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Synergistic effect of pedalitin and amphotericin B against *Cryptococcus neoformans* by in vitro and in vivo evaluation

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ABSTRACT

Cryptococcosis is an opportunistic fungal infection responsible for high morbidity and mortality in immunocompromised patients. Combination of antifungal substances is a promising way to increase the percentage of successful treatment. Pedalitin (PED) is a natural substance obtained from *Pterogyne nitens*. The aim of this study was to verify the efficacy of PED alone and in combination with amphotericin B (AmB) in vitro and in vivo against *Cryptococcus* spp. In the in vitro assay, minimum inhibitory concentrations (MICs) of 0.125 mg/L for AmB and 3.9 mg/L for PED were found when the substances were tested alone, whilst in the combination treatment the active concentration of both decreased, with MICs of 0.03 mg/L for AmB and 1 mg/L for PED. In the survival assay, fungal burden study and histopathological assays it was possible to study the efficacy of the substances alone and in combination. The efficacy of combination therapy was considered better than monotherapy as evaluated in a *Galleria mellonella* model and a murine model. Thus, the combination of PED and AmB is an interesting alternative for anticryptococcal fungal treatment. Moreover, a correlation was observed between the invertebrate and murine models for this antifungal treatment combination.

1. Introduction

Cryptococcus neoformans and *Cryptococcus gattii* are the main pathogenic species causing cryptococcosis, an opportunistic fungal infection caused by inhaling basidiospores or dissected yeasts present in the environment, affecting the respiratory tract of the host and causing severe pneumonia and respiratory insufficiency [1,2]. Cryptococcosis mainly affects immunocompromised patients, in whom the yeast can reach other sites of infection such as the central nervous system, causing meningitis and meningoencephalitis. Cryptococcosis is responsible for high morbidity and mortality in human immunodeficiency virus (HIV)-infected patients [3–5].

Therapy for cryptococcosis comprises the combination of 5-flucytosine and amphotericin B (AmB), which presents high toxicity. Maintenance therapy is recommended with fluconazole, but the development of resistant strains has been described [6–8]. Natural substances have been shown to be an interesting alternative in the search for new antifungals, serving as templates for the synthesis of new compounds with fewer side effects and better biological activity [9].

Pterogyne nitens belongs to the family Fabaceae and is found distributed from the northeast to the south of Brazil as well as in Paraguay, Bolivia and Argentina. The crude extract of its leaves has been studied, culminating in the isolation and characterisation of various pure substances, including the flavonoid pedalitin (PED) [10]. Chromatographic (gel permeation chromatography and high-performance liquid chromatography) and spectroscopic [nuclear magnetic resonance (NMR)] techniques were carried out with crude extract of the leaves of *P. nitens* to afford the isolation and characterisation of the flavonoids kaempferol, afzelin, lespedin or kaempferitrin, pteroginoside, quercetin and isoquercetin and the flavone PED [11]. The PED structure was elucidated by NMR spectrometry and high-resolution mass spectrometry [12].

Combination of antifungal substances is a promising way to increase the percentage of successful treatment [13]. In vitro data showed that low doses of AmB might be used in combination with

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voriconazole without resulting in decreased clinical response [14]. The drugs used in cryptococcosis treatment are being widely investigated to find the best combination with high efficiency, reduced resistance development, reduced toxicity and shorter duration of therapy [15].

Because of ethical issues, scientists need to find alternative animal tests that can be used for screening and only subsequently use mammalian models in more focused studies, minimising the number of animals used [16]. Invertebrate animal models are valuable tools in different approaches. *Galleria mellonella* is a useful model to evaluate virulence as well as efficacy and toxicity of antifungal drugs. Studies using *G. mellonella* and *Cryptococcus* yeast are increasing, and there are reports on the use of this invertebrate model to study virulence and the efficacy of antifungal therapy against *Cryptococcus* [17,18]. Thus, the use of an alternative animal model is an option in the search for new natural antifungal compounds, allowing screening for their toxicity and efficacy before launching more targeted studies with mammalian models.

The aim of this study was to demonstrate the in vitro antifungal activity of PED and its synergistic effect with AmB and, moreover, to verify the efficacy of the synergistic combination of PED and AmB in vivo using the alternative animal model *G. mellonella* first for screening, followed by correlation with a mammalian model.

2. Materials and methods

2.1. Pedalitin and antifungal agent

PED was provided by Chemical Institute–UNESP (Araraquara, Brazil) and AmB was from Sigma-Aldrich (St Louis, MO).

2.2. Fungal culture and inoculum

Cryptococcus neoformans strain ATCC 90112 was maintained in Sabouraud agar (Difco Laboratories, Detroit, MI) at 37 °C. For the experiments, the yeast was cultivated in Sabouraud broth (Difco) at 37 °C (150 rpm) for 24 h.

2.3. Susceptibility testing

The microdilution method described by the Clinical and Laboratory Standards Institute (CLSI) [19] with modifications [20] was used to determine the minimum inhibitory concentration (MIC), which was assessed visually as the lowest concentration between 0.48 and 250 mg/L that inhibited visible growth. Plates were incubated at 37 °C (150 rpm) for 48 h.

The minimum fungicidal concentration (MFC) was determined by transferring an aliquot to a Sabouraud agar plate (Sigma-Aldrich) plus 100 mg/mL ampicillin (Sigma-Aldrich), which was incubated at 37 °C for 48 h. The MFC was determined as the lowest concentration of the compound that did not permit visual fungal growth.

