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Antifungal potentials of 2,4-dichlorobenzyl on *Aspergillus* species using *Drosophila melanogaster* (UAS-Diptericin) as a Model

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ABSTRACT

Antimicrobial resistance (AMR) is one of the most significant global public health threats, reducing the effectiveness of existing antimicrobial agents and limiting treatment options. Furthermore, many currently available antifungal drugs are associated with adverse side effects. Consequently, the development of new, effective, and safe antimicrobial agents is essential. *Drosophila melanogaster* (fruit fly) has become a valuable model organism in biomedical research because of its genetic tractability, short life cycle, and availability of advanced genetic tools. This study evaluated the antifungal potential of 2,4-dichlorobenzyl against *Aspergillus* species using *D. melanogaster* (UAS-Diptericin) as an in vivo model. The purified 2,4-dichlorobenzyl compound was obtained from the Department of Chemistry, University of Lyon, France. Antifungal susceptibility was assessed using the agar well diffusion method, while the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were determined using the broth dilution method. Flies were infected with different *Aspergillus* species through ingestion and treated with diets containing varying concentrations of the purified compound or itraconazole (100 µg/mL). Survival was monitored for seven days, and mortality was recorded. The purified compound exhibited concentration-dependent antifungal activity with varying efficacy against the tested species. *Aspergillus terreus* was the most susceptible, with inhibition zones ranging from 11.90 ± 0.27 mm to 31.33 ± 2.40 mm, followed by *A. niger* (10.67 ± 0.67–30.30 ± 0.76 mm), while *A. fumigatus* was the least susceptible (4.27 ± 0.18–24.23 ± 0.92 mm). MIC and MFC results supported these findings, with *A. terreus* exhibiting the lowest MIC (20 µg/mL) and MFC (40 µg/mL), followed by *A. niger* (40 and 60 µg/mL, respectively). Survival analysis showed significant differences among treatment groups ($P \leq 0.05$). At 100 µg/mL, the purified compound produced survival rates of 81.39% and 73.91% in flies infected with *A. niger* and *A. fumigatus*, respectively, compared with 86.67% for itraconazole. However, survival of flies infected with *A. terreus* was lower (31.11%) than that achieved with itraconazole (86.67%). These findings suggest that 2,4-dichlorobenzyl possesses promising antifungal activity against *Aspergillus* species and that duplication of the chlorine atom on the N-benzyl group enhances its antifungal efficacy.

Keywords: 2,4-dichlorobenzyl, antifungal activity*, Aspergillus, Drosophila melanogaster*, survival rate.

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INTRODUCTION

Fungal infections caused by *Aspergillus* species pose a significant challenge in clinical practice, particularly among immunocompromised patients. Aspergillosis, an infection caused by *Aspergillus* fungi, can manifest in various forms, ranging from allergic reactions to invasive diseases that may be life-threatening (Hoenigl et al., 2024; Branda et al., 2025). Among the different *Aspergillus* species, *Aspergillus fumigatus*, *Aspergillus niger*, and *Aspergillus terreus* are particularly notable for their pathogenicity and resistance to standard antifungal

treatments (Wang et al., 2020). Traditional antifungal agents, such as azoles and polyenes, have long been the cornerstone of treatment. However, the emergence of drug-resistant strains, coupled with the adverse effects associated with these drugs, has necessitated the exploration of novel antifungal compounds (Kim et al., 2022).

2,4-Dichlorobenzyl alcohol, an organic compound with known antimicrobial properties, has shown promise as a potential antifungal agent. Previous studies have indicated that halogenated benzyl alcohol derivatives can disrupt fungal cell membranes and inhibit fungal growth (He et al., 2023). Despite these promising findings, comprehensive studies are still needed to evaluate the antifungal efficacy of 2,4-dichlorobenzyl alcohol against *Aspergillus* species, particularly using *in vivo* models that can provide valuable insights into its therapeutic potential and safety profile.

