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In vivo toxicological evaluation of 3-benzylideneindolin-2-one: antifungal activity against clinical isolates of dermatophytes

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Abstract

Background Dermatophytes, the primary causative agents of superficial cutaneous fungal infections in humans, present a significant therapeutic challenge owing to the increasing prevalence of recurrent infections and the emergence of antifungal resistance. To address this critical gap, this study was designed to investigate the antifungal potential of 3-benzylideneindolin-2-one against dermatophytes and assess its in vivo toxicological profile using brine shrimp and zebrafish embryo models.

Methods The antifungal activity of 3-benzylideneindolin-2-one was evaluated against 30 clinical isolates of dermatophyte species, including *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Microsporum gypseum*, *Microsporum canis*, and *Epidermophyton floccosum*, by determining the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) using the broth microdilution method. The fungicidal activity was evaluated using time-kill assays. Toxicological effects were investigated using the brine shrimp lethality assay to determine *Artemia salina* nauplii mortality after 48 h of exposure, and the fish embryo acute toxicity test, which assessed lethality and developmental abnormalities in zebrafish (*Danio rerio*) embryos over a 96 h post-fertilization period.

Results 3-Benzylideneindolin-2-one exhibited consistent fungicidal activity across all dermatophyte species, with MICs ranging from 0.25 to 8 mg/L and MFCs ranging from 1 to 32 mg/L. Time-kill assays revealed a concentration-dependent fungicidal effect on the microconidia. The compound exhibited moderate toxicity to *A. salina* nauplii, with LC50 values of 69.94 mg/L and 52.70 mg/L at 24 and 48 h, respectively, while showing no significant lethality within the MIC range. In zebrafish embryos, concentrations below 7.5 mg/L did not significantly affect lethality, hatchability, or induce morphological abnormalities. However, at a concentration of 10 mg/L, the compound induced mild toxicity in embryos, evidenced by a significant increase in mortality and the presence of morphological anomalies such as yolk-sac and pericardial edema compared to the control group.

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Conclusions The consistent antifungal activity of 3-benzylideneindolin-2-one against clinically significant dermatophyte species, combined with its low toxicity within the therapeutic window, underscores its potential as a promising lead compound for the development of effective therapeutics for dermatophytosis.

Keywords 3-benzylideneindolin-2-one, Oxindole, Antifungals, Dermatophytes, Superficial fungal infections, Zebrafish, Brine shrimp

Background

Superficial fungal infections, affecting approximately 20–25% of the global population, represent a significant dermatological and public health concern due to their persistent nature, frequent recurrence, and rising resistance to available antifungals [1, 2]. These infections are mainly caused by dermatophytes, a group of filamentous fungi or yeasts such as *Candida* species and *Malassezia* species. Among these, dermatophytes are the primary causative agents of superficial mycoses, colonizing keratinized tissues such as skin, hair, and nails by metabolizing keratin as a nutrient source [1, 3]. Dermatophyte species from the genera *Trichophyton*, *Microsporum*, and *Epidermophyton* are recognized as the most clinically significant human pathogens, affecting various body sites with diverse clinical manifestations [2]. Among these, onychomycosis remains the most difficult to cure, while other forms such as tinea capitis, tinea corporis, and tinea pedis, are highly prevalent in communities with prevalence varying by region [2, 3].

Current antifungals for the treatment of fungal infections are limited to a few classes: allylamines, polyenes, azoles, echinocandins, and other agents such as griseofulvin and 5-flucytosine, which target a restricted set of cellular pathways, including fungal membrane sterols, nucleic acids, and cell division processes. In managing dermatophytosis, treatment options are particularly limited, with azoles and allylamines being the most frequently used [1]. Although antifungal resistance in dermatophytes was once considered uncommon, the increasing use of topical antifungal agents for treating superficial dermatophytoses has raised concerns about resistance, particularly with reports from India highlighting widespread terbinafine-resistant dermatophytosis [4]. Additionally, some azole and allylamine antifungals have been reported to cause adverse effects, such as liver toxicity, neurotoxicity or interactions with other medications [5, 6]. On the other hand, while topical formulations such as 5% amorolfine and 8% ciclopirox offer reduced toxicity [5], they have demonstrated low clinical efficacy [7]. Thus, developing novel antifungal agents with enhanced therapeutic indices, reduced toxicity profiles, and mechanisms of action that circumvent existing resistance pathways has become critically important [1].

Indolin-2-one scaffolds, commonly referred to as oxindoles, are well-known pharmacophores in medicinal chemistry due to their presence in various biologically active natural and synthetic compounds. These molecules exhibit diverse pharmacological activities, including antiproliferative, antimicrobial, α-glucosidase inhibitory, antileishmanial, antioxidative, tyrosinase inhibitory, and anti-rheumatoid arthritis properties [8]. Notably, oxindole derivatives functionalized at the C-3 position of the indolin-2-one nucleus have been disclosed as potent kinase inhibitors, exemplified by the marketed anticancer agent sunitinib (SU11248, SutentTM; Pfizer, Cairo, Egypt) and several other anticancer candidates currently in clinical trials [8]. In recent years, studies have proposed that molecules with kinase inhibitory properties could also serve as effective antifungal agents, given the critical role of kinases in microbial growth and survival [9]. While the antibacterial properties of 3-substituted benzylideneindolin-2-one derivatives have been well-documented [10, 11], their antifungal potential remains relatively underexplored, with most research focusing on activity against *Candida* species. This represents an important research gap, particularly in the context of the rising prevalence of fungal infections and drug resistance.

As part of ongoing efforts to develop biologically active compounds incorporating benzylideneindolin-2-one core, a previous report showed that 3-alkenyl oxindole derivatives exhibited significant antifungal activity against pathogenic *Candida* species, suggesting their utility as a novel class of antifungal agents [12]. Prompted by this finding, the present study investigated the antifungal efficacy of 3-benzylideneindolin-2-one against dermatophytes species, while concurrently evaluating its toxicological profile in brine shrimp and zebrafish embryo models. By integrating a comprehensive evaluation of antifungal activity with toxicity assessments, this study was intended to identify novel therapeutic leads that overcome the limitations of existing antifungals and provide more effective therapeutic options to combat the growing threat of fungal infections.

