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Original article

Antifungal activity of altenusin isolated from the endophytic fungus *Alternaria* sp. against the pathogenic fungus *Paracoccidioides brasiliensis*

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ABSTRACT

Background: Altenusin is a biphenyl derivative isolated from different species of fungi, which presents several biological activities.
Aims: We report the antifungal activity of the altenusin isolated from the endophytic fungus *Alternaria* sp., against clinical isolates of *Paracoccidioides brasiliensis*, and its action on cell walls of *P. brasiliensis* and the nonpathogenic yeast *Schizosaccharomyces pombe*.
Methods: In vitro antifungal activity of altenusin was evaluated using the broth microdilution method against 11 strains of *P. brasiliensis* and one strain of *S. pombe*. The effects of the altenusin on the cell wall were estimated using the sorbitol protection assay.
Results: The altenusin presented strong activity against *P. brasiliensis* with MIC values ranging between 1.9 and 31.2 μg/ml, and 62.5 μg/ml for *S. pombe*. Our results demonstrated that the MIC values for altenusin were increased for *P. brasiliensis* Pb18 and for *S. pombe* when the medium was supplemented with sorbitol. Additionally, *S. pombe* cells treated with altenusin were more rounded in shape than untreated cells.
Conclusions: Altenusin showed activity against clinical strains of *P. brasiliensis* at the concentration tested, and this compound probably affects fungal cell walls. These findings suggest that altenusin could act through the inhibition of cell wall synthesis or assembly in *P. brasiliensis* and *S. pombe*, and could be considered as a lead compound for the design of new antifungals.

Actividad antifúngica de la altenusina aislada del hongo endofítico *Alternaria* sp. frente al hongo patógeno *Paracoccidioides brasiliensis*

Palabras clave:
Actividad antimicótica
Altenusina
Paracoccidioides brasiliensis
Schizosaccharomyces pombe
Pared celular

RESUMEN

Antecedentes: La altenusina es un derivado bifenilo aislado de diferentes especies de hongos, que presenta una diversidad de actividades biológicas.
Objetivos: Describimos la actividad antifúngica de la altenusina aislada del hongo endofítico *Alternaria* sp. frente a aislamientos clínicos de *Paracoccidioides brasiliensis*, y su acción sobre las paredes celulares de *P. brasiliensis* y la levadura no patógena *Schizosaccharomyces pombe*.
Métodos: Se valoró la actividad antifúngica de la altenusina in vitro usando un método de microdilución en caldo frente a 11 cepas de *P. brasiliensis* y una cepa de *S. pombe*. Los efectos de la altenusina sobre la pared celular se estimaron utilizando un análisis de protección con sorbitol.
Resultados: La altenusina presentó una potente actividad frente a *P. brasiliensis* con valores de concentración inhibitoria mínima (CIM) que variaron entre 1,9 y 31,2 μg/ml, y de 62,5 μg/ml para *S. pombe*. Los resultados del presente estudio demostraron que los valores CIM de la altenusina aumentaron para Pb18 de *P. brasiliensis* y para *S. pombe* cuando el medio se suplementó con sorbitol. Además, las células de *S. pombe* tratadas con altenusina adoptaron una forma más redondeada que las no tratadas.

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Conclusiones: Con la concentración examinada, la altenusina demostró actividad frente a las cepas clínicas de *P. brasiliensis*, y es probable que este preparado afecte a las paredes de las células micóticas. Estos hallazgos sugieren que la altenusina podría actuar a través de la inhibición de la síntesis o ensamblado de la pared celular en *P. brasiliensis* y *S. pombe* y podría considerarse la molécula inicial para el diseño de nuevos antimicóticos.

Altenusin is a biphenyl derivative isolated from different species of fungi, which presents antioxidant properties and the ability to inhibit several enzymes, such as myosin light chain kinase,[13] sphingomyelinase,[25] acetylcholinesterase,[10] and HIV-1 integrase.[19] Altenusin also exhibits broad antimicrobial activity against several drug-resistant pathogens with minimum inhibitory concentration (MIC) values of 31.2 to 125 μg/ml.[9] Cota et al.[2] isolated altenusin from the endophytic fungus *Alternaria* sp. UFMGCB 55 associated with the plant *Trixis vauthieri* DC (Asteraceae), and this compound presented the ability to inhibit the enzyme trypanothione reductase (TryR) from *Trypanosoma cruzi*, an enzyme involved in the protection of *Trypanosoma* and *Leishmania* species against oxidative stress.[5]