Drosophila melanogaster, commonly known as the fruit fly, has emerged as a valuable model organism in biomedical research because of its genetic tractability, short life cycle, and the availability of sophisticated genetic tools (Hanson et al., 2025). The UAS-Diptericin system in *Drosophila* provides a robust model for studying immune responses and infection dynamics *in vivo* (Hanson et al., 2023). Therefore, the use of *Drosophila melanogaster* (UAS-Diptericin) as a model to evaluate the antifungal potential of 2,4-dichlorobenzyl alcohol against *Aspergillus* species offers a novel approach to understanding the efficacy and mechanism of action of this compound in a whole-organism context.

This study aims to investigate the antifungal potential of 2,4-dichlorobenzyl alcohol against *Aspergillus fumigatus*, *Aspergillus niger*, and *Aspergillus terreus* using *Drosophila melanogaster* (UAS-Diptericin) as a model organism. Specifically, the study will examine survival rates, infection dynamics, and the efficacy of the compound compared with standard antifungal treatments, thereby providing insights into its potential as a novel therapeutic agent against aspergillosis.

MATERIALS AND METHODS

Source of purified 2,4-dichlorobenzyl alcohol

Purified 2,4-dichlorobenzyl alcohol was obtained from the Department of Chemistry, University of Lyon, France.

Reconstitution of purified 2,4-dichlorobenzyl alcohol for antifungal activity testing

Purified 2,4-dichlorobenzyl alcohol was reconstituted by dissolving it in 20% Tween 20 according to the modified method described by Elumalai et al. (2009) to obtain concentrations of 100 µg/mL, 80 µg/mL, 60 µg/mL, 40 µg/mL, and 20 µg/mL. The reconstituted compound was stored under refrigerated conditions at 4°C until required for the experiment (Eloff et al., 2008).

Source of microorganisms

Standard isolates of the fungi *Aspergillus niger*, *Aspergillus terreus*, and *Aspergillus fumigatus* were obtained from the National Veterinary Research Institute (NVRI), Vom, Nigeria. The organisms were supplied in Sabouraud broth (SB) suspension. They were subsequently subcultured on Sabouraud Dextrose Agar (SDA) to obtain pure cultures, which were examined microscopically for re-identification.

Antifungal susceptibility testing

Agar well diffusion technique

Antifungal susceptibility testing was performed using the agar well diffusion technique as described by Wuyep et al. (2020). The fungal inoculum was prepared from subcultures by suspending spores from three-day-old fungal cultures in broth, and the turbidity was adjusted to a 0.5 McFarland standard.

The pour plate method was used to inoculate the organisms onto SDA. One millilitre (1 mL) of the spore suspension was dispensed into a sterile Petri dish, after which 20 mL of molten SDA was added. The contents were gently swirled to ensure even distribution and allowed to solidify. Wells measuring approximately 6 mm in diameter were aseptically punched into the agar using a sterile cork borer, with five wells prepared per plate. Each well was filled with 100 µL of the different concentrations of the purified compound.

The plates were allowed to stand for 30 minutes at room temperature to facilitate diffusion of the compound into the agar before incubation at 37°C for 24 hours. The diameters of the zones of inhibition were measured to the nearest millimetre (mm).

Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

The minimum inhibitory concentration (MIC), defined as the lowest concentration of the compound that inhibits visible fungal growth, was determined using the broth dilution method. One millilitre (1 mL) of the standardized fungal suspension was inoculated into a test tube containing 5 mL of sterile broth. Thereafter, 200 µL of the reconstituted compound at the various concentrations was added to the respective test tubes. The tubes were incubated at 37°C for 24–48 hours and examined for fungal growth based on the presence or absence of

turbidity. The test tube containing the lowest concentration of the compound that showed no visible growth upon visual inspection was considered the MFC value (Zacchaeus et al., 2024).

Drosophila melanogaster fly stock selection

Drosophila melanogaster (UAS-Diptericin) flies were obtained from the National Species Stock Centre, Switzerland. The flies were maintained and reared on cornmeal medium at a temperature of 23 ± 1°C and 60% relative humidity under a 12-hour light/dark cycle. All experiments were conducted using the same *D. melanogaster* (UAS-Diptericin) strain.

