (EAC) suppressed *gliP* as a crucial gene involved in gliotoxin biosynthesis in *A. fumigatus* and efficiently inhibited the major virulence factors of the fungus in a dosedependent manner, including the growth and morphogenesis, cell membrane ergosterol content, protease activity, and gliotoxin production. Given the increasing resistance of *A. fumigatus*, as the major cause of life-threatening invasive aspergillosis, to antimicrobial agents and increasing public interest in non-chemical and herbal-based treatments, plant extracts such as *A. cepa* could open up a promising horizon for combating *Aspergillus*-related infections. Further studies are recommended to isolate and identify *A. cepa* bioactive metabolites and their *in vivo* activity in animal experimental models.

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