Methods

Synthesis of the compound and characterization by NMR analysis

The active compound 3-benzylideneindolin-2-one was synthesized using a modified version of the Knoevenagel condensation procedure previously reported [13]. A mixture of oxindole (1 mmol) (97% purity, CAZ Number 59-48-3, Sigma-Aldrich Co, St. Louis, MO) and benzaldehyde (1 mmol) (99% purity, CAZ 100-52-7, Techno

PharmChem, Haryana, India) in 5 mL of ethanol (99.9% purity, CAZ Number 67-56-1, Park Scientific Pvt. Ltd, Northampton, UK) was treated with the catalyst piperidine (0.2 mL) (CAZ Number 110-89-4, Sigma-Aldrich Co, St. Louis, MO) and refluxed at 80 °C for 2.5 h. After confirmation of the completion of the reaction by thin layer chromatography, ethanol was cooled evaporated under reduced pressure, and the crude product was washed with methanol: hexane (1:9). The product was purified by silica gel column chromatography using a mixture of hexane: ethyl acetate (1:1). The product was dissolved in chloroform-D (CDCl3-D), and ^{1}H-NMR and ^{13}C-NMR spectroscopy were analyzed using Bruker Ascend 400 NMR spectrometer (Bruker, USA) [Additional file 1]. The spectral data were compared with the values reported in the literature [12, 14, 15] for structural confirmation (Fig. 1).

The Knoevenagel condensation protocol utilized in our study yielded a yellow-solid with a 72% yield after purification with silica gel column chromatography. The chemical shifts (δ) in the NMR spectra are reported in part per million (ppm) and coupling constants (J) in Hertz (Hz). The spectral data are interpreted as follows: s = singlet, d = doublet, t = triplet, q = quartet, and m = multiplet. ^{1}H-NMR (400 MHz, CDCl3-D): δ8.99 (s, 1H, NH-1), 7.86 (s, 1H, H-vinyl), 7.69–7.64 (m, 3 H, H-2′,6′,4) 7.50–7.39 (m, 3 H, H-3′,5′,4′), 7.22 (dt, J = 0.80, 7.60 Hz, 1H, H-6), 6.94 (d, J = 7.60 Hz, 1H, H-7), 6.87 (dt, J = 0.80, 7.60 Hz, 1H, H-5). ^{13}C-NMR (100 MHz, CDCl3-D): δ 170.6(s), 141.7 (s), 137.7 (s), 134.8 (s), 130.0 (s),129.9 (s), 129.7 (s), 129.3 (s), 128.7 (s), 128.4 (s), 127.6 (s),123.1 (s), 121.9 (s), 121.7 (s), 110.3 (s).

In vitro antifungal activity against clinical isolates of dermatophytes

Microorganisms and reagents

In the present study, 30 different strains of dermatophyte clinical isolates, recovered from skin, hair and nail specimens were utilized. The dermatophyte clinical isolates, comprising *Trichophyton mentagrophytes* (18), *Trichophyton rubrum* (03), *Microsporum gypseum* (04), *Microsporum canis* (03), and *Epidermophyton floccosum* (02), were provided by the mycology culture collection of the Faculty of Medicine, University of Ruhuna (Galle, Sri Lanka). *Candida albicans* ATCC® 10,231 reference strain was used as quality control. All fungal cultures were maintained at -20 °C and sub-cultured on Sabouraud dextrose agar (SDA) (HiMedia, Maharashtra, India) supplemented with cycloheximide (300 mg/L) (94% purity, CAZ Number 66-81-9, SRL Pvt. Ltd., Maharashtra, India) and chloramphenicol (50 mg/L) (≥ 98% purity, CAZ Number 56-75-7, Sigma-Aldrich Co, St. Louis, MO) and incubated at 26 °C when required. Itraconazole (ITZ) (99% purity, CAS Number 84625-61-6, Sigma-Aldrich Co, St. Louis, MO) was used as the standard reference antifungal agent. Roswell Park Memorial Institute 1640 (RPMI 1640) broth with L-glutamine and 2% glucose, but without sodium bicarbonate and buffered with 3-(N-morpholino) propanesulfonic acid, monosodium salt (MOPS) at pH 7.0 (HiMedia, Maharashtra, India), was used as the culture medium for microbroth dilution assay and time-kill assay.