The increase in frequency of immunocompromised individuals among the world's human population has resulted in an ever-growing number of serious fungal infections, which will require new antimicrobial therapy.[16] The pathogenic fungus *Paracoccidioides brasiliensis* is the etiologic agent of paracoccidioidomycosis (PCM), a human systemic mycosis for which the portal of entry of the fungus is the respiratory tract via the inhalation of airborne propagules. Although geographically confined, paracoccidioidomycosis constitutes one of the most prevalent deep mycoses in Central and Southern America.[7] Antifungals used in cases of PCM are sulfonamides, amphotericin B, or azoles, mainly itraconazole. Extended periods of treatment are necessary and relapses of the disease commonly occur.[23] In the present work we report the antifungal activity of altenusin isolated from the endophytic fungus *Alternaria* sp. UFMGCB 55, against clinical strains of *P. brasiliensis*, and we also show the action of altenusin on cell walls of *P. brasiliensis* and the nonpathogenic yeast *Schizosaccharomyces pombe*.

Materials and methods

Isolation of altenusin

The biphenyl derivative altenusin (Fig. 1) was isolated from ethyl acetate extract of the endophytic fungus *Alternaria* sp. UFMGCB 55, which was recovered from leaves of the bioactive plant *Trixis vauthieri* DC (Asteraceae) as described by Cota et al.[2] This compound was stored at −20 °C at 20 mg/ml.

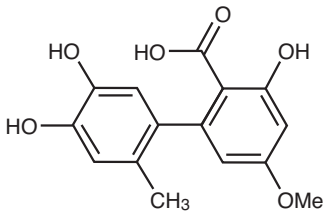

Fig. 1. The biphenyl derivative altenusin isolated from the endophytic fungal strain *Alternaria* sp. UFMGCB 55.

Table 1
Antifungal activity[d] of altenusin against 11 strains of *Paracoccidioides brasiliensis* and one of *Schizosaccharomyces pombe.*

Fungal strains	Altenusin	Amphotericin B	Trimethropin–sulfamethoxazole
ED01[a]	1.9	0.031	75
Pb 01[a]	3.9	0.12	300
Pb 2[b]	1.9	0.031	75
Pb B339[b]	1.9	0.015	75
Pb 11[b]	3.9	0.12	150
Pb 14[b]	1.9	0.125	75
Pb 8[b]	1.9	0.062	300
Pb 18[b]	15.6	0.062	300
Pb 3[c]	31.2	0.015	300
Pb 1578[a]	1.9	0.062	75
Pb 4[c]	31.2	0.062	150
S. pombe	62.5	0.025	–

[a] *Paracoccidiodes brasiliensis* isolate representative of the new phylogenetic species Pb-01-like.

[b] *Paracoccidiodes brasiliensis* isolates representative of the phylogenetic species S1 (Pb-18, Pb-B339, Pb-14, Pb-8 and Pb-11) (reported by Matute et al.[11] as B17, B18, B22, B25, B21, Table S1).

[c] *Paracoccidiodes brasiliensis* isolates representative of the phylogenetic species PS2 (Pb-2, Pb-3 and Pb-4) (reported by Matute et al.[11] as V2, B26, B23 Table S1).

[d] Values were expressed in μg/ml.

Antifungal activity assays

Maintenance of P. brasiliensis *and* S. pombe strains

Eleven clinical *P. brasiliensis* strains, of two established cryptic phylogenetic species (S1 and PS2) and one possibly new cryptic species, were used in the biological assays (Table 1). Isolates Pb18, PbB339, Pb14, Pb8 and Pb11 belong to the cryptic species S1, and isolates Pb03, Pb2 and Pb4 belong to the PS2 cryptic phylogenetic species.[11] Isolates Pb01, ED01 and Pb1578 are considered representative of a new phylogenetic species "Pb-01-*like*".[1] The strains of *P. brasiliensis* were maintained at Departamento de Microbiologia of Universidade Federal de Minas Gerais, Brazil, by weekly transfer in solid YPD medium (yeast extract, peptone and dextrose) at 37 °C. The wild type of *S. pombe* (PN556) was maintained on Sabouraud dextrose agar (Oxoid, Basingstoke, UK).

