NPC Natural Product Communications

2015
Vol. 10
No. 9
1589 - 1592

# Antifungal Activity of Pyranonaphthoquinones Obtained from *Cipura paludosa* Bulbs

**Adriana Campos[a], Greice Maria Rodrigues Souza[b], Franco Delle Monache[a], Estefanía Butassi[c], Susana Zacchino[c] and Valdir Cechinel Filho[a*]**

[a]*Programa de Pós-Graduação em Ciências Farmacêuticas and Núcleo de Investigações Químico-Farmacêuticas (NIQFAR), Universidade do Vale do Itajaí – UNIVALI, Itajaí, Santa Catarina, Brazil*

[b]*Núcleo da Saúde - Centro Universitário Euro-Americano (UNIEURO), Brasília-DF, Brazil*

[c]*Farmacognosia, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, CP2000, Rosario, Argentina*

*cechinel@univali.br*

**Received: March 27th, 2015; Accepted: June 6th, 2015**

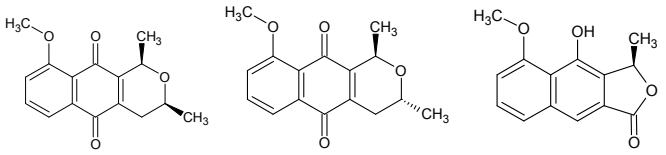
Previous studies with the bulbs of *Cipura paludosa* (Iridaceae) showed the presence of pyranonaphthoquinones, including eleutherine, isoeleutherine and eleutherol. The aim of this study was to evaluate the antifungal properties of these compounds. The activity was tested against the clinically relevant yeasts *Candida albicans*, *C. tropicalis*, *Saccharomyces cerevisiae* and *Cryptococcus neoformans* with the microbroth dilution method, following the guidelines of CLSI. Eleutherine, isoeleutherine and eleutherol all presented significant antifungal activity, especially the first two, the major components, with MIC values between 7.8 and 250 µg/mL. In conclusion, these results demonstrate that *C. paludosa* bulbs produce active principles with relevant antifungal potential, contributing, at least in part, to the antimicrobial effect evidenced for this plant and justifying its popular use against infections.

**Keywords:** *Cipura paludosa*, Antifungal activity, Pyranonaphthoquinones.

In the last years, considerable attention has been focused on natural products with antifungal properties, substantially increasing the number of antifungal drugs in this century [1]. *Cipura paludosa* Aubl. (Iridaceae), known as "batata-roxa", "alho-do-mato" and "cebolinha-do-campo", is widely found in the Amazon rainforest, in northern Brazil [2], and has been traditionally used to treat various conditions such as inflammation, infections and pain. Previous studies have shown biological activities of *C. paludosa*, including antinociceptive, anti-inflammatory and neuroprotective effects [3-5]. Regarding its chemical composition, previous studies demonstrated the presence of eleutherine, isoeleutherine and hongkonin, as well as a new component, 11-hydroxyeleutherine, in the dichloromethane extract of the bulbs. The main components (eleutherine and isoeleutherine) exhibit pronounced activity in different *in vivo* models of inflammation and hypernociception [2], and also promising antiproliferative activity. Screening of Brazilian medicinal plants had shown that the extracts of *C. paludosa* bulbs had promising antimicrobial action (unpublished results), and hence we have investigated the effects of eleutherine, isoeleutherine and eleutherol against some human pathogenic fungi.

The antifungal effect of the pyranonaphtoquinones eleutherine (**1**), isoeleutherine (**2**) and eleutherol (**3**) (Figure 1) isolated from *C. paludosa* bulbs, was analyzed against four pathogenic fungi: *Candida albicans, C. tropicalis, Saccharomyces cerevisiae* and *Cryptococcus neoformans*. The selection of these species to evaluate the antifungal activity was associated with the clinical relevance of their infections and previous studies, which indicated MIC values (µg/mL) of 125, 250, 62.5 and 15.6 for the dichloromethane fraction of this plant against these fungi, respectively (Cechinel Filho, Personal communication).

Values of MIC and MFC of compounds **1-3** are shown in Table 1. MIC values between 250 and 125 µg/mL were indicative of low




**(1)** **(2)** **(3)**

**Figure 1:** Structure of eleutherine (**1**), isoeleutherine (**2**) and eleutherol (**3**) isolated from *C. paludosa* bulbs.

activity, between 62.5 and 31.25 µg/mL moderate activity and ≤ 15.6 µg/mL, high activity. The percentage inhibition of fungal growth produced by the three compounds was calculated with the assistance of a microplate reader and the results are shown in Figure 2.