2.4. Chequerboard assays

The interaction of the compounds was assessed by the chequerboard broth microdilution method [21,22]. Each compound and/or antifungal agent four times more concentrated than the final concentration was dispensed following the conditions used in the microdilution test. The compounds were also tested alone. The combinatorial test result was the lowest concentration of each combination that completely inhibited sample growth.

The fractional inhibitory concentration index (FICI) was calculated using the equation: $\Sigma FIC = FIC_A + FIC_B$, where the FIC is the ratio of the MIC of the drug in combination with the MIC alone [21,22]. A combination was considered synergistic at an FICI ≤0.5, indifferent at FICI >1 and ≤4 and antagonistic at FICI >4.0. The same formula was used to calculate the fractional fungicidal concentration index (FFCI), using MFC values instead of MIC [22].

2.5. Cryptococcus neoformans *time–kill assay*

Cryptococcus neoformans (5×10^5 cells/well) was treated with PED at 4 mg/L, AmB at 0.125 mg/L and the combination of PED at 1 mg/L and AmB at 0.03 mg/L for 0, 0.5, 1, 2, 4, 8, 10, 12, 24 and 48 h at 37 °C. The suspensions were inoculated on Sabouraud agar plates supplemented with 100 mg/mL ampicillin and were incubated at 37 °C for 48 h. The number of CFU was determined by estimating the decrease in viable cell number after different times in contact with the different treatments.

2.6. Cell culture

The tumoural cell lineages U87-MG (human glioblastoma) and HepG-2 (human hepatoma) as well as MRC-5 (human pulmonary fibroblast), which were obtained from the Rio de Janeiro cell bank, and the lineage NOK (keratinocyte oral mucosa), which was donated by Dr Karl Munger (Department of Medicine, Harvard Medical School, Boston, MA) were used. Cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich) supplemented with 10% heat-inactivated foetal bovine serum at 37 °C in a 5% CO_2 atmosphere.

2.6.1. Cytotoxicity assay using MTT

The cell monolayer formed was exposed to concentrations of the compounds in the range 0.12–62.5 mg/L for 24 h. The MTT method was performed using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tretazolium bromide (Sigma-Aldrich) at 5 mg/mL. Untreated cells (viable cells) and cells treated with hydrogen peroxide (Sigma-Aldrich) (dead cells) were used as controls. The IC_{50} values indicate the concentration at which 50% of the cells were killed.

The selective index (SI) was calculated using the equation $SI = IC_{50}/MFC$ [23]. Compounds with SI values ≥10 were considered to be more active against the yeast and less cytotoxic to the host, which is indispensable for treatment safety [24].

2.6.2. Influence of antifungal treatment on infection with Cryptococcus neoformans *in cell culture*

U87-MG and MRC-5 cell lineages (1×10^5 cells/well) were infected with *C. neoformans* (2.5×10^4 cells/well) and were incubated at 37 °C (4 h for MRC-5 cell lineage and 1 h for U87-MG cell lineage). The wells were washed twice with phosphate-buffered saline (PBS) and were treated with PED at 4 mg/L, AmB at 0.125 mg/L and the combination of PED at 1 mg/L + AmB 0.03 mg/L. According to the time–kill assay, the incubation times were 30 min, 1 h and 2 h at 37 °C. Following treatment, the wells were washed twice with PBS, were subjected to trypsin treatment and were inoculated on Sabouraud plates supplemented with 100 mg/mL ampicillin. After 48 h at 37 °C, the percentage of CFU in each situation was determined.

2.7. Galleria mellonella *growth and experimental conditions*

Galleria mellonella was maintained and grown in the laboratory. The larval phase of *G. mellonella* was fed with wax and pollen and was maintained at 28 °C. For the experiments, control and treatment larvae weighing 150–200 mg and without colour alterations were selected and were incubated at 37 °C overnight, protected from light and without food. Before the experiment, the pro-leg region was cleaned with 70% ethanol. For the inoculation, a Hamilton syringe (Hamilton Co., Reno, NV) was used to inject 10 μL of inoculum and/or treatment solution through the last left larvae pro-leg. The control groups were untouched larvae and larvae injected

with PBS. Larvae were incubated at 37 °C and death was monitored daily for 7 days by visual inspection based on lack of movement after touching with forceps. Each experiment was repeated at least three times with 16 larvae for each condition.

2.7.1. Toxicity assay

Larvae were injected with dimethyl sulphoxide (DMSO) (5%, 10% and 20%), AmB (0.5, 1, 2 and 4 mg/kg) and PED (6.25, 12.5, 25, 40, 50, 100 and 200 mg/kg) and were incubated at 37 °C for 7 days. Death was monitored by visual inspection based on lack of movement after touching. The AmB doses used were in accordance with therapeutic doses recommended by cryptococcal clinical guidelines [6], and PED doses were calculated considering the MIC and the larval weight. The doses were increased to determine the toxicity of the substance.

2.7.2. Cryptococcus neoformans *survival curve*

Three fungal concentrations were used to determine the ideal inoculum concentration able to kill the larvae within 7 days of the experiment (5×10^6, 1×10^6 and 5×10^5 cells/larvae) at 37 °C, evaluating survival daily. The experiment time was considered appropriate for this model because of the insect life cycle.

