

## Effects of Capsaicin on the Growth and Virulence Factors of *Aspergillus fumigatus*

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### ABSTRACT

**Introduction:** Emerging drug-resistant fungal pathogens and the limitations of current antifungal therapies necessitate the development of alternative therapeutic strategies. This study aimed to investigate the effects of capsaicin on the growth and virulence factors of *Aspergillus fumigatus* AF293.

**Methods:** The impact of capsaicin on fungal growth, ergosterol content, and gene expression was evaluated at concentrations up to 25 µg/mL, and on biofilm formation at concentrations up to 100 µg/mL. Expression levels of the *PLB1*, *PLB2*, *PLD1*, and *PLD2* genes were quantified using quantitative real-time PCR (qRT-PCR). **Results:** Capsaicin exhibited dose-dependent antifungal activity, inhibiting fungal growth (15.33–89.78%) and reducing ergosterol content (42.35–82.59%) at concentrations of 3.125–25 µg/mL. The half-maximal inhibitory concentration ( $IC_{50}$ ) for fungal growth was estimated to be between 6.25 and 12.5 µg/mL. At the highest concentration tested (100 µg/mL), biofilm formation was significantly suppressed by 88.34%. *PLB1* and *PLB2* gene expression was downregulated in a dose-dependent manner, with maximal reductions of 56% and 63%, respectively, at 25 µg/mL. In addition, the expression of *PLD1* and *PLD2* genes decreased by 31.4% and 48.13%, respectively, at the highest tested concentration.

**Conclusion:** Capsaicin demonstrates potent antifungal activity against *A. fumigatus* AF293 by inhibiting fungal growth, disrupting ergosterol biosynthesis, and reducing key virulence factors, including biofilm formation as well as *PLB* and *PLD* genes expression. These findings suggest that capsaicin is a promising lead compound for antifungal drug development.

### INTRODUCTION

*Aspergillus fumigatus* (*A. fumigatus*) is a ubiquitous saprophytic fungus that thrives in soil and decaying organic materials, producing vast quantities of airborne conidia (asexual spores). Consequently, humans are estimated to inhale hundreds to thousands of conidia daily, resulting in near-constant exposure to this fungus [1].

In nature, *A. fumigatus* primarily reproduces asexually, releasing abundant conidia into the environment. In immunocompetent individuals, these conidia are usually cleared by the immune system, thereby preventing infection. However, in immunocompromised individuals, conidia can germinate and differentiate into hyphae that invade tissues, a process that can lead to life-threatening invasive aspergillosis. The pathogenicity of *A. fumigatus* is mediated by key virulence factors, including biofilm formation, phospholipase B (PLB), and phospholipase D

(PLD). Furthermore, ergosterol biosynthesis is essential for membrane integrity and fungal survival [2–5].

Biofilm formation is a complex process that proceeds through several stages, including attachment, microcolony formation, matrix production, maturation, and dispersal. These structured communities represent a significant clinical challenge because they exhibit marked resistance to both host immune defenses and antifungal treatments [6, 7]. Additionally, ergosterol, the primary sterol of the fungal cell membrane, is essential for maintaining membrane integrity, fluidity, and stability under stress conditions. Consequently, disrupting its synthesis leads to membrane dysfunction or cell lysis [8]. PLB and PLD are membrane-associated enzymes that promote the pathogenicity of *A. fumigatus* by hydrolyzing host cell membranes and modulating host cell signaling,

thereby facilitating tissue damage and fungal invasion [9, 10].

While antifungal drugs are the mainstay of treatment for fungal infections, conventional therapies, including echinocandins, triazoles, and amphotericin B, can cause significant side effects. Additionally, their extensive use has led to the emergence of antifungal resistance in *A. fumigatus*, posing a serious clinical challenge [11-15]. This growing resistance makes the development of novel antifungal agents and therapeutic targets imperative. Therefore, identifying natural compounds with potent antifungal activity, favorable safety profiles, rapid action, and potential for synergy with existing antifungals is a promising strategy for addressing these challenges [16, 17].