Data presented in Table 1 demonstrate that **1** and **2** showed the best activity against all the tested species, especially against *S. cerevisiae* and *C. neoformans*. Analyzing the $MIC_{50}$ and $MIC_{80}$, both compounds showed high activity against *C. neoformans*, and **1** also demonstrated high $MIC_{100}$ against this species demonstrating that **1** was more active than **2**. They are epimeric isomers and possess a naphthoquinone 1,4- moiety with the methyl group (β-methyl for **1** and α-methyl for **2**) as the only structural difference, Therefore, the higher activity of **1** seems to be related to the chirality at its pyran ring with the β-methyl group.

The prevalence of systemic fungal infections has increased significantly in the last two decades, especially in immunocompromised patients and those with critical illnesses, having enormous impact on morbidity and mortality [6,7]. The population also presents drug resistance due to the long use. There is, therefore, an urgent need for new antifungal chemical structures as alternatives to the existing ones [8].

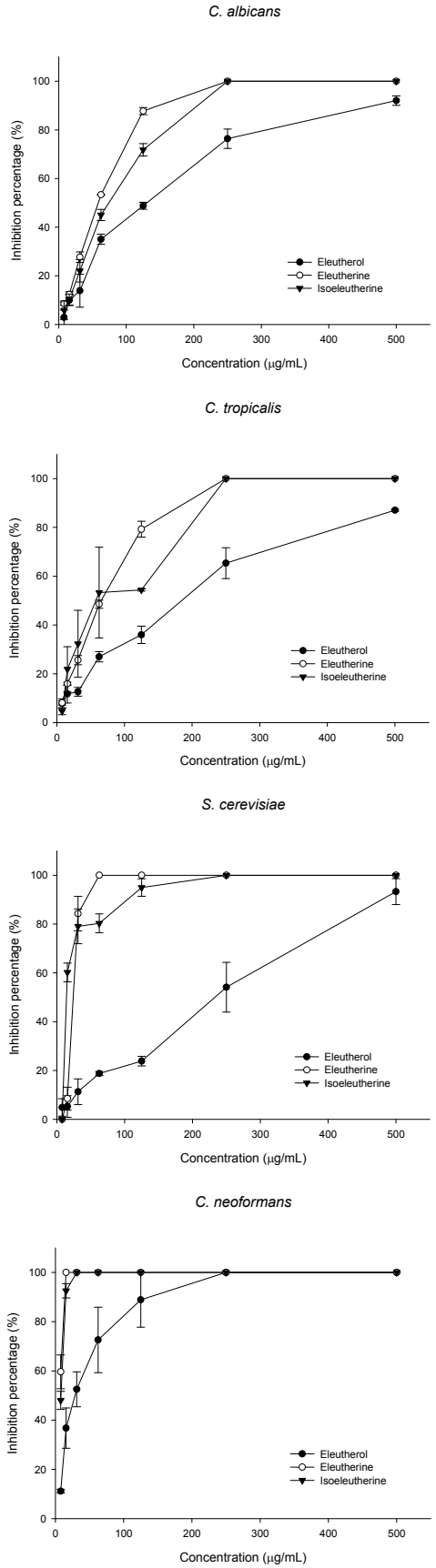



**Figure 2:** Growth inhibition of: *Candida albicans*, *C. tropicalis, Saccharomyces cerevisiae* and *Cryptococcus neoformans* by eleutherol, eleutherine and isoeleutherine, isolated from *C. paludosa* bulbs.