2.8. Treatment efficacy in Galleria mellonella infected with Cryptococcus neoformans

2.8.1. Survival time

Larvae were infected with *C. neoformans* (1×10^6 cells/larvae) and then 1 h later the following treatments were conducted: AmB doses of 0.5, 1, 2 and 4 mg/kg; PED at 6.25, 1.5, 25, 40, 50, 100 and 200 mg/kg; and the combination of AmB 0.3 mg/kg + PED 10 mg/kg.

2.8.2. Fungal burden

Three larvae from each group, i.e. untreated and treated with AmB 4 mg/kg, PED 40 mg/kg and the synergistic dose of AmB 0.3 mg/kg + PED 10 mg/kg, were collected during Days 0 to 3 of the experiment. Larvae were sliced, were mixed in PBS with glass beads, and different dilutions were plated on Sabouraud plates supplemented with 100 mg/mL ampicillin. After 48 h at 37 °C, the CFU percentage of each situation was evaluated to determine efficacy.

2.8.3. Histopathological analysis

Three larvae in each group, i.e. untreated and treated with AmB 4 mg/kg, PED 40 mg/kg and the synergistic dose of AmB 0.3 mg/kg + PED 10 mg/kg, were collected at times 0, 1, 2 and 3 days. The larvae were fixed in 10% formalin for 24 h and were dehydrated with increasing concentrations of ethanol, were washed with xylol and, finally, were embedded in paraffin. They were sectioned serially at a thickness of 5 μm and were stained using Periodic acid–Schiff. The images were analysed using an optical microscope with a 40 × objective.

2.9. Rearing of BALB/c mice and experimental conditions

All animal protocols were approved and performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee of the Faculdade de Ciências Farmacêuticas, Universidade Estadual Paulista (UNESP) (Araraquara, Brazil). BALB/c mice were anaesthetised with ketamine/xylazine (80/10 mg/kg). Animals were inoculated intratracheally with 50 μL of 5×10^6 yeast cells/mL and survival was monitored daily.

2.10. Treatment assay in BALB/c mice infected with Cryptococcus neoformans

2.10.1. Survival time

Infected animals were treated intraperitoneally daily for 40 days with AmB 1 mg/kg, PED 40 mg/kg or the combination treatment of AmB 0.3 mg/kg + PED 10 mg/kg. To determine the survival time, the animals were checked daily. The toxicity of these doses was evaluated (data not shown).

2.10.2. Fungal burden

Lungs and brain were excised after 7 days and 14 days and were homogenised in PBS using a tissue grinder [25]. The suspension was plated on Sabouraud plates supplemented with 100 mg/mL ampicillin and was incubated at 37 °C for 48 h. The CFU/organ of each condition was evaluated.

2.10.3. Histopathological assay

Lungs were excised from mice after 14 days of treatment and were fixed in formalin for 48 h at room temperature. The tissues were dehydrated and were embedded in paraffin. Tissue sections were stained with haematoxylin and eosin. The images were analysed under an optical microscope with a 20 × objective.

2.11. Statistical analysis

All statistical analyses were performed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA). The representative graphics of treatment of infection by *C. neoformans* in cell culture and cytotoxicity was analysed by two-way analysis of variance (ANOVA) with multiple comparison by Bonferroni post-test. Survival curves were analysed by log-rank (Mantel–Cox) test. Fungal burden was analysed using the *t*-test. A *P*-value of <0.05 was considered statistically significant.

3. Results

3.1. In vitro antifungal activity and cytotoxicity

Table 1 shows the MIC and MFC values of PED as well as PED in combination with AmB. The MFC was equal to the MIC for the antifungal combination. The combinations tested decreased the MIC value by four-fold compared with AmB and PED. The FICI and FFCI values were considered promising, showing a synergistic effect. In the evaluation of compound safety, PED presented IC_{50} values higher than the MIC and MFC values found in the susceptibility test. PED

Table 1
Susceptibility values of pedalitin (PED) and amphotericin B (AmB) alone and in combination against *Cryptococcus* spp., and selectivity index and IC_{50} values of PED in different cell lines.

Susceptibility test	Chequerboard assay		
MIC/MFC	MIC[a] (AmB/PED)	FICI/effect	FFCI/effect
AmB, 0.125/0.125	0.03/1	0.49/Syn	0.49/Syn
PED, 3.9/3.9			
HepG2	MRC-5	NOK	U87-MG
Selectivity index			
33.7	49.1	30.5	44.9
IC_{50} (mg/L)			
HepG2	MRC-5	NOK	U87-MG
131.7	191.7	119	175

MIC, minimum inhibitory concentration; MFC, minimum fungicidal concentration; FICI, fractional inhibitory concentration index; FFCI, fractional fungicidal concentration index; Syn, synergistic; IC_{50}, concentration at which 50% of the cells were killed; HepG2, human hepatoma; MRC-5, human pulmonary fibroblast; NOK, keratinocyte oral mucosa; U87-MG, human glioblastoma.

[a] MIC in the combination (mg/L).