Minimal inhibitory concentrations determination

The bioassays with all clinical strains of *P. brasiliensis* and *S. pombe* (PN556) were performed following the CLSI M27-A2 guidelines[12,14] with the modifications suggested by Johann et al.[8] Amphotericin B (AMB) (Sigma, St Louis, USA) and trimethoprim/sulfamethoxazole (SMT/TMP) were included as positive antifungal controls, being the stock solutions prepared in DMSO and water, respectively. Twofold serial dilutions were prepared exactly as outlined in CLSI document M 27-A2.[14]

Minimal fungicidal concentrations determination

The microtiter plate used to determine MIC values was also used to determine MFC values. The in vitro minimal fingicidal concentrations (MFC) of each compound tested was determined by streaking 10 μl from each well that showed complete inhibition (100% inhibition or a clear well), from the last positive well (growth similar to that of the growth control well), and from the growth

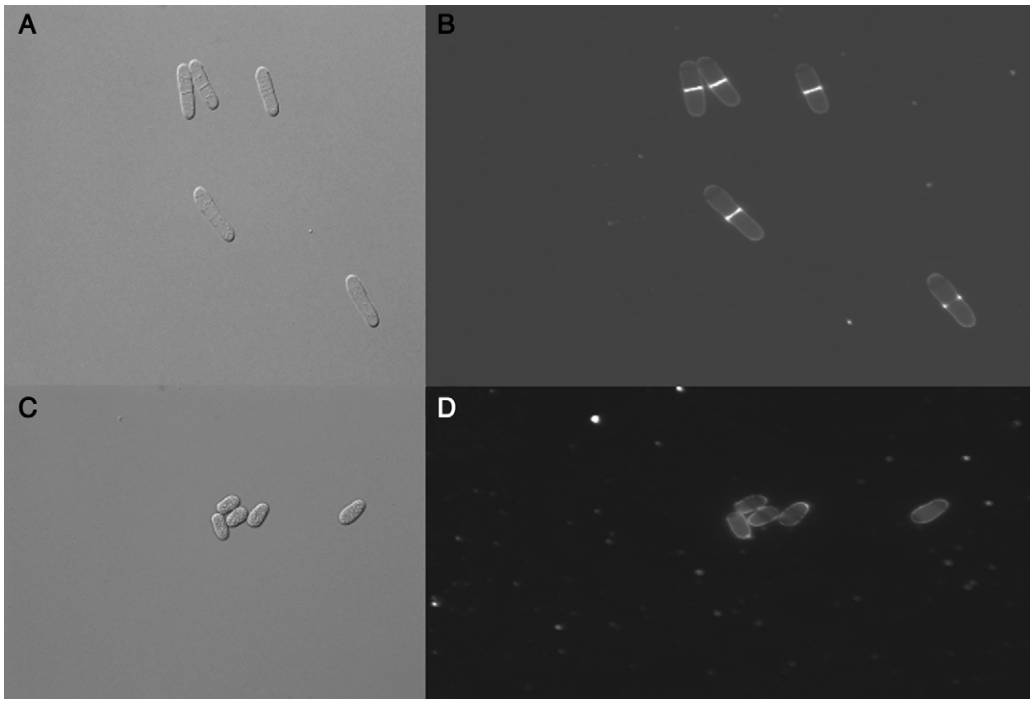

Fig. 2. *Schizosaccharomyces pombe* morphology after treatment with altenusin. A: Differential interphase contrast (DIC) micrographs of *S. pombe* cells in the absence of altenusin; B: Fluorescence micrographs of *S. pombe* stained with calcofluor white in the absence of altenusin; C: *S. pombe* DIC in the presence of altenusin (31.2 μg/ml); D: *S. pombe* stained with calcofluor white in the presence of altenusin (31.2 μg/ml).

control well onto YPD plates. The MFC was determined as the lowest drug concentration at which counts lower than three colonies were recovered after cultivation on YPD agar for 10 days at 37 °C.[4,18]

Sorbitol protection assays

MIC values were determined using *P. brasiliensis* strain Pb18 and *S. pombe* by the standard broth microdilution procedure as described above. Duplicate plates were prepared: one of them contained altenusin plus 0.8 M sorbitol as an osmotic support and the other one contained only altenusin. MICs were determined after 14 days for *P. brasiliensis* and 48 h for *S. pombe*.[3]

Cell morphology analysis

The model organism for cell morphology analysis was the nonpathogenic yeast *S. pombe*. *S. pombe* cell morphology was analyzed by fluorescence microscopy after cell staining with Calcofluor white. The *S. pombe* cells were grown in YES-yeast extract plus supplements: adenine, leucine, histidine, or uracil (100 mg/ml; Sigma) liquid medium to mid log-phase to an A_{600} of 0.6.[21] Images were captured with a Leica DM 4000B fluorescence microscope coupled to a cooled Leica DC 300F camera and IM_{50} software. For analyses of vacuoles, the cells of *S. pombe* were stained with CDCFDA (carboxydichlorofluorescein diacetate; Molecular Probes) and observed under fluorescence microscopy.