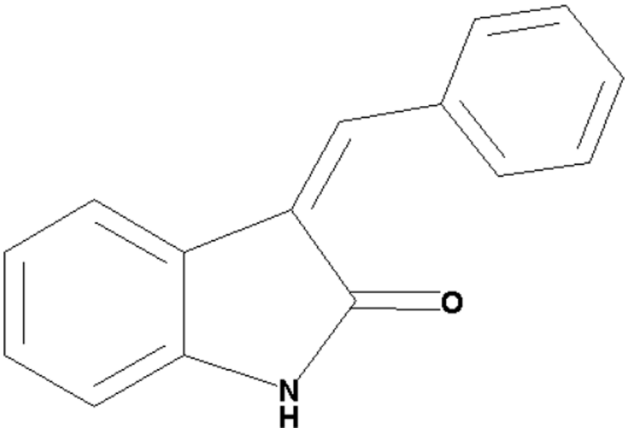

Fig. 1 Chemical structure of 3-benzylideneindolin-2-one

Determination of MIC and MFC: broth microdilution assay

The MIC of 3-benzylideneindolin-2-one was determined against all the dermatophyte isolates using the broth microdilution method proposed by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for dermatophytes [16]. Briefly, starting inoculum suspension of dermatophyte was prepared by gently washing the surface of a 7-day old dermatophyte colony with sterile water supplemented with 0.01% Tween 20 (99% purity, CAZ Number 9005-64-5, HiMedia, Maharashtra, India) and the spore count of the resulting suspension was adjusted to $2–5 \times 10^{6}$ CFU/mL using a haemocytometer. To prepare the working inoculum, the starting suspension was further diluted 1:10 with PBS to yield a final spore count of $2–3 \times 10^{5}$ CFU/mL. The compound was dissolved in DMSO and diluted in RPMI 1640 medium supplemented with Tween 20 to facilitate the solubility of the test compound in the culture medium. Serial two-fold dilutions were then performed to obtain 10 different solutions with double the final concentrations (64 mg/L to 0.06 mg/L). The final concentration of DMSO and Tween 20 were 1% (v/v) and 0.1% (v/v) respectively. Test solutions (100 µL) were added to columns 1–10 of a flat bottom 96 well microtiter plate and inoculated with respective fungal inoculum (100 µL). Three replicates were made for each fungal strain. Colum 11 received drug-free medium (RPMI 1640 with 1% DMSO and 0.1% Tween 20) (100 µL), and corresponding fungal inoculum (100 µL) (growth control),

while column 12 received drug free medium (100 µL) and distilled water (100 µL) (negative control). ITZ solutions ranging from 4 mg/L to 0.008 mg/L were tested against *C. albicans* ATCC 10,231 as quality control. Microtiter plates were incubated for 4–5 days at 26 °C. On the day of reading, 20 µL of resazurin sodium salt (0.002% w/v) (CAZ number 62758-13-8, Sigma-Aldrich, St. Louis, MO) was added to each well and further incubated for 4 h at 37 °C [17]. The change of colour from blue to pink indicated viable fungal growth. The MIC was interpreted as the lowest concentration (mg/L) of the drug that completely inhibited growth (100% inhibition), indicated by the first well that remained blue.

Following the MIC testing, the MFC assay was performed by transferring 20 µL of sample from each well that exhibited blue colour (100% inhibition), the last pink well and, the growth control well onto SDA plates. The plates were incubated at 26 °C until growth appeared in the growth control subculture. The MFC was defined as the lowest concentration of the drug (mg/L) that showed no fungal growth or fewer than three colonies, indicating 99.9% killing activity [18].

Time-kill assay

The time-kill assays were performed with modifications to the method described by Gupta et al. [19] using RPMI 1640 buffered with MOPS as the growth medium. A single isolate of each dermatophyte species, *T. mentagrophytes* (MIC = 8 mg/L), *T. rubrum* (MIC = 8 mg/L), *M. gypseum* (MIC = 8 mg/L), and *M. canis* (MIC = 4 mg/L) was selected for testing based on their MIC values. Prior to the study, isolates were subcultured twice on SDA medium, and inocula containing 1×10^6 microconidia per milliliter were prepared as described in the MIC testing procedure. For the assay, 1 mL of each conidial suspension was added to 9 mL of the growth medium alone or to a solution of RMPI medium containing the compound at 0.5, 1, 2, 4, and 8 times of the MIC for the respective clinical isolate. This resulted in a starting suspension of microconidia of $1–1.5 \times 10^5$ CFU/mL. At predetermined time intervals (0, 2, 4, 6, 12, 24 and 48 h), 0.1 mL of each sample taken and diluted 1:100 in PBS, and 100 µL of the dilution was spread on SDA medium. When colony counts were anticipated to be fewer than 1000 CFU/mL, 100 µL of the test solution was directly sampled and plated without dilution. The number of colonies were recorded following 5 days of incubation at 26 °C. Two separate experiments were performed in duplicate for each species, with the detection threshold for the tested strains set at 10 CFU/mL.

Brine shrimp lethality assay (BSLA)

Toxicity screening of 3-benzylideneindolin-2-one was performed using *Artemia salina* model, adopting the guidelines outlined by Johari et al. [20]. For the hatching process, 1 g of *A. salina* cysts (West Aquarium, Colombo, Sri Lanka) were dispersed in an artificial seawater (ASW) system prepared with 38 g/L of commercial sea salt (Red Sea Aquarium salt) dissolved in deionized water (pH 8.0). The system was maintained at 25 °C with continuous aeration under a 16-h light and 8-h dark cycle. After 24 h, newly hatched nauplii were collected with a glass pipette, and instar I nauplii were selected under a microscope for the toxicity assays. Ten nauplii were carefully introduced into each well of a 24-well plate filled with 2 mL of a given test solution. The exposure group was treated with six drug concentrations: 5 mg/L, 10 mg/L, 25 mg/L, 50 mg/L, 100 mg/L, and 150 mg/L (the maximum soluble concentration), dissolved in ASW with 1% DMSO and 0.1% Tween 80 (CAZ Number 9005-65-6, SRL Pvt. Ltd., Maharashtra, India) as solvents. In parallel, the toxicity was assessed in potassium dichromate (99.5% purity, CAZ Number 7778-50-9, SRL Pvt. Ltd., Maharashtra, India)) solutions at concentrations ranging from 5 mg/L to 100 mg/L (positive control), ASW with 1% DMSO and 0.1% Tween 80 (solvent control), and ASW (blank control). Prior studies conducted in our laboratory confirmed that 1% DMSO and 0.1% Tween 80 had no significant effect on nauplii motility compared to clear ASW. All the experimental groups were incubated at 25 °C, and the number of immobilized nauplii was recorded 24 h and 48 h post-exposure. Nauplii were considered immobilized if they failed to display forward movement within 10 s. Three independent toxicity assays, each with three replicates (30 nauplii per test), were conducted, and the average mortality (%) was calculated for each concentration.

Zebrafish embryo toxicity test

The developmental toxicity of 3-benzylideneindolin-2-one on zebrafish (*Danio rerio*) embryos was assessed in accordance with the Organization for Economic Cooperation and Development Technical Guide 236 (OECD TG 236) [21]. The assay was performed using fertilized eggs from the Medical Research Institute (Colombo, Sri Lanka). Zebrafish embryos for the experiments were obtained from the natural mating of wild-type adult zebrafish (female: male, 2:1) placed in a 1.5 L clean fish tank filled with dechlorinated water [22] under controlled conditions: temperature 26 °C, pH 7.5, and oxygen level equal to or above 85% saturation, and a light-dark (14:10 h) photoperiod. Immediately post-spawning, the embryos were collected using a sieve, rinsed in de-chlorinated water, and screened under an inverted optical microscope to select fertilized eggs, excluding unfertilized embryos or those exhibiting external anomalies such as asymmetries or vesicles.

