The effect of conocarpan on susceptibility of *Candida albicans* to phagocytosis and digestion by macrophages

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**Abstract.** *Piper solmsianum* C. DC. compounds exhibit several properties, including antimicrobial activity. The aim of the present study was to investigate whether conocarpan alters *Candida albicans* growth or killing of the yeast by macrophages. Conocarpan showed strong activity against the yeast with minimal inhibitory concentration (MIC) of 20 µg/mL and minimal fungicidal concentration (MFC) of 30 µg/mL. Mice peritoneal cells (macrophages) were cultured for 24 and 48 hours in supplemented RPMI 1640 medium. Cellular activation was assessed by determining MTT reduction and nitric oxide production. Standardized tests were conducted to select the optimal parameters for the subsequent killing test. Results showed that conocarpan exhibited antifungal activity and that *C. albicans* cultivated in the presence of the compound had greater susceptibility to death by macrophages. These findings suggest that conocarpan may have potential as an antimicrobial agent for *C. albicans* infections, promoting macrophagic immune support by altering growth of the yeast.

**Keywords:** Macrophage; Conocarpan; *Candida albicans*; Phagocytosis; *Piper solmsianum*.
Introduction

Candida species are major causes of infections affecting either body surfaces or the deep tissues. Candida is a complex pathogen and the immune system uses various cells, cell surface receptors and signaling pathways to trigger an efficient host defense. Host-Candida interaction can result either in rapid elimination of the pathogen or the persistence of the pathogen in immunocompromised patients, leading to either chronic mucocutaneous candidiasis or invasive candidiasis (Netea and Maródi, 2010).

Horn et al. (2009) conducted an epidemiological study that showed Candidemia as an important cause of mortality and morbidity in the health care system. Candidemia incidence caused by Candida albicans was 45.6% while incidence caused by non-Candida albicans was 54.4%; among them it was mentioned C. parapsilosis and C. krusei.

A prospective study conducted in Brazil reported a high mortality and the most common species were C. albicans (40.9%), in addition to C. tropicalis (20.9%) and C. parapsilosis (20.5%) (Colombo et al., 2006).

Candida albicans is a commensal microorganism in healthy individuals but is capable of causing serious infections if the protective mucosal barrier is breached. Therefore, immune discrimination between Candida colonization and invasion is of particular significance (Cheng et al., 2012). Candida albicans filaments’ virulence factors are thought to be important for Candida invasion in infected organs and, probably, for mediating fungus-induced tissue immunopathology (Felk et al., 2002). Candida is also able to efficiently adhere to and invade epithelial and endothelial cells via induced endocytosis and active penetration; both adhesion and invasion facilitate Candida dissemination (Filler and Sheppard, 2006).

The response of the host towards the potentially dangerous Candida albicans includes the recruitment of phagocytes from blood and tissues, considered as the first line of defence (Amulic et al., 2012).

The development of novel drugs derived from natural products as raw materials offers a number of advantages. These benefits include the richness in diversity of chemical structures and the ability to use these products as raw materials in the synthesis of new candidates for prototype drugs.

The genus Piper is one of the largest and most important aromatic and medicinal plants in the Piperaceae Family (Parmar et al., 1997; Kato and Furlan, 2007). Piper species are used in traditional remedies and folklore medicine worldwide for their numerous activities, particularly antifungal (Lago et al., 2004; Pessini et al., 2005; Terreaux et al., 2010). One of the main bioactive compounds with antifungal properties is the neolignan conocarpan (Figure 1). In previous studies, conocarpan has shown activity against filamentous fungi and yeast (Freixa et al., 2001; Campos et al., 2005). In addition, in vitro studies (Baumgartner et al., 2011) have shown that isolated lignan derivatives exhibit inhibition of NF-κB, cyclooxygenase-1 and -2,5-lipoxygenase, and of microsomal prostaglandin E₂ synthase-1, as well as antioxidant properties.
Figure 1. Chemical structure of neolignan conocarpan isolated from *Piper solmsianum* var. *solmsianum*.

The aim of this study was to analyze whether conocarpan alters growth of the yeast or killing of *C. albicans* by macrophages, suggesting the pathogen might be sensitized by the compound and rendered more susceptible to macrophage activity.