Results

Preliminary results demonstrated that altenusin was not active against *Candida albicans* (>250.0 μg/ml) (data not showed), but *P. brasiliensis* strains were susceptible to altenusin, with MIC values between 1.9 and 31.2 μg/ml. *P. brasiliensis* strains Pb ED01, 2, B339, 11, 14, 8, and 1578 were the most susceptible with MIC values of 1.9 μg/ml whereas the strains Pb03 and Pb04 were less susceptible to altenusin with an MIC value of 31.2 μg/ml. MIC values found for amphotericin B were between 0.031 and 0.12 μg/ml for the *P. brasiliensis* strains tested, with better activity against the isolates Pb ED01 and Pb2. The drug trimethropin–sulfamethoxazole presented high MIC values, ranging between 75.0 and 300.0 μg/ml. For all strains of *P. brasiliensis* the MFC values obtained for altenusin were equal to the MIC values. In addition, altenusin also presented activity against *S. pombe* with a MIC value of 62.5 μg/ml.

Altenusin modified MIC values to *P. brasiliensis* and *S. pombe* after addition of sorbitol to the culture medium. The MIC value against *P. brasiliensis* (strain Pb18) in culture medium treated with altenusin and supplemented with sorbitol was 62.5 μg/ml whereas the MIC was 15.6 μg/ml in the same medium without the addition of sorbitol. MIC values obtained against *S. pombe* were 62.5 μg/ml and 125.0 μg/ml in absence and presence of sorbitol in the medium, respectively. These results suggested that the antifungal activity of altenusin could affect fungal cell walls. When *S. pombe* cells stained with calcofluor white were observed in fluorescence microscopy, cells treated with altenusin were more rounded than untreated cells (Fig. 2). Tests to observe whether vacuoles of the cells treated with altenusin were affected by the compound were negative. Differences between control *S. pombe* cells and cells treated with altenusin were not observed (Fig. 3).

Discussion

We assayed the altenusin against fungal isolates of *P. brasiliensis* of two distinct cryptic phylogenetic species: S1 (Pb18, PbB339, Pb14, Pb8 and Pb11) and PS2 (Pb03, Pb2 and Pb4).[11] The cryptic species S1 was more susceptible than the cryptic species S2, with MIC values of 1.9–15.5 μg/ml for the first and MIC values of 1.9–31.2 μg/ml for the second. Altenusin was also tested against *P. brasiliensis* isolated Pb01, ED01 and Pb1578, which are considered representative of a new phylogenetic species "Pb-01-*like*".[1] Teixeira et al.[22] recommended the formal description of the "Pb-01-*like*" (Pb 01, 1578 and ED01) cluster as a new species, *Paracoccidioides lutzii*.[22] The isolates of the *P. lutzii* group treated with altenusin presented the highest susceptibility, with MIC values ranging between 1.9 and 3.9 μg/ml. On the basis of the MIC values for altenusin,