3-Benzylideneindolin-2-one was dissolved in dechlorinated water (dilution water) to prepare a series of seven concentrations (0.25 mg/L, 0.5 mg/L, 1 mg/L, 2.5 mg/L, 5 mg/L, 7.5 mg/L and 10 mg/L) covering the range up to the maximum observed MIC for dermatophytes and considering the compound's maximum solubility in water without solvents. Within 2 h post-fertilization (hpf), embryos were individually introduced into 20 wells of a 24-well plate (Bibby Steriline Ltd., Statts, UK), each containing 2 mL of a given drug solution, with the remaining four wells containing only dilution water (internal control). Twenty embryos per concentration were exposed to the test compound. In parallel, groups of twenty embryos were exposed to 4 mg/L of 3,4-dichloroaniline (positive control) (98% purity, CAZ Number 95-76-1, Sigma-Aldrich Co, St. Louis, MO) and to dilution water (negative control). All plates were maintained at 26 °C under a 14/10 h light/dark photoperiod for 96 hpf. Approximately 90% of exposure solutions were renewed daily and the chemical was freshly prepared each day. Embryos were observed at 24, 48, 72, and 96 hpf under an inverted optical microscope at 40x magnification, and four apical indicators of lethality including the coagulation of eggs, non-detachment of tail bud from the yolk sac, lack of somite formation, and lack of heartbeat were recorded. Additionally, sublethal effects such as spinal curvature, tail deformity, pericardial edema, yolk-sac edema, heart rate (beats per minute) and hatching embryos in control and treatment groups were also monitored. All experiments were conducted in triplicate for each concentration.

Statistical analysis

The median lethal concentration (LC50) for brine shrimp was determined using a probit analysis model in SPSS 26.0 software (version 26; IBM, Chicago, USA) with a 95% confidence level (CI). One-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test, was conducted to compare differences between treatment and control groups using GraphPad Prism 10.3.1 software (GraphPad Software Inc., Boston, USA, trial version). Statistical significance was defined as a p-value less than 0.05 ($p < 0.05$). The data were expressed as mean ± standard error of mean (SEM).

Results

In vitro antifungal activity against clinical isolates of dermatophytes

The antifungal effect of 3-benzylideneindolin-2-one was determined against 30 dermatophyte clinical isolates from patients with superficial fungal infections. The MIC and MFC values for the tested dermatophyte species are summarized in Table 1. The compound effectively inhibited the growth of all the dermatophytes strains tested, with MICs ranging from 0.25 to 8 mg/L, and the MFCs from 1 to 32 mg/L. The MFC values were approximately 2–4 times higher than the respective MICs for most isolates. The compound demonstrated the strongest inhibitory activity against *E. floccosum* (MIC = 0.25-1 mg/L, MFC = 0.5-1 mg/L). In contrast, MIC values for other dermatophytes, including *T. mentagrophytes*, *T. rubrum*, *M. canis* and, *M. gypseum*, ranged from 2 to 8 mg/L. In addition, analysis of MIC/MFC ratios indicated that the compound exhibited fungicidal activity (MIC/MFC = 1–4) across the tested isolates. However, a fungistatic effect (MIC/MFC = 8) was also observed in a small subset of *T. mentagrophytes* and *M. gypseum* strains.

The combination of solvents DMSO (1% v/v) and Tween 20 (0.1% v/v), used to dissolve the drug compound, showed no detectable inhibitory activity at the tested concentrations in broth microdilution assays.

Table 1 Antifungal activity of 3-benzylideneindolin-2-one against clinical isolates of dermatophytes ($n = 30$) from patients with superficial fungal infections

Clinical isolate	MIC				MFC			
	MIC range	GM MIC ± SEM	MIC50	MIC90	MFC range	GM MFC ± SEM	MFC50	MFC90
Trichophyton mentagrophytes ($n = 18$)	2–8	4.16 ± 0.59	4	8	4–32	14.81 ± 2.72	16	32
Trichophyton rubrum ($n = 3$)	4–8	6.35 ± 1.33	8	8	16	16.0 ± 0.00	16	16
Microsporum gypseum ($n = 4$)	4–8	5.66 ± 1.15	4	8	16–32	26.91 ± 4.00	32	32
Microsporum canis ($n = 3$)	4–8	5.04 ± 0.0	4	8	16–32	20.16 ± 5.33	16	32
Epidermophyton floccosum ($n = 2$)	0.25-1	0.5 ± 0.38	0.25	1	1–4	2.0 ± 1.50	1	4

Minimum inhibitory concentration (MIC): the lowest concentration (mg/L) of the compound that inhibited visible fungal growth

Minimum fungicidal concentration (MFC): the lowest concentration (mg/L) of the compound that killed at least 99.9% of the initial inoculums

Geometric mean: (GM); Standard error of mean: (SEM)

MIC50: the lowest concentration (mg/L) of the compound at which 50% of isolates are inhibited

MIC90: the lowest concentration (mg/L) of the compound at which 90% of isolates are inhibited

MFC50: the lowest concentration (mg/L) of the compound at which 50% of isolates are killed

MFC90: the lowest concentration (mg/L) of the compound at which 90% of isolates are killed