Preparation of fly food containing purified 2,4-dichlorobenzyl alcohol

Fly food was prepared by incorporating the purified compound into the diet to obtain final concentrations of 100 µg/mL, 80 µg/mL, 60 µg/mL, 40 µg/mL, and 20 µg/mL.

Establishment of virulence and re-identification of the standard isolates

Stock cultures of the fungal isolates were streaked onto formulated Yeast Agar Glucose (YAG; Sigma-Aldrich, USA) plates and incubated at 37°C for 3-7 days. Subcultures were prepared, and colonies were identified based on macroscopic colony morphology, micromorphological characteristics, and their ability to grow, thereby confirming their virulence (Wuyep et al., 2020).

Preparation of fungal inoculum

Spores were harvested from the surface of the agar plates using a sterile inoculating needle and suspended in 3-4 mL of sterile distilled water. The suspension was homogenized, and larger particles were allowed to settle. The resulting homogeneous suspension was adjusted to a 0.5 McFarland standard using a spectrophotometer at 530 nm, corresponding to a final concentration of approximately 0.4-5 × 10^6 CFU/mL for moulds.

Sexing and sorting of the flies

While anaesthetized on ice, male and female flies were differentiated based on their genitalia, body size, abdominal shape and pigmentation, abdominal stripes or bands, and the presence of sex combs (bristles) on the forelegs. Virgin female flies were identified by the presence of a dark mark on the ventral abdomen, representing an embryonic residue that is expelled from the gastrointestinal tract 8-12 hours after eclosion (Lionakis and Kontoyiannis, 2010). Female flies aged 2-4 days were consistently used because they exhibit significantly lower mortality following infection than flies aged 10-15 days.

Establishment of infection in the flies

Flies were collected and starved for 8–10 hours before being introduced into Yeast Agar Glucose (YAG) medium containing three-day-old cultures of the fungal isolates. The flies were allowed to feed for 6 hours, after which they were transferred to their respective treatment groups. Survival rates were monitored throughout the experimental period.

Experimental groups

Group A: Uninfected flies fed a normal diet (vehicle control).
Group B: Infected flies that received no treatment (negative control).
Group C: Flies infected with *Aspergillus terreus* and treated with different concentrations of purified 2,4-dichlorobenzyl alcohol.
Group D: Flies infected with *Aspergillus niger* and treated with different concentrations of purified 2,4-dichlorobenzyl alcohol.
Group E: Flies infected with *Aspergillus fumigatus* and treated with different concentrations of purified 2,4-dichlorobenzyl alcohol.
Group F: Flies infected with each fungal pathogen and treated with the standard antifungal drug, itraconazole (100 µg/mL).

Data analysis

Data obtained were subjected to analysis of variance (ANOVA) and results were presented as means ± standard error of means using Graph Pad Prism version 8 software. The results obtained were tested for significant difference at 5% level.

RESULTS

In vitro antifungal activity of 2,4-dichlorobenzyl alcohol

The antifungal activity of 2,4-dichlorobenzyl alcohol against *Aspergillus fumigatus*, *Aspergillus terreus*, and

Aspergillus niger is presented in Table 1. The compound exhibited concentration-dependent antifungal activity against all three fungal species, with increasing concentrations producing progressively larger zones of inhibition.

Among the test organisms, *Aspergillus terreus* was the most susceptible to the compound, with inhibition zones increasing from 11.90 ± 0.27 mm at 20 µg/mL to 31.33 ± 2.40 mm at 100 µg/mL. *Aspergillus niger* also demonstrated marked susceptibility, with inhibition zones ranging from 10.67 ± 0.67 mm to 30.30 ± 0.76 mm over the same concentration range. In contrast, *Aspergillus fumigatus* exhibited the lowest susceptibility, with inhibition zones increasing from 4.27 ± 0.18 mm at 20 µg/mL to 24.23 ± 0.92 mm at 100 µg/mL.