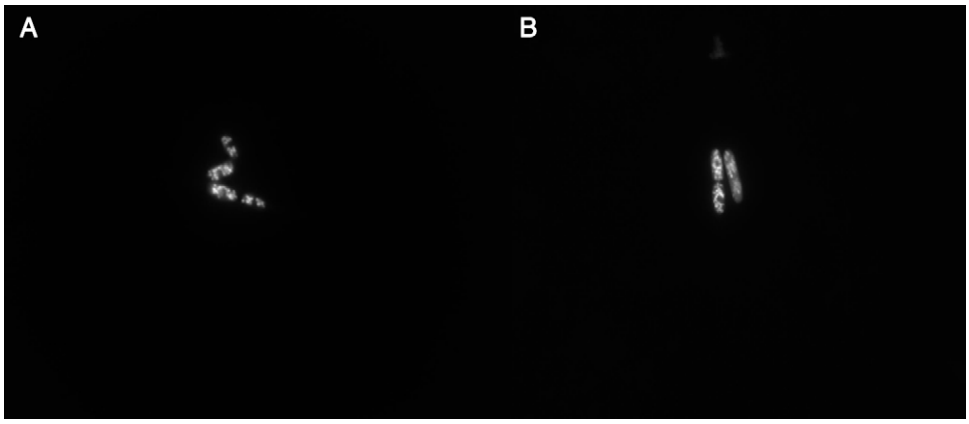

Fig. 3. *Schizosaccharomyces pombe* stained with CDFDA (carboxydichlorofluorescein diacetate) for observation of vacuoles. A: *S. pombe* in presence of altenusin (31.2 μg/ml); B: *S. pombe* in the absence of altenusin.

isolates of *P. brasiliensis* were around 50 times more susceptible to this compound than to trimethropin–sulfamethoxazole in vitro assays. Sulfonamides are the first class of drugs available for treating patients with PCM, but long periods of treatment may be required (more than 2 years).[24] In addition, the identical MFC and MIC values presented by altenusin may be important as the altenusin could kill the pathogen in situations when infection occurs in sites not easily accessed by host defenses.

According to Frost et al.,[6] a distinctive feature of the specific inhibitors of the fungal cell wall is that the antifungal effect is reversed in media containing an osmotic stabilizer such as sorbitol. Cell wall disruptive and osmotic destabilizing agents lead to cell wall rearrangements that enable fungal cells to survive.[14] Our results suggest that altenusin could act through the inhibition of cell wall synthesis or assembly in *P. brasiliensis* and *S. pombe* cells. Cell morphology analysis was performed with *S. pombe*, a yeast that is an excellent model organism for the study of cell walls. The major *S. pombe* glucose polymers of the cell wall are similar to those of *P. brasiliensis*, with presence of β-D-(1,3) glucan and α-D-glucan.[15,17,20] In the present work, the *S. pombe* cells treated with altenusin in sub-inhibitory concentration were smaller in size and more rounded than control (altenusin absence) cells. It is known that β-D-(1,3) glucan synthase inhibitors produce hallmark changes in the morphologies of yeasts and filamentous fungi.[3,17] According to Frost et al.,[6] the target in cell walls is unknown when *C. albicans* presents smaller rounded cells. Altenusin showed interesting in vitro activity against clinical strains of both phylogenetically cryptic species of *P. brasiliensis*, with low MIC values when compared to trimethropin–sulfamethoxazole. This compound could be considered as a lead compound for the design of new antifungals. Our results suggested that the antifungal activity of altenusin affects fungal cell walls, but new assays will be performed to establish its specific action mechanism.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Acknowledgments