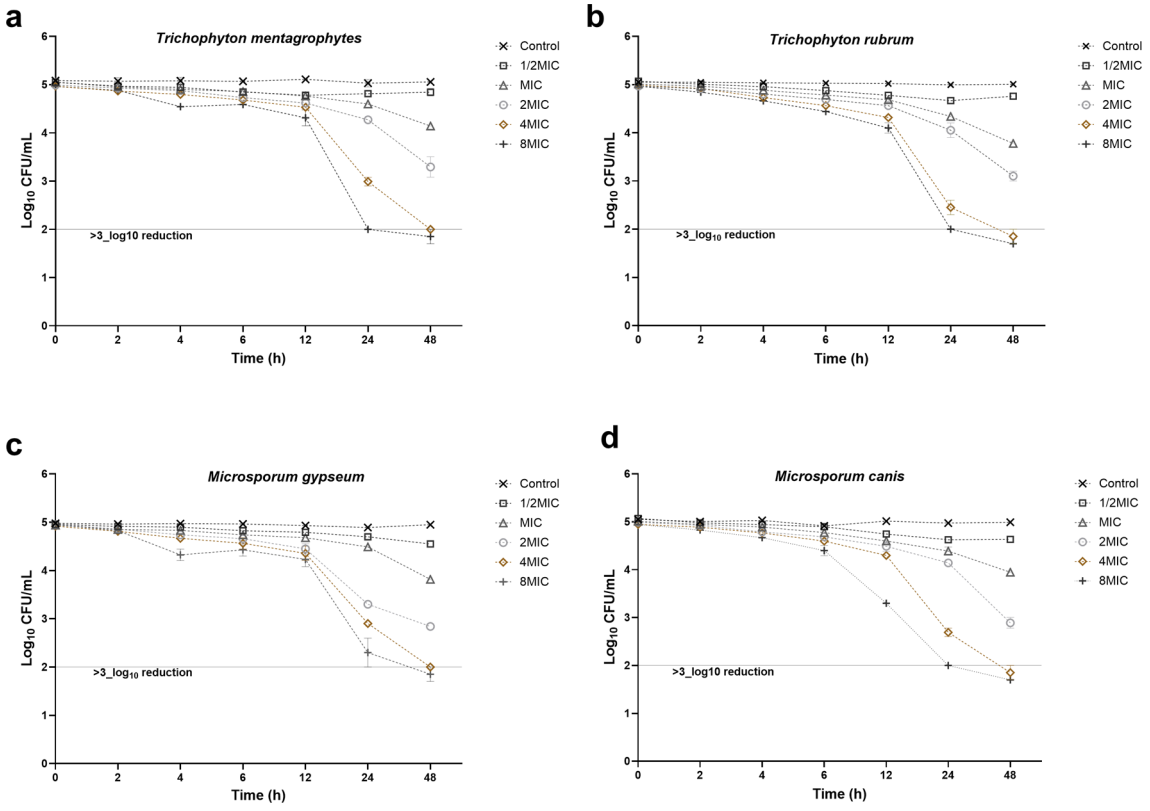

Fig. 2 Time-kill curves for 3-benzylideneindolin-2-one against representative dermatophyte isolates (**a**) *Trichophyton mentagrophytes* (**b**) *Trichophyton rubrum* (**c**) *Microsporum gypseum* and (**d**) *Microsporum canis*. Each data point represents the mean $\log_{10}$ of colony forming units (CFU) per millilitre from two independent experiments performed in duplicate

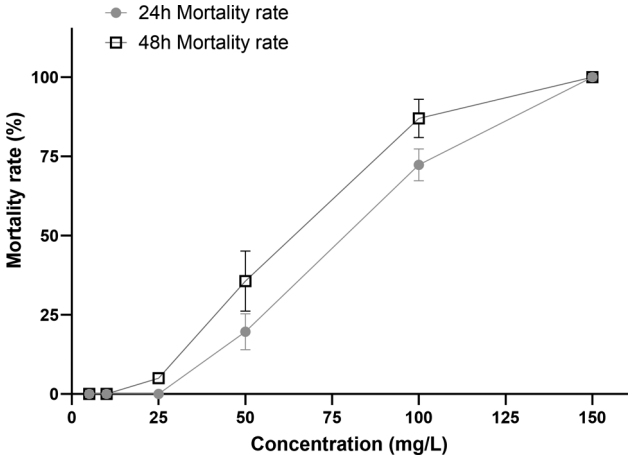

Fig. 3 Dose-response curve of mortality rate for *Artemia salina* nauplii exposed to 3-benzylideneindolin-2-one after 24-h and 48-h. Data presented mean mortality rates (and SEM) from three independent experiments, each performed in triplicate with 10 nauplii per group

Time-kill assay

Time-kill curves of 3-benzylideneindolin-2-one for *T. mentagrophytes*, *T. rubrum*, *M. gypseum* and *M. canis* are shown in Fig. 2. All four dermatophyte isolates showed a 50% reduction in viable counts within 6 h of exposure to 4x MIC and 8x MIC, and 12 h at 2x MIC. Time-kill curves also confirmed the fungicidal activity of the compound against the tested dermatophyte isolates, achieving a 99.9% reduction in CFU per millilitre (≥ 3 $\log_{10}$ CFU/mL) relative to the starting inoculum. The fungicidal endpoint for the dermatophytes strains was achieved after 48 and 24 h at 4x MIC and 8x MIC respectively. The differences in the time taken to reach these endpoints at different concentrations revealed the concertation-dependent fungicidal activity of the compound across the tested dermatophyte species ($p < 0.05$).

Brine shrimp lethality assay

In toxicity testing of 3-benzylideneindolin-2-one using *A. salina* as the model organism, the compound did not exhibit a lethality effect on nauplii at concentrations up to 25 mg/L at 24 h (Fig. 3). However, the mortality rate increased significantly with concentrations from 50 to 150 mg/L, with an LC50 value of 69.94 mg/L (95% CI: 64.31–76.33 mg/L). Extending the exposure period to 48 h resulted in increased toxicity towards the brine shrimp nauplii, reducing the LC50 value to 52.70 mg/L (95% CI: 48.44–57.43 mg/L). The highest mortality rate was observed at a concentration of 150 mg/L. The LC50 values for potassium dichromate (positive control) were 34.21 mg/L (95% CI: 26.70-52.69 mg/L) and 17.57 mg/L

(95% CI: 15.80-19.72 mg/L) after 24 h and 48 h of exposure, respectively. The mortality rate of negative control and solvent control at 48 h was 0% which was comparable to the guidelines proposed in Johari et al. [20].