Capsaicin is a vanilloid alkaloid found in *Capsicum* spp., where it is concentrated in the placental tissue and seeds. This compound is responsible for the pungency of peppers, a sensation mediated by activation of transient receptor potential vanilloid 1 (TRPV1) receptors. In addition to its analgesic effects, capsaicin possesses various bioactive properties, including antioxidant and antifungal activities [18]. Therefore, this study aimed to investigate the antifungal effects of capsaicin against *A. fumigatus* by evaluating its impact on fungal growth and key virulence factors, including biofilm formation, ergosterol biosynthesis, and phospholipase gene expression.

## MATERIAL AND METHODS

**Fungal isolate and culture conditions.** *A. fumigatus* AF293, obtained from the Pathogenic Fungi Culture Collection (PFCC) at the Pasteur Institute of Iran, was cultured on Sabouraud dextrose agar (SDA) for 7 days at 30°C. Following incubation, conidia were harvested by flooding the agar surface with sterile 0.1% (v/v) Tween 80 solution, followed by gentle scraping of the colony surface.

**Growth inhibition assay.** The harvested conidia were suspended to a final concentration of  $1 \times 10^5$  conidia/mL in 100-mL Erlenmeyer flasks containing 30 mL of Sabouraud dextrose broth (SDB). Capsaicin (Sigma-Aldrich, St. Louis, MO, USA) stock solution (10 mg/mL) was prepared in methanol. The medium was supplemented with capsaicin at final concentrations ranging from 3.125 to 25 µg/mL. Cultures were incubated at 35°C in an orbital shaker at 180 rpm for 72 h. Subsequently, the fungal biomass was harvested by vacuum filtration through pre-weighed filter paper and dried at 80°C to a constant weight. All experiments were performed in triplicate. Growth inhibition was calculated relative to a vehicle control (containing an equivalent volume of methanol without capsaicin), and the IC<sub>50</sub> value was determined by dose-response curve fitting [19].

**Biofilm inhibition assay.** Biofilm formation was initiated by adding *A. fumigatus* AF293 conidia to the

wells of 96-well microtiter plates to a final concentration of  $5 \times 10^6$  conidia/mL in a total volume of 100 µL of RPMI 1640 medium. Plates were incubated under static conditions at 37°C for 24 h with or without capsaicin at concentrations ranging from 3.125 to 100 µg/mL. Wells containing conidia and vehicle (methanol) but no capsaicin served as the biofilm growth control, and wells with RPMI medium alone served as the sterility control. Following incubation, non-adherent material was removed by washing the wells twice with distilled water. Adherent biofilms were then stained with a 0.1% (w/v) crystal violet solution for 15 min at room temperature. Excess dye was subsequently removed by washing the wells three times with distilled water. To quantify biofilm formation, the bound crystal violet was solubilized with 150 µL of 95% (v/v) ethanol for 10 min, and absorbance was measured at 600 nm using a microplate reader [20].

**Ergosterol quantification.** Ergosterol content was quantified from the dried mycelial biomass obtained from the growth inhibition assay as previously described [19] with minor modifications. Briefly, after incubation, the mycelia were harvested by filtration, washed three times with distilled water, and dried to a constant weight. For saponification, a specified amount from each sample was transferred into a glass test tube, followed by the addition of 3 mL of a 25% (w/v) alcoholic potassium hydroxide (KOH) solution and vortex mixing for 1 min. The samples were then incubated in an 85°C water bath for 1 h. After cooling to room temperature, non-saponifiable sterols were extracted by adding 1 mL of distilled water and 3 mL of *n*-hexane, followed by vigorous vortexing for 3 min. The upper hexane phase was carefully collected after phase separation, transferred to a clean test tube, and stored at -20°C overnight to precipitate any residual impurities. Prior to analysis, the sterol extracts were diluted fivefold with absolute ethanol. Absorbance spectra of the extracts were recorded between 200 and 300 nm using a Perkin-Elmer EZ 301 spectrophotometer to quantify ergosterol content. The ergosterol content, expressed as µg per mg of mycelial dry weight, was calculated using the following equations:

$$\% \text{ergosterol} + \% 24(28)\text{-dehydroergosterol (DHE)} = [(A_{281.5}/290) \times F]/\text{mycelial dry weight (mg)}$$

$$\% 24(28)\text{-DHE} = [(A_{230}/518) \times F]/\text{mycelial dry weight (mg)}$$

$$\% \text{ ergosterol} = [\% \text{ergosterol} + \% 24(28)\text{-DHE}] - [\% 24(28)\text{-DHE}]$$

Where F is the ethanol dilution factor; 290 and 518 are the empirically derived extinction coefficients for crystalline ergosterol and 24(28)-DHE, respectively.

**Gene expression analysis.** Conidia ( $1 \times 10^5$  conidia/mL) were cultured in 30 mL of RPMI 1640 medium supplemented with various concentrations of capsaicin (6.25, 12.5, and 25 µg/mL) in 100 mL Erlenmeyer flasks, along with a vehicle-treated control (methanol without capsaicin). Following incubation at

35°C in an orbital shaker at 180 rpm for 72 h, the mycelia were harvested, washed three times with distilled water, immediately snap-frozen in liquid nitrogen, and ground to a fine powder. Total RNA was extracted from the pulverized mycelia using a guanidinium isothiocyanate (GITC)-based reagent. First-strand complementary DNA (cDNA) was synthesized from 1 µg of total RNA using RevertAid M-MuLV reverse transcriptase (Thermo Fisher Scientific) and random hexamer primers according to the manufacturer's protocol.

Quantitative real-time PCR (qRT-PCR) was performed in a 12 µL volume using SYBR Green master mix (Amplicon, Odense, Denmark) and specific primers for

*PLB1*, *PLB2*, *PLD1*, and *PLD2* (Table 1). Reactions were run on a CFX96 Real-Time system (Bio-Rad, Hercules, CA, USA). The thermal cycling protocol consisted of an initial denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 30s and annealing/extension at 60°C for 30s. No-template controls (NTCs; water substituted for cDNA) and no-reverse-transcriptase controls (−RT controls) were included in each run. All reactions were performed in triplicate. Product specificity was confirmed by melt curve analysis, which verified the presence of a single melting peak. The relative fold change in gene expression was calculated using the  $2^{-\Delta\Delta Ct}$  method, with *β-tubulin* serving as the endogenous reference gene.

**Table 1.** Oligonucleotide primer sequences used for qRT-PCR analysis in *A. fumigatus*

Target gene	Direction	Sequence (5'-3')	References
<i>PLB1</i>	Forward	ATACCACTGCACCCGGTGA	[9]
	Reverse	GGGAATTGCAGGAAAGGAA	
<i>PLB2</i>	Forward	CTCGATCCCCTTCTTCAGC	[9]
	Reverse	GCCAGTCGCGTTTGAACTA	
<i>PLD1</i>	Forward	GATATCGCGAGCATTGT	[21]
	Reverse	AAATTCCACTGCTCCAATCG	
<i>PLD2</i>	Forward	TCCAAGGTCAAGGTTTGAG	[21]
	Reverse	CTCCATCCAATATCGCACT	
<i>β-tubulin</i>	Forward	GGAACCTCTCCGGATCTTG	[19]
	Reverse	GAGCCCTTCCGACCTGAT	

**Statistical analysis.** All data are presented as the mean ± standard error of the mean (SEM) and were analyzed using GraphPad Prism version 6 (GraphPad Software, Inc., San Diego, CA, USA). Data from the fungal growth, ergosterol, and biofilm formation assays were analyzed using a one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test to compare each treatment group against the vehicle control. Gene expression data were analyzed by Two-way ANOVA to assess the effects of capsaicin concentration and target gene as factors. Differences were considered statistically significant at  $P < 0.05$ .