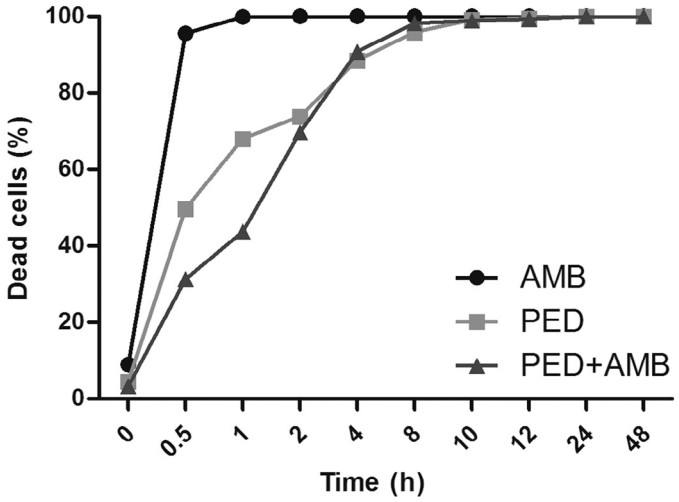

Fig. 1. Time–kill analysis of *Cryptococcus neoformans*. *Cryptococcus neoformans* was treated with 0.125 mg/L amphotericin B (AmB), 4 mg/L pedalitin (PED) and a synergistic combination (1 mg/L PED + 0.03 mg/L AmB) and death was observed in relation to contact time (0, 0.5, 1, 2, 4, 8, 10, 12, 24 and 48 h). Rates are representative of the percentage of dead cells referring to the mean count of CFU after plating.

showed the highest IC_{50} value for all the cell types; moreover, SI values of ≥10 were found.

3.2. Cryptococcus neoformans *in vitro time–kill assay*

AmB at 0.125 mg/L showed strong activity, with >95% killing in 30 min of contact with *C. neoformans*. Treatment with PED at 4 mg/L killed 50% of the yeast cells in 30 min and 100% after 8 h. The combined treatment (AmB 0.03 mg/L + PED 1 mg/L) killed a high number of yeast cells within 30 min (31%) and >98% after 8 h (Fig. 1).

3.3. *Influence of synergistic treatment in infection by* Cryptococcus neoformans *in cell culture*

A significant reduction in infection was observed at all times ($P < 0.05$). After 30 min, 50% reduction in infection was observed with the treatments in both cell types assayed. After 2 h of treatment, 60% and 40% infection levels were observed in lung fibroblasts and in glioblastoma cells, respectively. Comparing treatments with AmB and PED showed significant differences at 30 min ($P < 0.05$), whilst similar activity of these two compounds was observed after 1 h and 2 h. When the data of PED were compared with the synergistic combination, there was difference just at 30 min for glioblastoma cells ($P < 0.05$) (Fig. 2). AmB and the synergistic combination were different just for lung fibroblasts at 30 min ($P < 0.05$).

3.4. *Efficacy of treatment with pedalitin alone and combined with amphotericin B in a* Galleria mellonella *model*

3.4.1. *Survival curve of* Cryptococcus neoformans

The fungal suspension of 1×10^6 cells/larvae was chosen for the following tests. On Days 2, 3 and 4, 75%, 25% and 0% of the larvae were alive, respectively (Fig. 3A).

3.4.2. *Toxicity assay of diluents and substances*

The DMSO concentration used was 5% for solubilisation of agents, as higher concentrations caused larval death. For AmB, no tested doses showed toxicity (100% survival). For PED, toxicity was not observed below 40 mg/kg; however, there was a high larval mortality rate between 50 mg/kg and 200 mg/kg (Fig. 3B).

3.4.3. *Efficacy of synergistic treatment*

All of the AmB doses (1, 2 and 4 mg/kg) increased larvae survival ($P < 0.05$) (Fig. 4). The dose of 4 mg/kg was selected as a control for the subsequent experiments. Regarding PED, all of the doses (6.25, 12.5, 25 and 40 mg/kg) were able to increase the survival of infected larvae ($P < 0.05$); however, the dose with the highest survival percentage was 40 mg/kg. At this dose, 37% of the larvae were alive at the end of the experiment (Fig. 4B). To evaluate the synergistic efficacy in vivo, the antifungal combination of 0.3 mg/kg AmB + 10 mg/kg PED, as well as the substances alone at the same dose, were evaluated. Treatment with 0.3 mg/kg AmB led to 50% and 18.7% survival of larvae on the fifth and sixth days of the experiment. When analysing 10 mg/kg PED treatment, 26% and 0% survival of larvae was observed on the fifth and sixth days of the experiment. However, for the combination treatment, >56% of larvae were alive at the end of the experiment (Fig. 4C).

There was large decrease in *C. neoformans* CFUs treated with AmB (4.0 mg/kg), PED (40 mg/kg) and the combination of the two substances (AmB 0.3 mg/kg + PED 10 mg/kg). On Day 1, reductions of 88%, 87% and 93% were observed after treatment with AmB, PED or the combination therapy, respectively, and on Day 4 there was almost 100% reduction of the fungal burden (Fig. 4D–F).