**Materials and methods**

**Chemicals and media**

The solvents and chemicals used were: PBS (phosphate buffer saline); Sabouraud dextrose agar from BD®; Sabouraud-dextrose agar from Biobrás®; LPS (from *Escherichia coli* K-235), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) from Sigma®. RPMI 1640 medium and FBS (fetal bovine serum) were from Gibco®. All other chemicals were of analytical grade.

**Conocarpan**

Conocarpan was isolated from *Piper solmsianum* with high-purity grade, as previously reported by our group (Campos et al., 2005).

**Animals**

Swiss (male and female) *Mus musculus* mice (25-30 g) were housed at 18 °C-22 °C under 12/12 h light/dark conditions. The experiments were conducted according to the protocols reviewed and approved by the Animal Experiment Ethics Committee of the University of the Vale do Itajaí, Brazil (approval under ID: 4216).

**Microorganism and inoculum**

*Candida albicans* (ATCC 10231) from the American Type Culture Collection (Rockville, MD, USA) was cultivated on Sabouraud-dextrose agar (Merck, 5438) for 48 h at 35 °C. Cell suspension in sterile distilled water was adjusted to yield a final concentration of 1x10⁶ to 5x10⁶ yeast cells/mL, standardized with 0.5 on the McFarland scale (520 nm) (Espinel-Ingroff et al., 1995).

**Susceptibility testing**

Broth microdilution testing of *C. albicans* was performed as outlined at Clinical and Laboratory Standards Institute (CLSI) document M27-S4 (formerly the Nacional Committee for Clinical Laboratory Standards, NCCLS). The concentrations of conocarpan tested ranged from 10 to 100 µg/mL. The minimum inhibitory concentration (MIC) endpoint was determined after 48 h of incubation. MIC was defined as the
lowest concentration evidencing no visible yeast growth after the incubation period. Minimum fungicidal concentration (MFC) was determined using subcultures of the well with inhibition on Sabouraud dextrose agar plates. When the microorganism failed to grow, the result was expressed by fungicide. Drug-free solution was used as a control (blank well). The tests were repeated at least in triplicate.

**Macrophage cells**

Macrophage cells were obtained from the peritoneal cavities of the mice. Peritoneal cells at 1.5 and 10x10^5 cells/well (96-well microtiter plates) were cultured in RPMI 1640 medium supplemented with 10% FBS, glutamine (1%), sodium bicarbonate (2.7 mM), streptomycin (100 μg/mL) and penicillin (100 UI/mL) at 37 °C (5% CO₂). In all experiments, cell viability exceeded 94% (as assessed by the trypan blue dye exclusion test).

**MTT assay cytotoxicity**

The MTT assay was performed as described by Mosmann, 1983. Cell viability was assessed by the mitochondria-dependent reduction of MTT to formazan crystals. MTT (500 μg/mL) dissolved in PBS (pH 7.4) was added to each well. After 3 h of incubation, 100 μL of SDS 10% (wt/vol) in HCl 1mM were added to the wells to dissolve the dark blue crystals. The absorbance was read at 560 nm with a microtiter plate reader.

**Nitrite assay**

Secretion of nitric oxide (NO) by macrophages was measured by determining nitrite and nitrate, stable end products of NO oxidation, as described previously by Miranda et al. (2001). Supernatants from macrophage cultures were mixed with an equal volume of Griess reagent and absorbance at 540nm measured and compared with a NaNO₂ (1-100 mM) standard curve.

**Phagocytosis assay**

Peritoneal cells with LPS (0.5, 1 or 5 μg/mL) and without LPS were cultured for 24 and 48 h. Thereafter, 10 μL of a yeast suspension, treated and untreated with conocarpan, was added to the wells. After 3 h of incubation, the wells were washed 5 times and 10 μL of Triton X-100 added to each well to lyse the macrophage. Subsequently, 10 μL was then seeded onto RPMI 1640 and cultivated for a further 18 h to check for yeast growth.