At the highest concentration tested (100 µg/mL), the antifungal activity of the purified compound against *A. fumigatus* (24.23 ± 0.92 mm) was comparable to that of itraconazole (24.25 ± 0.25 mm). However, the compound produced larger inhibition zones against *A. terreus* (31.33 ± 2.40 mm) and *A. niger* (30.30 ± 0.76 mm) than the standard antifungal drug, which produced inhibition zones of 22.26 ± 0.21 mm and 23.53 ± 0.21 mm, respectively.

Analysis of variance revealed a statistically significant difference ($P < 0.0001$) in the antifungal activity of the purified compound among the three *Aspergillus* species (Table 1).

Table 1. Antifungal activity of purified 2,4-dichlorobenzyl alcohol on *Aspergillus* species.

Fungal pathogens	20 µg/ml	40 µg/ml	60 µg/ml	80 µg/ml	100 µg/ml	Itracanazole (100 µg/ml)
A. fumigatus	4.27± 0.18[b]	7.67± 0.44[b]	12.93± 0.58[b]	14.90± 0.59[b]	24.23± 0.92[b]	24.25± 0.25[a]
A. terreus	11.90± 0.27[a]	15.33± 0.67[a]	22.00±2.00[a]	28.00±1.16[a]	31.33±2.40[a]	22.26±0.21[a]
A. niger	10.67± 0.67[a]	13.00±1.24[a]	22.20±1.17[a]	26.77±0.41[c]	30.30± 0.76[c]	23.53±0.21[a]
L.S.D	2.80					
P-value	<0.0001 ****					

At P≤0.05 there was a significant difference in the antifungalactivity of the purified 2,4-dichlorobenzyl alcohol on *Aspergillus* species. Values are presented as mean ± standard error of means. Ranking was done across the organisms and values with the same super script are not significant.

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of 2,4-dichlorobenzyl alcohol against the three *Aspergillus* species are presented in Table 2.

Aspergillus terreus exhibited the lowest MIC (20 µg/mL) and MFC (40 µg/mL), indicating the greatest susceptibility to the purified compound. *Aspergillus niger* showed intermediate susceptibility, with an MIC of 40 µg/mL and an MFC of 60 µg/mL. *Aspergillus fumigatus* was the least susceptible, requiring the highest concentrations for inhibition and fungicidal activity, with an MIC of 80 µg/mL and an MFC of 100 µg/mL.

Survival of *Drosophila melanogaster* following fungal infection and treatment

Kaplan–Meier survival analysis demonstrated that treatment with 2,4-dichlorobenzyl alcohol significantly improved the survival of *Drosophila melanogaster* (UAS-Diptericin) infected with *Aspergillus* species. Survival was dependent on both fungal species and treatment concentration (Figures 1–3).

For flies infected with *Aspergillus niger*, treatment with 100 µg/mL of the purified compound resulted in a survival rate of 81.39% after seven days, which was comparable to the 86.67% survival observed in flies treated with itraconazole. Survival declined progressively with decreasing concentrations, reaching 57.78%, 35.56%, 8.89%, and 0% at concentrations of 80, 60, 40, and 20 µg/mL, respectively (Figure 1).

Similarly, flies infected with *Aspergillus fumigatus* showed the highest survival rate (73.91%) when treated with 100 µg/mL of the purified compound. Lower concentrations resulted in reduced survival rates of 53.33%, 15.56%, 0%, and 0% at 80, 60, 40, and 20 µg/mL, respectively (Figure 2). Survival following treatment with 100 µg/mL of the purified compound was comparable to that observed with itraconazole (86.67%).

In contrast, flies infected with *Aspergillus terreus* exhibited lower survival across all treatment concentrations (Figure 3). Although survival improved with increasing concentrations of the purified compound, the highest survival rate achieved at 100 µg/mL (31.11%) remained considerably lower than that observed with itraconazole (86.67%).

Log-rank analysis indicated significant differences ($P \leq 0.05$) in the survival distributions of infected flies treated with different concentrations of the purified compound compared with the untreated control groups. Overall, the highest concentration (100 µg/mL) consistently produced the greatest survival benefit across all fungal species, whereas lower concentrations were associated with

Table 2. The Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of purified 2,4-dichlorobenzyl alcohol on *Aspergillus* species.