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References

1. Carrero LL, Nino-Vega G, Teixeira MM, Carvalho MJ, Soares CM, Pereira M, et al. New *Paracoccidioides brasiliensis* isolate reveals unexpected genomic variability in this human pathogen. Fungal Genet Biol. 2008;45:605–12.
2. Cota BB, Rosa LH, Caligiorne RB, Rabello ALT, Alves TMA, Rosa CA, et al. Altenusin, a biphenyl isolated from the endophytic fungus *Alternaria* sp., inhibits trypanothione reductase from *Trypanosoma cruzi*. FEMS Microbiol Lett. 2008;285:177–82.
3. Escalante A, Gattuso M, Pérez P, Zacchino S. Evidence for the mechanism of action of the antifungal phytolaccoside B isolated from *Phytolacca tetrâmera* Hauman. J Nat Prod. 2008;71:1720–5.
4. Espinel-Ingroff A, Boyle K, Sheehan DJ. *In vitro* antifungal activities of voriconazole and reference agents as determined by NCCLS methods: review of the literature. Mycopathologia. 2001;150:101–15.
5. Fairlamb AH. Future prospects for the chemotherapy of Chagas's disease. Medicina (B Aires). 1999;59:179–87.
6. Frost D, Brandt K, Cugier D. A whole-cell *Candida albicans* assay for the detection of inhibitors towards fungal cell wall synthesis and assembly. J Antibiot (Tokyo). 1995;48:306–10.
7. Gow N, Nino-Vega G. *Paracoccidioides brasiliensis* – the man-hater. Mycologist. 2002;16:77–8.
8. Johann S, Cisalpino PS, Watanabe GA, Cota BB, Siqueira EP, Pizzolatti MG, et al. Antifungal activity of extracts of some plants used in the Brazilian traditional medicine against the pathogenic fungus *Paracoccidioides brasiliensis*. Pharmac Biol. 2010;48:388–96.
9. Kjer J, Wray V, Edrada-Ebel RA, Ebel R, Pretsch A, Lin W, et al. Xanalteric acids I and II and related phenolic compounds from an endophytic *Alternaria* sp. isolated from the mangrove plant *Sonneratia alba*. J Nat Prod. 2009;72:2053–7.
10. Lin J, Zhang P, Zheng Z, Dong Y, Lu X. Study on acetylcholinesterase inhibitor F01-2076A produced from fermentation broth of fungus F01-2076. J Hebei Univ Nat Sci. 2006;26:47–50.
11. Matute DR, McEwen JG, Puccia R, Montes BA, San-Blas G, Bagagli E, et al. Cryptic speciation and recombination in the fungus *Paracoccidioides brasiliensis* as revealed by gene genealogies. Mol Biol Evol. 2006;23:65–73.
12. Nakai T, Uno J, Ikeda F, Jauregui A. *In vitro* antifungal activity of micafungin (FK463) against dimorphic fungi: comparison of yeast-like and mycelial forms. Antimicrob Agents Chemother. 2003;47:1376–81.
13. Nakanishi S, Toki S, Saitoh Y, Tsukuda E, Kawahara K, Ando K, et al. Isolation of myosin light-chain kinase inhibitors from microorganisms – dehydroaltenusin, altenusin, atrovenetinone, and cyclooctasulfur. Biosci Biotechnol Biochem. 1995;59:1333–5.
14. National Committee for Clinical Laboratory Standards 2002. M27-A2. Method for broth dilution antifungal susceptibility testing of yeast, 2nd ed. Wayne, USA: National Committee for Clinical Laboratory Standards.
15. Onishi J, Meinz M, Thompson J, Curoto J, Dreikorn S, Rosenbach M, et al. Discovery of novel antifungal (1,3)-β-D-glucan synthase inhibitors. Antimicrob Agents Chemother. 2000;44:368–77.
16. Pfaller MA, Sheehan DJ, Rex JH. Determination of fungicidal activities against yeasts and molds: lessons learned from bactericidal testing and the need for standardization. Clin Microbiol Rev. 2004;17:268–80.
17. Pérez P, Ribas JC. Cell wall analysis. Methods. 2004;33:245–51.
18. Portillo A, Vila R, Freixa B, Ferro E, Parella T, Casanova J, et al. Antifungal sesquiterpene from the root of *Vernonanthura tweedieana*. J Ethnopharmacol. 2005;97:49–52.
19. Singh SB, Jayasuriya H, Dewey R, Polishook JD, Dombrowski AW, Zink DL, et al. Isolation, structure, and HIV-1-integrase inhibitory activity of structurally diverse fungal metabolites. J Ind Microbiol Biotechnol. 2003;30:721–31.
20. San-Blas G, Suzuki S, Hearn V, Pinel C, Kobayashi H, Mendez C, et al. Fungal polysaccharides. J Med Vet Mycol. 1994;32:321–8.

21. Soto T, Villar-Tajadura MA, Madrid M, Vicente J, Gacto M, Perez P, et al. Rga4 modulates the activity of the fission yeast cell integrity mapk pathway by acting as a rho2 gtpase-activating protein. J Biol Chem. 2010;285:11516–25.
22. Teixeira MM, Theodoro RC, Carvalho MJ, Fernandes L, Paes HC, Hahn RC, et al. Phylogenetic analysis reveals a high level of speciation in the *Paracoccidioides* genus. Mol Phylogenet Evol. 2009;52:273–83.
23. Thomaz L, Apitz-Castro R, Marques AF, Travassos LR, Taborda CP. Experimental paracoccidioidomycosis: alternative therapy with ajoene, compound from *Allium sativum*, associated with sulfamethoxazole/trimethoprim. Med Mycol. 2008;46:113–8.
24. Travassos LR, Taborda CP, Colombo AL. Treatment options for paracoccidioidomycosis and new strategies investigated. Expert Rev Anti Infect Ther. 2008;6:251–62.
25. Uchida R, Tomoda H, Dong YS, Omura S. Altenusin, a specific neutral sphingomyelinase inhibitor, produced by *Penicillium* sp. FO-7436. J Antibiot. 1999;52:572–4.