## RESULTS

**Capsaicin inhibits the growth and ergosterol biosynthesis of *A. fumigatus*.** Capsaicin inhibited the growth of *A. fumigatus* AF293 in a dose-dependent manner. After 72 h of exposure, growth inhibition ranged from 15.33% at 3.125 µg/mL to 89.78% at 25 µg/mL, with an IC<sub>50</sub> value between 6.25 and 12.5 µg/mL. Furthermore, capsaicin significantly reduced ergosterol content in a dose-dependent manner, with inhibition ranging from 42.35% at 3.125 µg/mL to 82.59% at 25 µg/mL (Table 2).

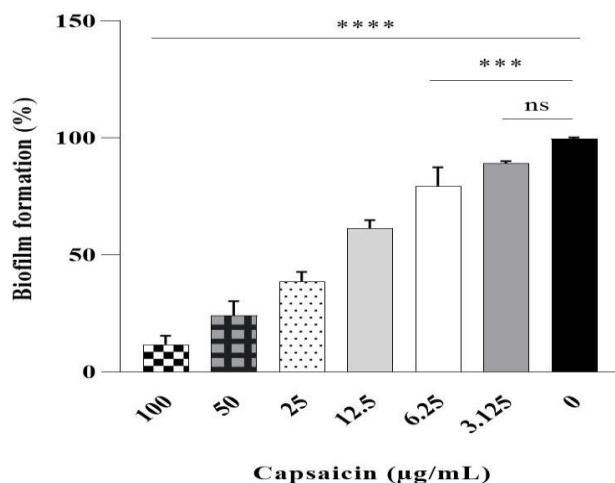
**Table 2.** Effect of capsaicin on the mycelial dry weight and ergosterol content of *A. fumigatus* AF293 after 72 h of incubation.

Capsaicin (µg/mL)	Fungal dry weight (mg/mL culture)	Growth inhibition (%)	Ergosterol content (µg/mg mycelial dry weight)	Ergosterol inhibition (%)
0 (Control)	0.274 ± 0.030	0.00	0.133 ± 0.020	0.00
3.125	0.232 ± 0.050*	15.33	0.076 ± 0.010*	42.35
6.25	0.165 ± 0.010**	39.05	0.075 ± 0.010*	43.25
12.5	0.089 ± 0.002***	67.51	0.043 ± 0.020***	67.74
25	0.028 ± 0.001****	89.78	0.023 ± 0.002****	82.59

**Note:** Data are presented as the mean ± SEM of three independent experiments. Asterisks indicate a statistically significant difference compared to the vehicle control (0 µg/mL) as determined by one-way ANOVA with Dunnett's post-hoc test (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ ).

**Capsaicin inhibits *A. fumigatus* biofilm formation.** Capsaicin significantly inhibited *A. fumigatus* AF293 biofilm formation in a dose-dependent manner (Figure 1). A maximum inhibition of 88.34% was observed at 100

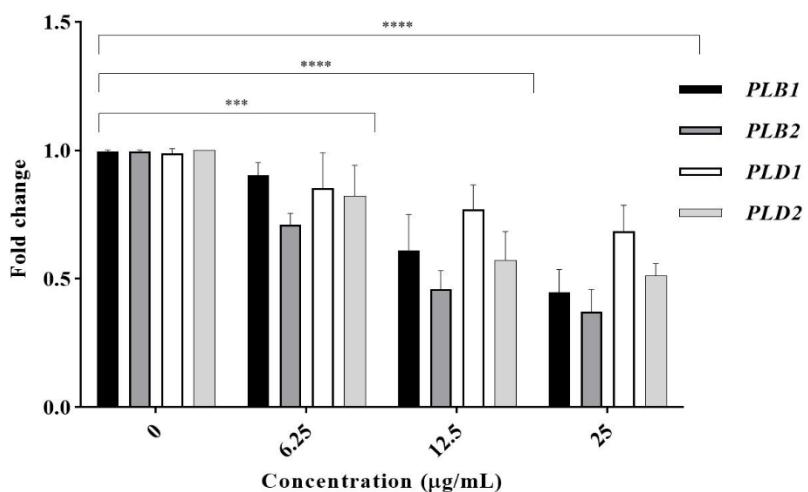
µg/mL compared to the vehicle control ( $P < 0.0001$ ). The IC<sub>50</sub> for biofilm inhibition was between 12.5 and 25 µg/mL.