**Table 1:** Minimum Inhibitory Concentration (MIC) (μg/mL) and Minimum Fungicide Concentration (MFC) (μg/mL) of eleutherine, isoeleutherine and eleutherol isolated from *C. paludosa* bulbs.

| Tested material | $MIC_{100}$ | $MIC_{80}$ | $MIC_{50}$ | MFC |
|---|---|---|---|---|
| **Eleutherine** | | | | |
| Ca | 250 | 125 | 62.5 | >500 |
| Ct | 250 | 125 | 62.5 | >500 |
| Sc | 62.5 | 31.25 | 31.25 | 125 |
| Cn | 15.6 | 15.6 | 7.8 | 500 |
| **Isoeleutherine** | | | | |
| Ca | 250 | 250 | 62.5 | >500 |
| Ct | 250 | 250 | 62.5 | >500 |
| Sc | 250 | 31.25 | 15.6 | 500 |
| Cn | 31.25 | 15.6 | 7.8 | 500 |
| **Eleutherol** | | | | |
| Ca | >500 | 500 | 125 | >500 |
| Ct | >500 | 500 | 250 | >500 |
| Sc | >500 | 500 | 250 | >500 |
| Cn | 250 | 125 | 31.25 | 250 |

*Candida albicans* (Ca); *Candida tropicalis* (Ct); *Saccharomyces cerevisiae (Sc); Cryptococcus neoformans* (Cn). Amphotericin B used as standard exhibited the following MICs (μg/mL): Ca= 1.0; Ct=0.5; Sc= 0.5 and Cn= 0.25.

The fungal pathogen *C. neoformans* is an encapsulated yeast that causes life-threatening infections, especially in immunocompromised patients. It is a very frequent cause of human disease and the number of cases of cryptococcosis worldwide is estimated at 1 million, with more than 600,000 deaths *per* year. So, new compounds with anticryptococcal activity are highly welcome [9-12]. Among natural substances, studies with naphthoquinone derivatives have demonstrated their importance in vital biochemical processes and their several known biological activities, such as antitumor, antiviral, antibacterial, and especially antifungal [13-18].

Sassaki *et al*. [13] evaluated the antifungal activity of naphthoquinone derivatives and demonstrated MIC values of 8 - 16 μg/mL against cultures of *C. tropicalis*, *C. parapsilosis* and *C. neoformans*. Rahmoun *et al*. [16] demonstrated that two naphthoquinone derivatives exhibited *in vitro* antibacterial activity with MICs ranging between 16 to 64 μg/mL.

To the best of our knowledge, this is the first report about the antifungal potential of these pyranonaphtoquinones against human pathogenic fungi, although preliminary studies have indicated that they are active against *Pyricularia oryzae* [19] and **1** exhibited antidermatophyte activity against *Trichophyton mentagrophytes* by agar diffusion assay [20].

These results demonstrate that the bulbs of the medicinal plant *C. paludosa* possess promising antifungal properties, related at least in part to the presence of pyranonaphthoquinones identified as eleutherine isoeleutherine and eleutherol, which contribute to the antimicrobial effects of the dichloromethane fraction from this plant. The current results are relevant and motivate new *in vitro* and *in vivo* research focusing on the development of new and effective antifungal agents.

## Experimental

***Plant material:*** *C. paludosa* was cultivated in the surroundings of Itajaí city (SC – Brazil), near to UNIVALI, collected in May 2013 and identified by Dr Oscar B. Iza (Universidade do Vale do Itajaí). A voucher specimen was deposited at the Barbosa Rodrigues Herbarium (Itajaí-SC) under number VC Filho 108.

***Isolation of pyranonaphtoquinones:*** Eleutherine (**1**), isoeleutherine (**2**) and eleutherol (**3**) (Figure 1) were isolated from *C. paludosa* bulbs according to previously described procedures [2].

**Antifungal evaluation**

***Microorganisms and media:*** For the antifungal evaluation, standardized strains from the American Type Culture Collection (ATCC, Rockville, MD, USA) were used in a first instance of screening: *C. albicans* (ATCC 10231), *C. tropicalis* (CCC191), *S. cerevisiae* (ATCC 9763) and *C. neoformans* (ATCC 32264). Strains were grown on Sabouraud-chloramphenicol agar slants for 48 h at 30°C, maintained on slopes of Sabouraud-dextrose agar (SDA, Oxoid) and sub-cultured every 15 days to prevent pleomorphic transformations. Inocula of cell suspensions were obtained according to reported procedures and adjusted to 1-5 x $10^3$ colony forming units (CFU)/mL [21].

***Minimum inhibitory concentration (MIC) determination:*** Minimum inhibitory concentration (MIC) of each compound was determined by using broth microdilution techniques following the guidelines of the CLSI for yeasts [21]. MIC values were determined in RPMI-1640 (Sigma, St. Louis, MO, USA) buffered to pH 7.0 with MOPS (Sigma). Microtiter trays were incubated at 35°C for yeasts and hyalohyphomycetes and at 28°C for dermatophyte strains in a moist, dark chamber; MICs were recorded at 48 h for yeasts, and at a time according to the control fungal growth, for the rest of the fungi. The susceptibilities of the standard drug amphotericin B (Sigma-Aldrich, St. Louis, MO, USA) were defined as the lowest concentration of drug which resulted in total inhibition of fungal growth.