Finally, the efficacy of the synergism was observed by histopathology of larvae from Days 0 to 3. Severe tissue destruction and

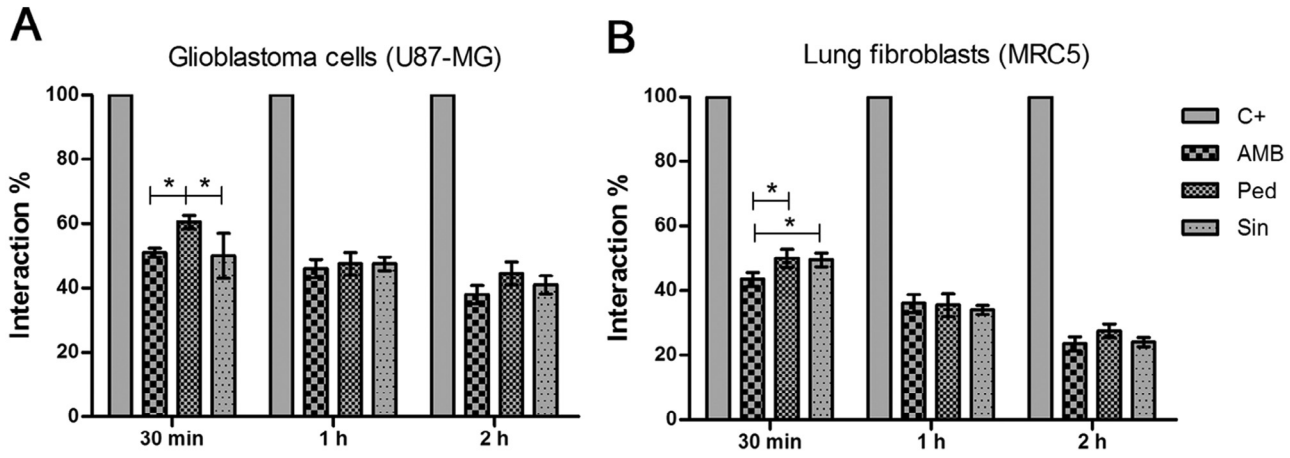

Fig. 2. Effects after treatment with 0.125 mg/L amphotericin B (AmB), 4 mg/L pedalitin (PED) and the synergistic combination of 1 mg/L PED + 0.03 mg/L AmB (Sin) for 30 min, 1 h and 2 h in (A) U87-MG and (B) MRC-5 cells infected with *Cryptococcus neoformans*. * $P < 0.05$.

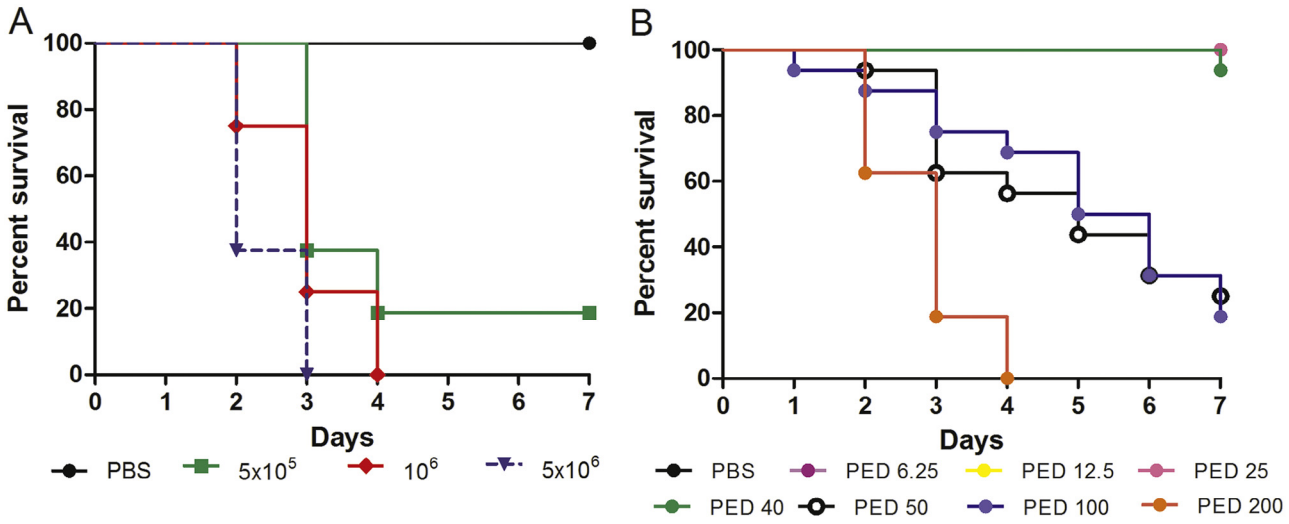

Fig. 3. Standardisation of *Galleria mellonella* conditions for efficacy tests. (A) Survival curve of *Cryptococcus neoformans* ATCC 90012 using concentrations of 5×10^5, 1×10^6 and 5×10^6 cells/mL. (B) Pedalitin (PED) toxicity using doses of 6.25, 12.5, 25, 40, 50, 100 and 200 mg/kg. PBS, phosphate-buffered saline.

large amounts of yeast were observed in the untreated larvae infected with *C. neoformans*. Treatment with AmB 4 mg/kg, PED 40 mg/kg or the combination of these substances (AmB 0.3 mg/kg + PED 10 mg/kg) reduced the number of yeast. Fig. 5 shows the histopathology of untreated and treated larvae on the third day of the experiment, illustrating the high efficacy of these treatments.