**Fig. 1.** Inhibitory effect of capsaicin on *A. fumigatus* AF293 biofilm formation. Biofilms were cultured for 24 h with the indicated concentrations of capsaicin, and the resulting biofilm biomass was quantified by crystal violet staining and absorbance measurement at 600 nm. Data are presented as the mean  $\pm$  SEM of three independent experiments and are expressed as a percentage relative to the vehicle control (0  $\mu$ g/mL). Statistical significance was determined by comparison to the vehicle control using one-way ANOVA followed by Dunnett's multiple comparisons test. \*\*\* $P$  < 0.001; \*\*\*\* $P$  < 0.0001; ns, not significant.

**Capsaicin selectively downregulates *PLB* gene expression in *A. fumigatus*.** To investigate the effect of capsaicin on virulence-related gene expression, the transcript levels of four key phospholipase genes (*PLB1*, *PLB2*, *PLD1*, and *PLD2*) were quantified by qRT-PCR. The expression of *PLB1* and *PLB2* was significantly downregulated in a dose-dependent manner. At the

highest concentration tested (25  $\mu$ g/mL), the expression of *PLB1* and *PLB2* was reduced by 56% and 63%, respectively, compared to the vehicle control ( $P$  < 0.05 for the overall treatment effect by two-way ANOVA). In addition, the expression of *PLD1* and *PLD2* genes represented a decrease (31.4% and 48.13%, respectively) at the highest tested concentration (Figure 2).



**Fig. 2.** Effect of capsaicin on the relative expression of phospholipase genes in *A. fumigatus*. Relative mRNA levels of the *PLB1*, *PLB2*, *PLD1*, and *PLD2* genes were quantified by qRT-PCR after culturing *A. fumigatus* for 72 h with the indicated concentrations of capsaicin. Expression levels of the target genes were normalized to the endogenous reference gene,  $\beta$ -tubulin, and were expressed as a fold change relative to the vehicle control (0  $\mu$ g/mL), which was set at 1. Data represent the mean  $\pm$  SEM of three independent experiments. Statistical significance was determined using two-way analysis of variance followed by Tukey's multiple comparisons. A  $P$  value < 0.05 indicates a significant effect of treatment on gene expression at different concentrations. A significant difference is observed between the control group (0  $\mu$ g/mL) and the all treated (6.25, 12.5, 25  $\mu$ g/mL) groups. \*\*\* $P$  < 0.001; \*\*\*\* $P$  < 0.0001; ns, not significant.

## DISCUSSION

The incidence of opportunistic fungal infections, particularly those caused by *A. fumigatus*, has increased significantly in recent decades. This trend is driven largely by the growing population of immunocompromised individuals and the widespread use of immunosuppressive drugs. A major challenge in treating these infections is the emergence of drug resistance in this fungus, which limits therapeutic options and poses a serious threat to clinical outcomes [22–26]. In response to this challenge, the present study investigated the potential of capsaicin, a natural compound derived from *Capsicum* species, as an alternative to conventional antifungal agents.