Fungal pathogens	MIC (µg/ml)	MFC(µg/ml)
A. fumigatus	80	100
A. terreus	20	40
A, niger	40	60

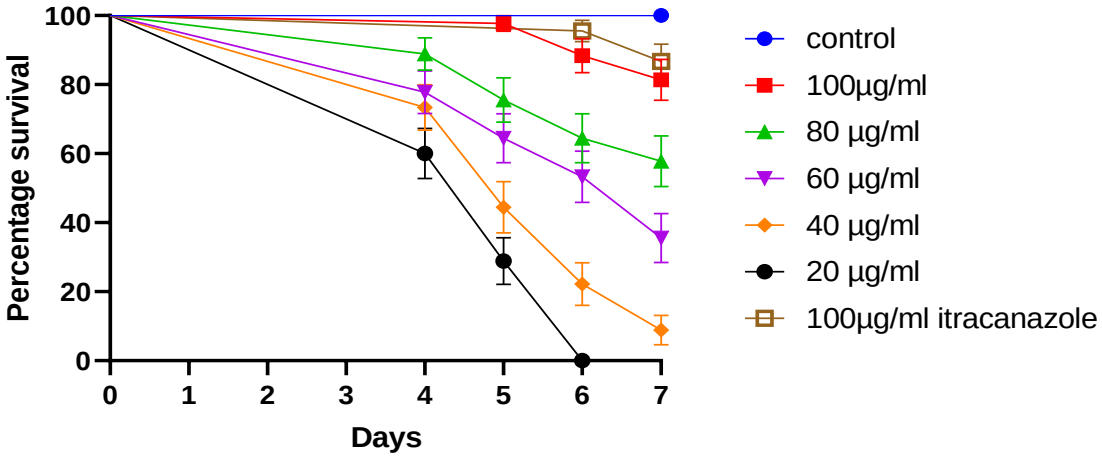

Figure 1. The Kaplan Meiers survival curve for purified 2,4-dichlorobenzyl alcohol on *D. melanogaster* (UAS-Diptericin) infected with *A. niger.*

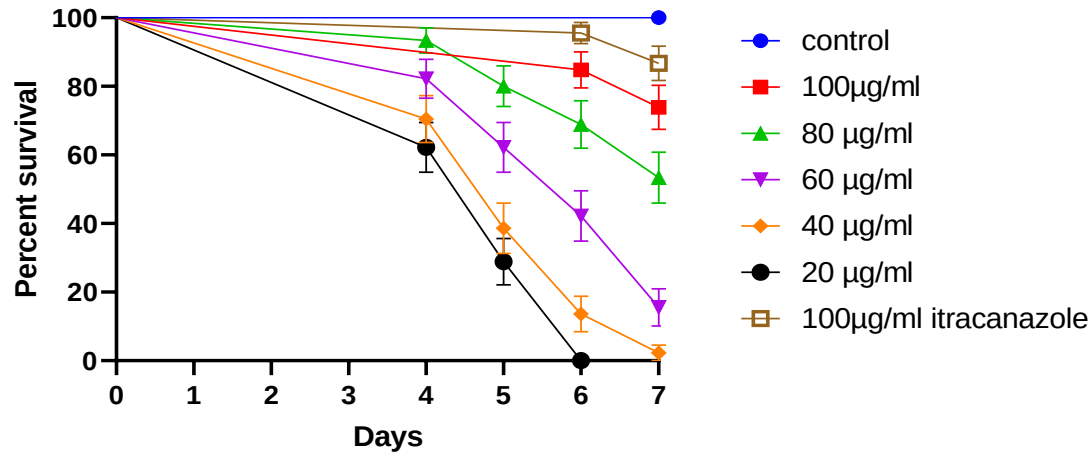

Figure 2. The Kaplan Meiers survival curve for purified 2,4-dichlorobenzyl alcohol on *D. melanogaster* (UAS-Diptericin) infected with *A. fumigatus.*

progressively reduced survival.