3.5. Efficacy of treatment with pedalitin alone and combined with amphotericin B in a murine model

Treatment with AmB 1 mg/kg, PED 40 mg/kg or the combination therapy of AmB 0.3 mg/kg + PED 10 mg/kg significantly prolonged the survival of mice ($P < 0.05$) compared with infected

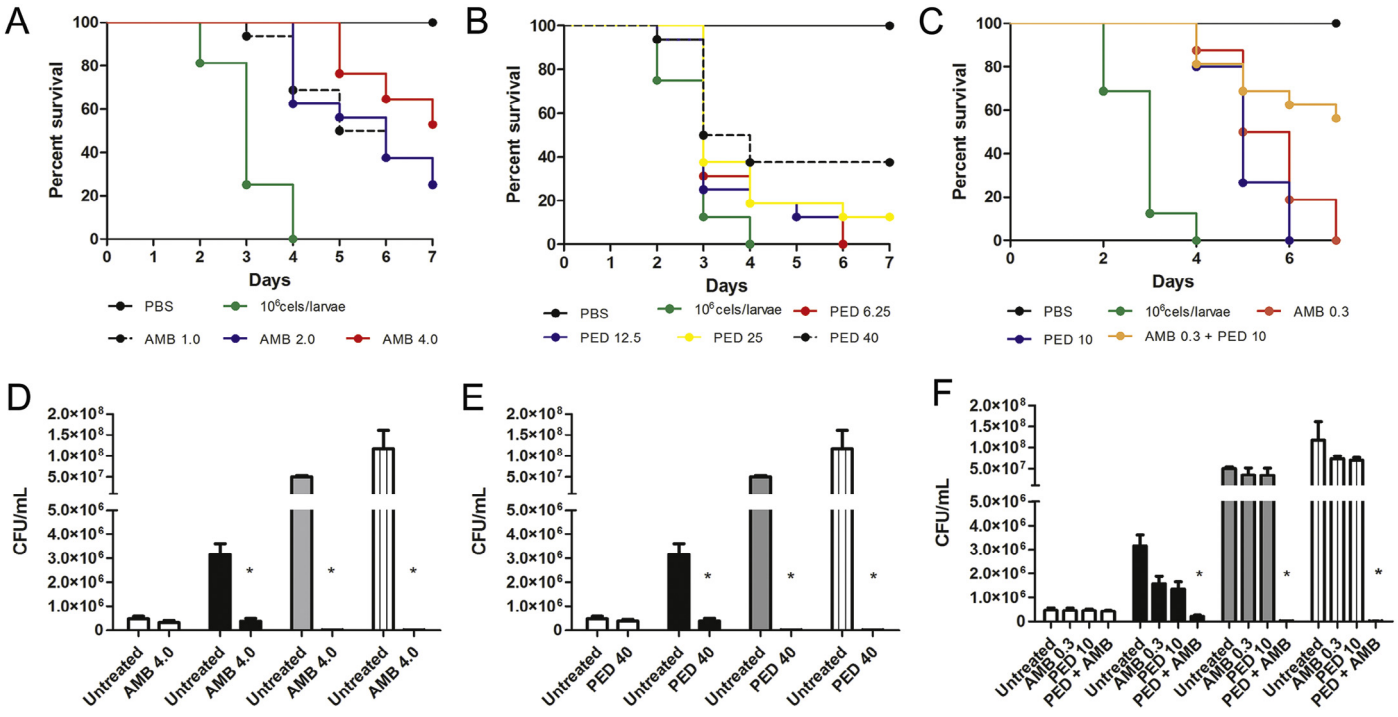

Fig. 4. Efficacy of the combination of amphotericin B (AmB) and pedalitin (PED) in the *Galleria mellonella* model. (A–C) Survival curves of larvae infected with *Cryptococcus neoformans* (1×10^6 cells/larva) and treated with AmB 1, 2 and 4 mg/kg (A), PED 6.25, 12.5, 25 and 40 mg/kg (B) and AmB 0.3 mg/kg, PED 10 mg/kg and AmB 0.3 mg/kg + PED 10 mg/kg (C). (D–F) Fungal burden of larvae infected with *C. neoformans* (1×10^6 cells/larvae) and treated with AmB 4 mg/kg (D), PED 40 mg/kg (E) and AmB 0.3 mg/kg, PED 10 mg/kg and AmB 0.3 mg/kg + PED 10 mg/kg (F). Colours represent Day 0 (white), Day 1 (black), Day 2 (grey) and Day 3 (striped). * $P < 0.001$.

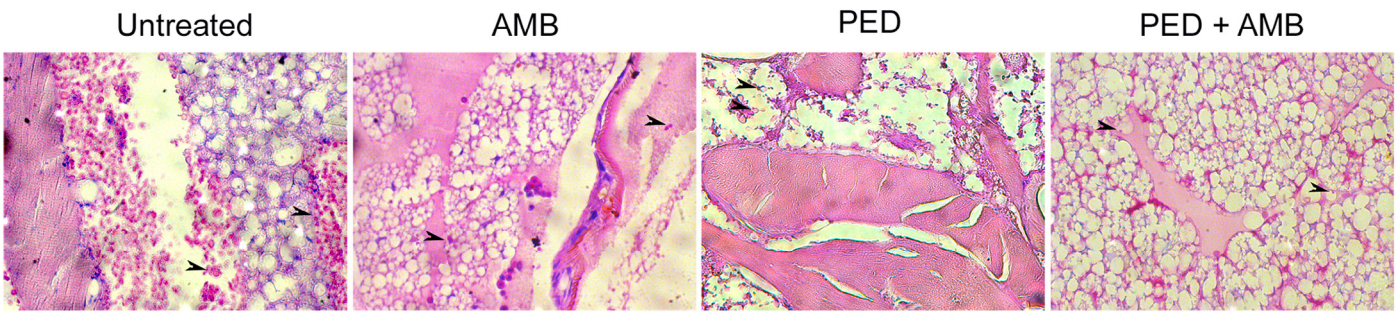

Fig. 5. Histopathology of untreated and treated *Galleria mellonella* larvae. Larvae infected with *Cryptococcus neoformans* (1×10^6 cells/mL), either untreated or treated with 4 mg/kg amphotericin B (AmB), 40 mg/kg pedalitin (PED) and AmB 0.3 mg/kg + PED 10 mg/kg on the third day of the experiment and stained with Periodic Acid–Schiff. Arrows indicate *C. neoformans* cells. Magnification of 400×.

and untreated mice. After 20 days and 40 days of the experiment, survival of 60% and 40% was observed with the combined therapy, respectively; moreover, no difference was observed between the treatments with the compounds alone at higher doses (Fig. 6).