Zebrafish embryo toxicity test

The zebrafish embryo toxicity test met the criteria described in OECD TG 236 [21], as demonstrated by a 96 hpf mortality rate of 70% in the positive control group and 1.7% in the negative control group. The cumulative mortality rates for each drug concentration over different times of exposure are depicted in Fig. 4a. The compound exhibited a time and concentration-dependent toxicity effect on zebrafish embryos, with increasing the concentrations from 1 mg/L to 10 mg/L. During the observation window from 24 to 96 hpf, embryos exposed to drug concentrations below 7.5 mg/L showed either no mortality or negligible mortality compared to the negative control group, with a statistically insignificant difference ($p > 0.05$). At the highest concentration tested (10 mg/L), the mortality was 5% at 24 hpf, and the lethality effect increased significantly over time, reaching a maximum mortality of 13.3% by 96 hpf.

In the negative control group, hatching started at 48 hpf (32%), and a hatching rate of 98% was achieved at 72 hpf. Thus, the 72 hpf time point was used to assess any premature or delayed hatching across tested drug concentrations. No premature hatching was observed at any drug concentration, and in all tested concentrations, hatching occurred within 48–72 hpf (Fig. 4b). Embryos exposed to the highest concentration (10 mg/L) exhibited a significantly reduced hatching rate (87%) at 72 hpf ($p < 0.001$), while no significant difference in hatchability was observed at concentrations between 0.25 mg/L and 7.5 mg/L compared to the control group.

The sublethal effect of the 3-benzylideneindolin-2-one on embryonic/larval developments were further evaluated through heart rate monitoring and the identification of morphological abnormalities such as spinal curvature, tail deformity, pericardial edema, and yolk-sac edema. Figure 5 displays the heart rate and morphological abnormalities observed in zebrafish larvae exposed to different concentrations of the drug. The control embryos demonstrated normal heart rate and typical development at 24 hpf, 48hpf, 72 hpf and 96 hpf. The heart rate of the treated embryos was not significantly affected by the drug at any of the tested concentrations. At 72 hpf, body malformations were observed in 5% and 6% of zebrafish larva in the 7.5 mg/mL and 10 mg/L treatment groups, respectively, with the pericardial edema and yolk-sac edema being the most prominent abnormalities ($p < 0.001$). No spinal curvature and tail deformity were observed in zebrafish embryos after 96 h of exposure to different drug concentrations.

Discussion

The frequency of treatment failures and recurrence of dermatophyte infections is increasingly reported, yet the global burden of these infections remains underestimated [4, 23]. The emergence of antifungal resistance and tolerance to the existing antifungal therapies raise concerns regarding a potential escalation antifungal treatment failure in the near future [1, 4]. Therefore, the development of novel antifungal compounds with minimum toxicity is an essential goal in anti-infective research.

In the present study, 3-benzylideneindolin-2-one uniformly demonstrated fungicidal activity against *T. mentagrophytes*, *T. rubrum*, *M. gypseum*, *M. canis*, and *E. floccosum*, which represent the most clinically significant cutaneous dermatophyte species. While no prior studies have reported the antidermatophytic properties

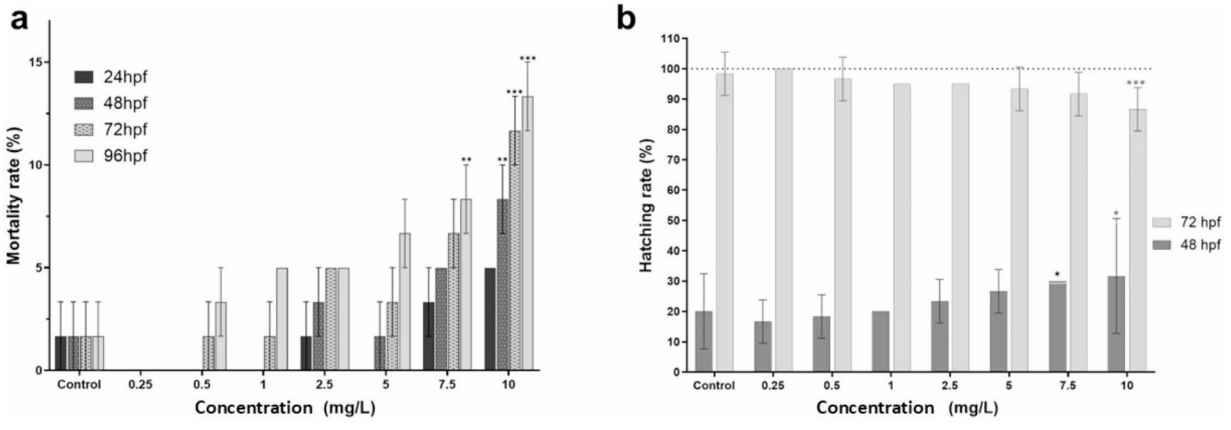

Fig. 4 Mortality and hatching rate of zebrafish embryos exposed to different concentrations of 3-benzylidene-indolin-2-one. **a**) Mortality rate (%) of zebrafish embryos at 24, 48, 72, and 96 h of post fertilization (hpf). **b**) Hatching rate (%) of zebrafish embryos at 48 and 72 hpf. Data are presented as mean ± SEM ($n = 20$ embryos/group) of three independent replicates. (*Indicates the significant level when compare to the respective negative control group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) by statistical analysis using ANOVA followed up by Dunnet's *post hoc* test)