For the assay, compound stock solutions were two-fold diluted with RPMI-1640 from 250 to 0.24 µg/mL (final volume = 100 µL) and a final DMSO (Sigma) concentration <1%. A volume of 100 µL of inoculum suspension was added to each well with the exception of the sterility control where sterile water was added to the well instead. MIC was defined as the minimum inhibitory concentration of the compound, which resulted in total inhibition of the fungal growth.

MIC values were determined through three endpoints: $MIC_{100}$, $MIC_{80}$ and $MIC_{50}$ (minimum concentration required to inhibit 100, 80 and 50% of fungal growth respectively). Minimum fungicide concentration (MFC), that is the concentration of compound that kills fungi rather than inhibits the fungal growth, was determined by plating duplicate 5 µL from each clear well of MIC determinations onto a 150 mm SDA plate. After 48 h at 37°C, MFCs were determined as the lowest concentration of each compound showing no growth in these plates.

***Fungal growth inhibition percentage determination:*** Broth microdilution techniques were performed in 96-well microplates according to the guidelines of the Clinical and Laboratory Standards Institute for yeasts (M27-A3) [21]. For the assay, compound test wells (CTWs) were prepared with stock solutions of each compound in DMSO (maximum concentration ≤ 1%), diluted with RPMI-1640 to final concentrations of 250-0.98 µg/mL. Inoculum suspension (100 µL) was added to each well (final volume in the well = 200 µL). A growth control well (GCW) (containing medium, inoculum, the same amount of DMSO used in CTW, but compound-free) and a sterility control well (SCW) (sample, medium and sterile water instead of inoculum) were included for each fungus tested. Microtiter trays were incubated in a moist, dark chamber at 30ºC for 48 h for *C. albicans*, *C. tropicalis*, *S. cerevisiae* and *C. neoformans*. Microplates were read in a VERSA Max microplate reader (Molecular Devices, Sunnyvale, CA, USA). Amphotericin B was used as positive control. Tests were performed in duplicate. Reduction of growth for each compound concentration was calculated as follows: % of inhibition = 100 - ($OD_{405}$ CTW - $OD_{405}$ SCW)/ ($OD_{405}$ GCW - $OD_{405}$ SCW). The mean ± SEM for individual tests and for 3 repeated tests were used for constructing the curves representing % inhibition vs concentration of each compound, SigmaPlot software was used.

**Acknowledgments** – The authors thank to RIBIOFAR/CYTED, CNPq, CAPES and UNIVALI (Brazil), and ANPCyT, PICT 2014-1170 and National University of Rosario (UNR) (Argentine).

**References**

[1] Negri M, Salci TP, Shinobu-Mesquita, CS, Capoci IRG, Svidzinski TIE, Kioshima ES. (**2014**) Early state research on antifungal natural products. *Molecules*, ***19***, 2925–2956.

[2] Tessele PB, Delle Monache F, Quintão NLM, Da Silva GF, Rocha LW, Lucena GM, Ferreira VM, Prediger RD, Cechinel-Filho V. (**2011**) A new naphthoquinone isolated from the bulbs of *Cipura paludosa* and pharmacological activity of two main constituents. *Planta Medica*, ***77***, 1035–1043.

[3] Lucena GM, Gadotti VM, Maffi LC, Silva GS, Azevedo MS, Santos AR. (**2007**) Antinociceptive and anti-inflammatory properties from the bulbs of *Cipura paludosa* Aubl. *Journal of Ethnopharmacology*, ***112***, 19–25.

[4] Lucena GM, Porto FA, Campos EG, Azevedo MS, Cechinel-Filho V, Prediger RD, Ferreira VM. (**2010**) *Cipura paludosa* attenuates long-term behavioral deficits in rats exposed to methylmercury during early development. *Ecotoxicology and Environmental Safety, **73***, 1150–1158.