This study demonstrated that capsaicin exhibits potent, dose-dependent antifungal activity against *A. fumigatus*. This finding is consistent with previous reports on other fungi, including the inhibition of conidial germination in *Colletotrichum capsici* [27]. Notably, the potency observed in this study, with a growth IC<sub>50</sub> value between 6.25 and 12.5 µg/mL, is considerably higher than that reported for other *Aspergillus* species such as *A. flavus* and *A. parasiticus*, which required substantially higher concentrations for inhibition [28]. While the potency of capsaicin may be species-dependent, as shown against *Penicillium expansum* [29], these results underscore capsaicin's strong activity against the primary human pathogen *A. fumigatus*.

In addition to its direct effects on fungal growth, this study showed that capsaicin strongly suppressed *A. fumigatus* biofilm formation, with a maximal reduction of 88.34%. This anti-biofilm activity is particularly significant because biofilms confer marked resistance to both host immune defenses and antifungal agents [30]. Thus, by disrupting biofilm formation, capsaicin targets a key mechanism underlying both virulence and drug resistance, highlighting capsaicin's potential to overcome challenges associated with biofilm-related infections [31].

To elucidate capsaicin's mechanism of antifungal action, this study investigated its effect on ergosterol biosynthesis. The results demonstrated a marked, dose-dependent reduction in the ergosterol content of *A. fumigatus*, with inhibition ranging from 42.35% to 82.59%. This finding is particularly significant because ergosterol is the primary sterol of the fungal cell membrane and a well-validated target for many clinically successful antifungal drugs, including azoles and polyenes. These results are consistent with the findings of Behbehani *et al.* (2023) [32], which also found that capsaicin caused a significant, dose-dependent decrease in ergosterol levels in *Candida albicans*. Collectively, these findings suggest that the disruption of ergosterol biosynthesis is a key mechanism underlying the antifungal activity of capsaicin.

In addition to disrupting membrane integrity via inhibition of ergosterol biosynthesis, this study revealed that capsaicin selectively modulates key virulence-

associated phospholipase gene expression. Specifically, significant dose-dependent downregulation of *PLB1* and *PLB2* expression was observed, with reductions of up to 56% and 63%, respectively, while the expression of *PLD1* and *PLD2* remained unaffected. The greater magnitude of *PLB* gene downregulation (56–63%) compared to *PLD* genes (31–48%) may suggest a degree of preferential effect on *PLB* expression; however, the mechanism underlying this differential response requires further investigation. This finding further demonstrates how capsaicin compromises the pathogenicity of *A. fumigatus*.

In conclusion, this study demonstrates that capsaicin exerts potent antifungal activity against *A. fumigatus* through a multi-targeted mechanism that includes inhibition of growth, disruption of biofilm formation, interference with ergosterol biosynthesis, and selective downregulation of *PLB* virulence genes. While these findings collectively establish capsaicin as a promising lead compound for developing new antifungal drugs that may offer favorable safety and efficacy profiles, it should be acknowledged that this study was limited to a single reference strain and *in vitro* assays. Therefore, future research should validate capsaicin's efficacy against diverse clinical isolates, including resistant strains, and evaluate capsaicin's therapeutic potential in an *in vivo* model of invasive aspergillosis to establish its clinical relevance.

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## CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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## AI DISCLOSURE

The authors declare that no Artificial Intelligence (AI) tools were used in the writing or data analysis for this manuscript.

## DATA AVAILABILITY

The summary data supporting the findings of this study are presented within the article. Raw data, including individual replicate measurements, are available from the corresponding author upon reasonable request.

## AUTHORS' CONTRIBUTIONS

HT: Investigation; Methodology; Writing – original draft; Writing – review & editing. ZJ: Conceptualization;

## ETHICS STATEMENT

This study did not involve human participants or animal subjects. All experimental procedures involving *A. fumigatus* were conducted in a Biosafety Level 2 (BSL-2) facility in accordance with the biosafety guidelines of Tehran Medical Sciences Branch, Islamic Azad University (approval code: IR.IAU.PS.REC.1403.585).

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