DISCUSSION

The *in vitro* antifungal activity of the purified compound (Table 1) demonstrated a significant concentration-dependent effect against the tested *Aspergillus* species. *Aspergillus terreus* was the most susceptible, exhibiting zones of inhibition ranging from 11.90 ± 0.27 mm to 31.33 ± 2.40 mm, followed by *Aspergillus niger*, with inhibition zones ranging from 10.67 ± 0.67 mm to 30.30 ± 0.76 mm. *Aspergillus fumigatus* was the least susceptible, producing inhibition zones ranging from 4.27 ± 0.18 mm to 24.23 ± 0.92 mm when compared with the standard antifungal drug, itraconazole. The observed

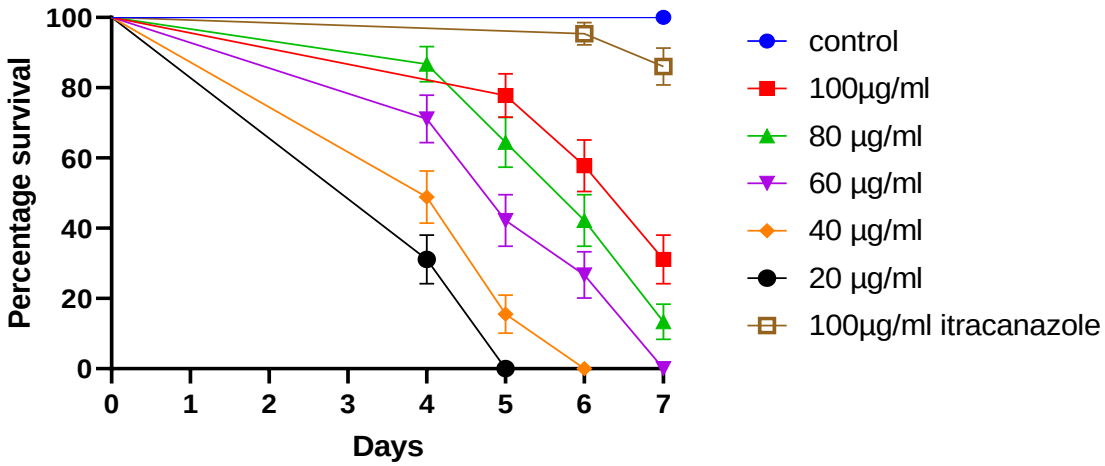

Figure 3. The Kaplan Meiers survival curve for purified 2,4-dichlorobenzyl alcohol on *D. melanogaster* (UAS-Diptericin) infected with *A. terreus.*

concentration-dependent antifungal activity is consistent with the findings of Manohar et al. (2021), who reported that plant-derived compounds exhibited similar concentration-dependent inhibitory effects against *Aspergillus* species. Similarly, Kamatou et al. (2020) highlighted the importance of concentration in their evaluation of essential oils against *A. niger*, demonstrating a proportional increase in antifungal activity with increasing concentrations.

The results of the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) (Table 2) further showed that *A. terreus* was the most susceptible organism, with an MIC of 20 µg/mL and an MFC of 40 µg/mL. This was followed by *A. niger*, which had an MIC of 40 µg/mL and an MFC of 60 µg/mL, while *A. fumigatus* was the most resistant, with an MIC of 80 µg/mL and an MFC of 100 µg/mL. These findings are consistent with those of Sharifzadeh et al. (2019), who reported varying susceptibility among *Aspergillus* species to different antifungal agents. Their study showed that *A. terreus* generally exhibited larger zones of inhibition and lower MIC values than other *Aspergillus* species when exposed to specific antifungal compounds.