A high fungal burden was observed in the lungs of untreated animals. Treatment with AmB, PED or the combination therapy significantly decreased the fungal burden in both organs. After 7 days of treatment, a significant reduction of 48% in the fungal burden of the lungs and brain was observed for all treatments (Fig. 7). Moreover, after 14 days of treatment, a reduction of >80% in the number of colonies was observed for all treatments for both organs.

For histopathology, mouse lungs were selected because this organ is the most strongly affected in cryptococcosis (Fig. 8). The histopathological findings revealed fungal cells with increased capsules. After 14 days of treatment with AmB, PED or combination therapy, a significant reduction was observed in the number of yeasts. These results indicate a higher efficiency of combination therapy compared with monotherapy.

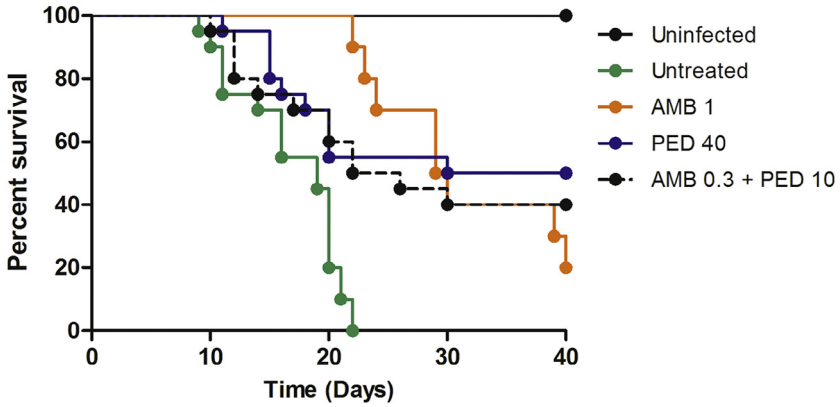

Fig. 6. Efficacy of the combination of amphotericin B (AmB) and pedalitin (PED) in a murine model. Survival curves of mice infected with *Cryptococcus neoformans* and treated with 1 mg/kg AmB, 40 mg/kg PED and AmB 0.3 mg/kg + PED 10 mg/kg.

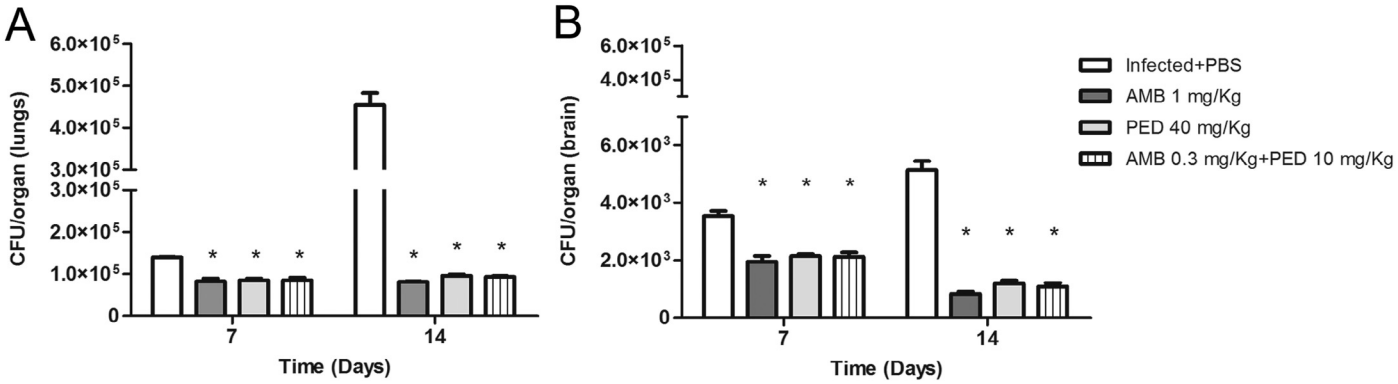

Fig. 7. Fungal burden (CFU/organ) of (A) lungs and (B) brain of mice infected with *Cryptococcus neoformans* and treated with amphotericin B (AmB), pedalitin (PED) or the combination therapy. * $P < 0.05$. Data are given as the mean ± standard deviation.