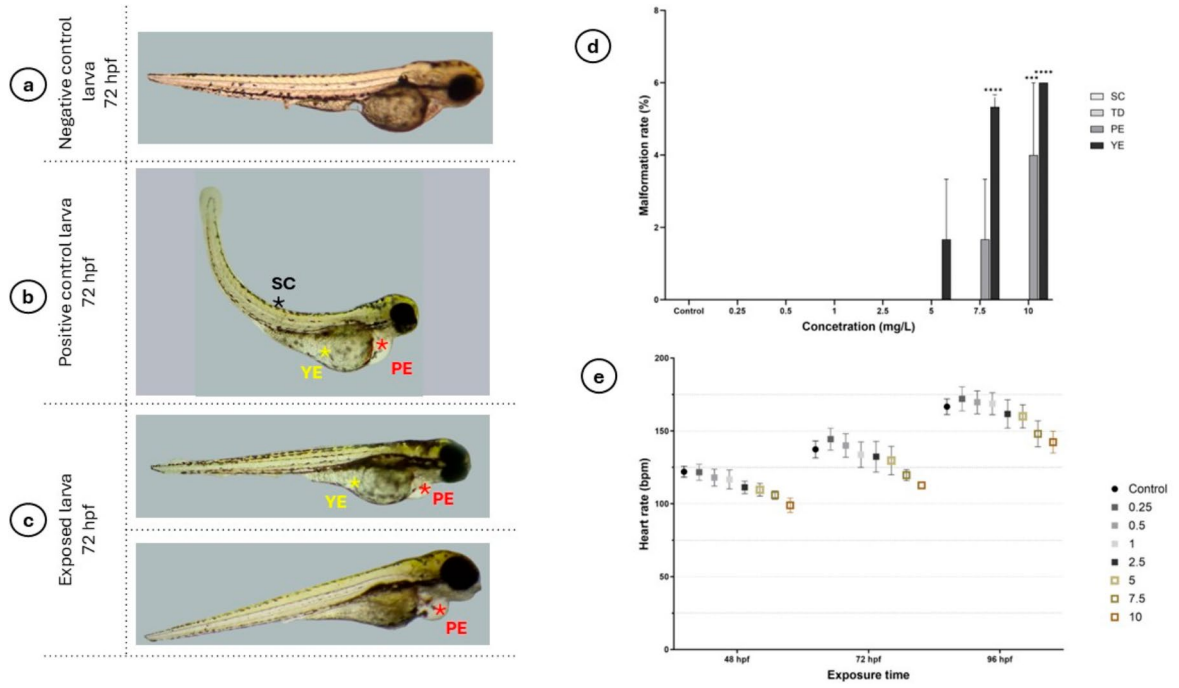

Fig. 5 Effect of 3-benzylideneindolin-2-one on the embryonic development of zebrafish. Representative images show sublethal alterations observed in zebrafish larvae at 72 hpf: (**a**) normal development in the negative control, (**b**) larvae exposed to 3,4-dichloroaniline (positive control) at 4 mg/L, and (**c**) larvae exposed to 3-benzylideneindolin-2-one at 10 mg/L. (**d**) Rate of malformations in zebrafish larva exposed to the different concentrations of the drug, SC: spinal curvature, TD: tail deformity, PE: pericardial edema, and YE: yolk-sac edema. **e**) Heart rate (bpm: beats per minute) of zebrafish embryos/larva at 48, 72, and 96 hpf exposed to different concentrations of the drug. Data are presented as mean ± SEM ($n = 20$ embryos/group) of three independent replicates. (*Indicates the significant level when compare to the respective negative control group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) by statistical analysis using ANOVA followed up by Dunnet's *post hoc* test)

of 3-benzylideneindolin-2-one, its antifungal efficacy against *Candida* species has been reported by Wijekoon et al. [12] against reference strains, including *C. albicans* (ATCC 25922), *C. parapsilosis* (ATCC 22019), *C. glabrata* (ATCC 90030), and *C. krusei* (ATCC 6258), using the broth microdilution method. The study reported a MIC and MFC range for *Candida* species (MIC: 2–8 mg/L and MFC: 2–16 mg/L), which align with the values observed against dermatophytes in the present study. The consistency of the compound's antifungal activity within therapeutic windows is a positive indication for the broad-spectrum antifungal potential of 3-benzylideneindolin-2-one targeting both dermatophytes and *Candida* species, the major causes of the cutaneous fungal infections.

The compound demonstrated fungicidal activity for dermatophytes which was both concentration and species-dependent. Notably, its fungicidal activity on the microconidia of key dermatophyte pathogens became evident as early as 24 h. In contrast, many currently used antifungals have demonstrated primarily fungistatic or inconsistent fungicidal effects against spores (microconidia/macroconidia or chlamydospores) and mycelial forms of dermatophytes. Azole drugs, such as miconazole, clotrimazole, itraconazole and ketoconazole, are typically regarded as fungistatic in nature [24]. Meanwhile, terbinafine, griseofulvin, and ciclopirox have shown fungistatic or heterogeneous fungicidal activity in time-kill analyses targeting spores and mycelia [25, 26]. For example, a study by Pannu et al. [26] examined the time-kill kinetics of antifungals ciclopirox, itraconazole, and terbinafine at 16× MIC concentrations against both microconidia and mycelial forms of *T. rubrum* in a suspension of 10^6 CFU/mL in water. The results indicated that none of these antifungals attained 99.9% colony counts for either dermatophyte form at any tested time point, with the only exception being ciclopirox (16× MIC), which demonstrated a 3-$\log_{10}$ unit reduction in *T. rubrum* mycelia, but not in microconidia after 8 h. Thus, 3-benzylideneindolin-2-one appears to be a more potent antifungal candidate, capable of effectively killing dermatophyte spores which are dormant cells often implicated in treatment failures and recurrent infections.

Using brine shrimp lethality testing, 3-benzylideneindolin-2-one was found to be mildly toxic to *A. salina* following the BSLA toxicity classification scale proposed by Clemen-Pascual, Macahig, & Rojas [27], which defined plant extracts with LC50 > 100 µg/mL as non-toxic. Toxic effects in BSLA are often indicative of the presence of pharmacologically relevant compounds, including those

with anticancer activities. BSLA outcomes frequently correlate with cytotoxic activities against cancer cell lines, as reported in previous studies [27]. Thus, the observed mild BSLA toxicity of 3-benzylideneindolin-2-one in the current study suggests its potential anticancer activity, aligning with cytotoxic effects exemplified by prior studies on cancer cell lines [14, 28]. These studies reported modest cytotoxicity of 3-benzylideneindolin-2-one, with an LC50 > 1 nM (~ 0.0022 mg/L) for human leukaemia cell lines MV4-11 [14] and > 25 µM (~ 5.53 mg/L) for Hepa1c1c7 cells [28]. Furthermore, in the present study, the LC50 of 3-benzylideneindolin-2-one was found to be approximately six times higher than its MIC (8 mg/L) and twice its MFC against dermatophytes, indicating a favorable therapeutic window with relatively low cytotoxicity.