Conversely, Singh et al. (2020) reported that *A. fumigatus* was more susceptible than *A. terreus* to certain synthetic antifungal agents, highlighting that the efficacy of antifungal compounds depends largely on their chemical composition. The differential susceptibility observed among the *Aspergillus* species may be attributed to inherent differences in cell wall composition and antifungal resistance mechanisms, as suggested by Muller et al. (2019). Their study demonstrated that *A. fumigatus* possesses robust defence mechanisms, resulting in higher MIC and MFC values than those observed for *A. terreus* and *A. niger*. These resistance patterns are further supported by Arendrup et al. (2020), who reported that *A. fumigatus* isolates frequently harbour mutations in the *Cyp51A* gene, contributing to increased resistance to triazole antifungal agents, including itraconazole.

The log-rank test (Table 1) showed that, at $P \leq 0.05$, there was a significant difference in the survival rates of *Drosophila melanogaster* (UAS-Diptericin) infected with the three fungal species and treated with different concentrations of the purified compound. At the highest concentration (100 µg/mL), the purified compound compared favourably with the standard drug, itraconazole, in flies infected with *A. niger* and *A. fumigatus*, producing survival rates of 81.39% and 73.91%, respectively, compared with 86.67% for itraconazole.

These findings are consistent with those of Yadav et al. (2021), who employed similar survival analysis methods to evaluate antifungal compounds in *D. melanogaster* infected with pathogenic fungi. Likewise, Sharma et al. (2020) reported significant improvements in the survival of *D. melanogaster* infected with *Candida albicans* following treatment with novel antifungal peptides. In contrast, flies infected with *A. terreus* exhibited significantly lower survival rates than those treated with itraconazole. The comparatively low survival rate (31.11%) observed for *A. terreus*-infected flies, relative to the standard drug (86.67%), highlights the difficulty in treating infections caused by this species, as similarly reported by Siedler et al. (2021). Their study suggested that certain fungal strains possess intrinsic resistance to conventional antifungal agents, thereby requiring higher doses or alternative therapeutic approaches.

The favourable performance of the purified compound at 100 µg/mL against *A. niger* and *A. fumigatus* further supports its potential as an effective antifungal agent. This observation agrees with the findings of Kang et al. (2019), who demonstrated that some naturally derived compounds were as effective as conventional antifungal drugs against *Aspergillus* infections in *D. melanogaster* when administered at higher concentrations.

A concentration-dependent increase in fly survival was also observed. Specifically, flies infected with *A. niger* exhibited survival rates of 57.78%, 35.56%, 8.89%, and 0% at concentrations of 80 µg/mL, 60 µg/mL, 40 µg/mL, and 20 µg/mL, respectively. Similarly, flies infected with *A. fumigatus* showed survival rates of 53.33%, 15.56%, 0%, and 0% at 80 µg/mL, 60 µg/mL, 40 µg/mL, and 20 µg/mL, respectively, after seven days of treatment. In contrast, flies infected with *A. terreus* exhibited survival rates below 50% at all tested concentrations.

The concentration-dependent improvement in survival observed in this study agrees with the findings of Ahmed et al. (2019), who reported increased survival of *D. melanogaster* treated with antifungal agents in a dose-dependent manner. Similarly, Zhang et al. (2020) demonstrated that plant-derived antifungal extracts produced concentration-dependent improvements in the survival of *D. melanogaster* infected with *Aspergillus* species. Furthermore, the differences in survival observed among flies infected with *A. niger*, *A. fumigatus*, and *A. terreus* indicate species-specific responses to antifungal treatment. This observation is consistent with the findings of Li et al. (2020), who reported that the efficacy of antifungal compounds varies among different *Aspergillus* species because of differences in their susceptibility profiles.

CONCLUSION

The results of the *in vitro* antifungal assays demonstrated that *Aspergillus terreus* was the most susceptible to the purified compound, exhibiting a clear concentration-dependent response. Furthermore, treatment with the purified compound increased the survival rates of infected *Drosophila melanogaster* in a concentration-dependent manner. These findings suggest that the purified compound possesses promising antifungal activity against *Aspergillus* species. Additionally, the duplication of the chlorine atom on the *N*-benzyl group appears to enhance the antifungal activity of the compound against *Aspergillus* species, indicating its potential for further development as a novel antifungal agent.

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