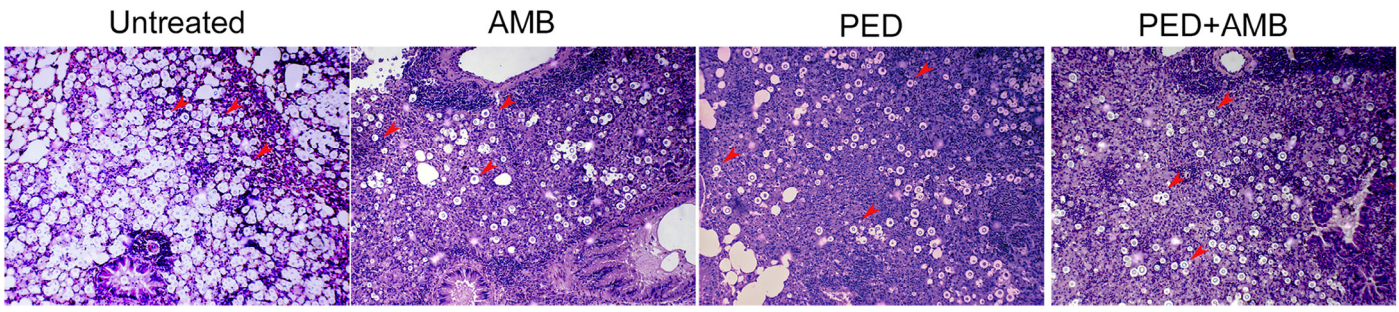

Fig. 8. Histopathological examination of lungs of mice infected with *Cryptococcus neoformans* and treated with amphotericin B (AmB), pedalitin (PED) or combination therapy by haematoxylin and eosin staining. Arrows indicate *C. neoformans* cells. Magnification of 200 ×.

4. Discussion

Because of the complications in the treatment of cryptococcosis, the study of plants and their derivatives as an alternative source of new antifungal compounds has been highlighted in recent years [26]. In vitro studies with PED showed antifungal activity against *C. neoformans*. Moreover, PED shows synergistic activity with AmB, reducing the necessary concentration of both substances by four-fold. PED also showed savings when the SI and IC_{50} were evaluated.

The combination of antifungal substances is a promising way to increase the percentage of successful treatment and reduces the required concentrations of the antifungals and consequently their toxicity [13]. Clinical isolates of *C. neoformans* were tested with combinations of AmB and different compounds resulting in a reduction of capsule induction [27]. Another study tested the combination of AmB and fluconazole against *C. gattii* strains resulting in synergistic to antagonistic activity depending on the strain and concentration tested [28].

In the current study, the effect of antifungal treatment on host–yeast interaction was analysed in MRC-5 and U87-MG cells. High inhibition of yeast–cell interaction was observed after treatment with the substances alone or in combination. Adhesion of pathogenic microorganisms to host tissues is considered indispensable for colonisation and progression of the infectious process, and the large number of tissues that fungi can colonise and infect suggests that they have a variety of surface molecules that allow the adhesion process [29].

To evaluate the in vitro efficiency, *C. neoformans* was subjected to treatments with PED, AmB and the combination treatment for different times (30 min to 48 h). The fungicidal effect of the compounds was evaluated by time–kill during 48 h of exposure. Significant results were obtained after 8 h of exposure to the tested substances; after contact with AmB and PED both alone and in combination, the yeast death rate was 100%. By comparison, Pereira de Sá et al. [30] evaluated the antifungal activity of 13 compounds against *C. neoformans* and *C. gattii* and selected 4 compounds. They conducted time–kill assays with these compounds and similar results to AmB were obtained. However, when the compounds were combined with AmB and/or fluconazole, no effect was found.

The *G. mellonella* model was used as an alternative animal for in vivo assays. Initially, *G. mellonella* was infected with different concentrations of *C. neoformans*, and 1×10^6 cells/larva was selected to correspond to a balance where not all larvae died at the beginning of the experiment but they also did not die too late. A previous study used a similar inoculum concentration [31]. *Galleria mellonella* has been used to evaluate the toxicity of compounds and solvents [32]. In the current study, doses of >5% DMSO and doses ≥50 mg/kg PED were not used in the efficacy experiments because they showed a toxic effect on larvae.

To evaluate the *G. mellonella*–*C. neoformans* in vivo efficacy of the combination of PED and AmB, three factors were evaluated: survival curve; fungal burden; and histological analysis. All doses of PED tested increased the survival of *C. neoformans*-infected larvae, and survival was highest for 40 mg/kg. Regarding the PED and AmB combination, the treatment presented the same effect on larvae survival as high doses of these compounds alone (4 mg/kg AmB or 40 mg/kg PED), indicating that the combination presents similar activity to the substances alone. A significant decrease in fungal burden was observed from macerated larvae and the histology images. The use of *G. mellonella* to verify in vivo antifungal efficacy and in vivo drug resistance has been reported for *C. neoformans* and other pathogenic fungi, and this alternative model appears appropriate for this purpose [33,34]. *Galleria mellonella* has also been described as an alternative animal model to screen new antifungal compounds [35,36].

After screening of the antifungal doses and evaluation of the synergistic combination efficacy using the alternative animal model *G. mellonella*, this effect was also evaluated in a murine model. As observed in *G. mellonella*, treatment with the combination of PED + AmB significantly increased the survival of the mice, and again the combined treatment was as efficient as AmB monotherapy at higher doses. Similar results were observed for the fungal burden and histopathological assays, proving the efficacy of this treatment. The correlation of fungal virulence and response to antifungal treatment between *G. mellonella* and mice has been described in different studies [37,38].

5. Conclusion

This study confirms the potential synergistic effect of combined therapy with PED and AmB against *C. neoformans* through various tests that validate the final results, again showing the effectiveness of the use of the *G. mellonella* alternative model as an excellent methodology for the optimisation of subsequent trials using the highly controversial murine model.

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