Consistent with its low toxic effect in the therapeutic range, 3-benzylideneindolin-2-one exhibited minimal toxic effects on early-life stages of zebrafish, with no induction of significant lethal effects, delays or inhibition in hatching. However, a significant increase in mortality and reduced hatchability were observed at the highest concentration (10 mg/L), suggesting a threshold effect for developmental toxicity. The zebrafish embryo test, conducted over 96 h, has been widely validated as a robust alternative model to traditional adult fish acute toxicity testing, providing comparable predictability of chemical toxicity [29]. According to the Global Harmonized System of Classification and Labelling of Chemicals (GHS Rev. 10) [30], substances with an LC50 below 1 mg/L are considered highly toxic to fish in acute fish toxicity assessments. Given the low mortality observed at 10 mg/L over 96 h (13.3%), our findings suggest that 3-benzylideneindolin-2-one exhibits a low toxicity level for zebrafish embryos, aligning with the GHS [30] classification criteria for category 3 with LC50 values between 10 mg/L and 100 mg/L. However, due to the compound's limited solubility in clear water, it was not possible to achieve full dissolution at concentrations higher than 10 mg/L, preventing the determination of a maximal lethal concentration or the LC50 for zebrafish embryos, as complete solubility is essential for reliable fish embryo testing. Additionally, at this concentration, embryo exposure led to only minimal occurrences of morphological anomalies such as yolk-sac edema and pericardial edema, suggesting a relatively low potential for developmental toxicity in zebrafish. Many antifungals, including widely used azole-based compounds, have shown LC50 values ranging from 1 to 10 mg/L in various fish models and are often associated with significant physiological and morphological abnormalities [31]. The present findings signify the potential application of 3-benzylideneindolin-2-one as a leading antifungal candidate with comparatively low toxic effect within the therapeutic window. The use of zebrafish embryos in primary chemical screening for developmental and organ-specific toxicology during the early stages of drug development is recognized as an ethically viable alternative to animal studies. Results from zebrafish-based chemical screens have demonstrated strong translational relevance to organisms, including humans [32]. The present study assessed the developmental toxicity of 3-benzylideneindolin-2-one in zebrafish embryos. Future studies are necessary to exploit its organ-specific toxicities, genotoxic effects, and the impact of long-term exposure using appropriate testing approaches.

While 3-benzylideneindolin-2-one demonstrated antifungal activity within the MIC window, the current study constrained by its poor water solubility which restricted the evaluation of toxicity effects on zebrafish embryos at higher concentrations. Furthermore, resistance profiles of the tested isolates to standard antifungals, (i.e. terbinafine or itraconazole) were not investigated and compared with the sensitivity patterns observed for 3-benzylideneindolin-2-one in the study.

Conclusions

3-Benzylideneindolin-2-one exhibits a broad spectrum of antifungal activity against clinically relevant dermatophyte species and *Candida* species. Its potential as a promising lead compound for the development of antifungal therapeutics is highlighted by its low toxicity in brine shrimp and zebra fish animal model experiments. Further studies are needed to evaluate safety, toxicity and mechanism of action which could pave the way for clinical application, offering a novel topical therapeutic option for treatment of dermatophyte infections.

Abbreviations

ANOVA	One-way analysis of variance
ASW	Artificial seawater
ATCC	American type culture collection
BSLA	Brine shrimp lethality assay
CFU	Colony forming units
DMSO	Dimethylsulfoxide
EUCAST	European committee on antimicrobial susceptibility
GM	Geometric mean
ITZ	Itraconazole
LC	Lethal concertation
MFC	Minimum fungicidal concentrations
MIC	The minimum inhibitory concentrations
MOPS	3-(N-morpholino) propanesulfonic acid, monosodium salt
NMR	Nuclear magnetic resonance spectroscopy
OECD	Organization for Economic Co-operation and Development
RMPI 1640	Roswell Park Memorial Institute 1640
SDA	Sabouraud dextrose agar
SEM	Standard error of mean

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40360-025-00850-1.

Supplementary Material 1

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Author contributions
RSR carried out all experimental work, data acquisition and analysis, literature review, and manuscript writing. SSN and TDCP were responsible for conceptualizing the study, designing the experiments, supervising the work, and revising the manuscript. PM and KGUR provided input for compound synthesis, conceptualizing the study, supervising the work, and contributed to manuscript revision. HHPMJ provided clinical isolates of dermatophytes and, as a supervisor, offered clinical insights for the manuscript. RJB, as a supervisor of the study, contributed to securing grant funding, study design, and data analysis. GGYH facilitated funding and contributed to investigations. KAAU assisted with the design, execution, and interpretation of toxicity assays. E was involved in study design, conceptualization, supervision, and critically evalution of the manuscript. All authors have read and approved the final manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
In accordance with the Council of Europe Directive 2010/63/EU and its guidelines on the protection of experimental animals, zebrafish embryos are not considered protected organisms until they reach the stage of independent feeding (up to 5 days post-fertilization/120 hours post-fertilization hpf), and therefore, do not fall into the regulatory frameworks dealing with animal experimentation. In adhering to the regulatory frameworks outlined in EU Directive 2010/63/EU, we ceased all the experiments at 96 hpf. As such, ethical approval was not required for the fish embryo toxicity experiment procedures conducted herein. Zebrafish embryos were maintained and used in accordance with all the international ethical guidelines and institutional standards. For this type of study informed consent is not required.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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