[5] Lucena GM, Matheus FC, Ferreira VM, Tessele PB, Azevedo MS, Cechinel-Filho V, Prediger RD. (**2013**) Effects of ethanolic extract and naphthoquinones obtained from the bulbs of *Cipura paludosa* on short-term and long-term memory: Involvement of adenosine A1 and A2A receptors. *Basic & Clinical Pharmacology & Toxicology, **112***, 229–235.

[6] Vicente MF, Basilio A, Cabello A, Peláez F. (**2003**) Microbial natural products as a source of antifungals. *Clinical Microbiology and Infection*, ***9***, 15–32.

[7] Chen S, Playford E, Sorrel, T. (**2010**) Antifungal therapy in invasive fungal infections. *Current Opinion in Pharmacology, **10***, 522–530.

[8] Carrasco H, Raimondi M, Svetaz L, Di Liberto M, Rodriguez MV, Espinoza L, Madrid A, Zacchino S. (**2012**) Antifungal activity of eugenol analogues. Influence of different substituents and studies on mechanism of action. *Molecules*, ***19***, 1002–1024.

[9] Park BJ, Wannemuehler KA, Marston BJ, Govender N, Pappas PG, Chiller TM. (**2009**) Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. *AIDS*, ***23***, 525–530.

[10] Alspaugh JA. (**2015**) Virulence mechanisms and *Cryptococcus neoformans* pathogenesis. *Fungal Genetics and Biology*, ***78***, 55-58.

[11] Azevedo MI, Ferreiro L, Da Silva AS, Tonin AA, Ruchel JB, Rezer JF, França RT, Zimmermann CE, Leal DB, Duarte MM, Lopes ST, Flores MM, Fighera R, Santurio JM. (**2014**) E-NTPDase and E-ADA activities in rats experimentally infected by *Cryptococcus neoformans*. *Veterinary Microbiology, **174***, 206–213.

[12] Vu K, Tham R, Uhrig JP, Thompson III GR, Na Pombejra S, Jamklang M, Bautos JM, Gelli A. (**2014**) Invasion of the central nervous system by *Cryptococcus neoformans* requires a secreted fungal metalloprotease. *MBio*, 5, 01101–14.

[13] Sasaki K, Abe H, Yoshizaki F. (**2002**) *In vitro* antifungal activity of naphthoquinone derivatives. *Biological and Pharmaceutical Bulletin*, ***25***, 669–670.

[14] Alves TMA, Kloos H, Zani CL. (**2003**) Eleutherinone, a novel fungitoxic naphthoquinone from *Eleutherine bulbosa* (Iridaceae). *Memórias do Instituto Oswaldo Cruz*, ***98***, 709–712.

[15] Ferreira VF, Ferreira SB, da Silva FDC. (**2010**) Strategies for the synthesis of bioactive pyran naphthoquinones. *Organic & Biomolecular Chemistry*, ***8***, 4793–4802.
[16] Rahmoun NM, Boucherit-Otmani Z, Boucherit K, Benabdallah M, Villemin D, Choukchou-Braham N. (**2012**) Antibacterial and antifungal activity of lawsone and novel naphthoquinone derivatives. *Médecine et Maladies Infectieuses*, ***42***, 270–275.
[17] Ferreira MPSBC, Cardoso MFC, da Silva FC, Ferreira VF, Lima ES, Souza JVB. (**2014**) Antifungal activity of synthetic naphthoquinones against dermatophytes and opportunistic fungi: preliminary mechanism-of-action tests. *Annals of Clinical Microbiology and Antimicrobials*, ***6***, 13–26.
[18] Hook I, Mills C, Sheridan H. (**2014**) Bioactive naphthoquinones from higher plants. *Studies in Natural Products Chemistry*, ***41***, 119–160.
[19] Xu J, Qiu F, Qu G, Wang N, Yao X. (**2005**) Studies on antifungal constituents isolated from *Eleutherine americana*. *Zhongguo Yaowu Huaxue Zazhi*, ***15***, 157–61.
[20] Kusuma IY, Arung ET, Rosamah E, Purwatiningsih H, Astuti J, Kim YU, Shimizu K. (**2010**) Antidermatophyte and antimelanogenesis compound from *Eleutherine americana* grown in Indonesia. *Journal of Natural Medicines*, ***64***, 223–226.
[21] CLSI - Clinical and Laboratory Standards Institute. (**2008**) Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard-3rd ed. CLSI Document M27-A3; CLSI: Wayne, PA, USA, 28(14), 1–25.