**Statistical analysis**

All experiments were repeated four times. Values are expressed as mean ± SD. All statistical analyses were performed using the SPSS statistical package (SPSS 20 software) and GraphPad Prism 5. Statistical significance was assessed by ANOVA (post-hoc Tukey test). Values of p < 0.05 were considered significant.

**Results and discussion**

**Determination of MIC and MFC**

In the present study, the determination of anti-*Candida* activity revealed MIC and MFC of 20 and 30 μg/mL, respectively. In the control well, substantial yeast growth was evident given there was no substance inhibiting its development. These activity values are in agreement with those reported by other authors (Campos et al., 2005).

The antifungal activity of conocarpan appears to be associated with the phenolic hydroxyl present in the phenyl-prophenyl-benzofuran structure, as well as with its position at carbon 4 in this structure and the unsaturation of carbons 7 and 8 (Freixa et al., 2001).

**Determination of macrophage activation**

It is known that activated macrophages increase their breathing capacity many times over in an event called respiratory burst. This is a potent
oxygen-dependent killing mechanism in phagocytic cells, such as monocytes/macrophages and neutrophils, and is regarded as a highly efficient non-specific cell defense mechanism (Bedard and Krause, 2007). In addition, mice peritoneal cells are a good source of macrophages given almost 90% of the peritoneal cells are macrophages (Steil, 1996). MTT reduction and nitric oxide production can be used as a measure of macrophage activation (Knowles and Moncada, 1994; Rao et al., 2003; Cruz et al., 2005).

As depicted in Figure 2, the peritoneal cells at 5 and 10$\times$10$^5$ cells/well exhibited the same degree of MTT reduction. MTT reduction by 5 and 10$\times$10$^5$ cells/well was significantly higher than the MTT reduction by 1 $\times$ 10$^5$ cells/well. The addition of LPS to the culture had no effect on MTT reduction. The same results were obtained for both cell culture times: 24 h (Figure 2A) and 48 h (Figure 2B). It is possible that the cells were already activated, explaining the lack of cell activation by LPS. Johnson and Sung (1987) showed that after culture in vitro with LPS, macrophages exhibited a marked decrease in their capacity to secrete high levels of respiratory burst products.

**Figure 2.** Reduction of MTT by mice peritoneal cells in culture with LPS for 24 h (A) or 48 h (B). Results expressed as mean ± SD of 4 assays.

LPS, or endotoxin, triggers the initiation of host responses to a bacterial insult where nitric oxide (NO) is a key intermediary signal in several different responses during this assay. NO is generated by constitutive nitric oxide synthase (cNOS) and inducible NOS (iNOS). iNOS is induced by inflammatory stimuli such as LPS in macrophages or hepatocytes, and is responsible for the production of most NO during endotoxemia. Endotoxin or cytokine administration increases iNOS activity (Hamada et al., 1999). The production of NO mediators can be assessed by determining the concentration of nitrite in cell culture supernatant (Stuehr and Nathan, 1989). In the present study, NO production was used to evaluate cell activation by LPS. The results of this study given in Figure 3 show that 5 and 10 $\times$ 10$^5$ cells/well produced significantly higher amounts of NO than 1 $\times$ 10$^5$ cells/well.
The addition of LPS to the cell cultures had no effect on NO production. Taken together, the data from the MTT reduction and NO production assays indicated that the addition of LPS appeared to have no effect on cell activity state or MTT reduction, and suggested that NO production was not the best parameter to measure the activation of macrophages. LPS is known to induce autophagy in macrophages (Xu et al., 2007). Thus, another possibility is that the macrophages were activated and produced a greater amount of NO, but some macrophages subsequently died. Consequently, both the amount of NO produced and MTT reduction were similar for the groups with and without LPS in the culture.

*Candida albicans* killing by macrophages

For the evaluation of *C. albicans* killing by macrophages, 5x10^5 peritoneal cell culture was used with 1 μg/mL of LPS for 48 h. Thereafter, *C. albicans* were added at a rate 1:5 (macrophages: *Candida*), as used by Vonk et al., 2002. The yeast cells were treated with and without conocarpan. The results depicted in Figure 4 shows the yeasts were removed when the plate was washed, as evidenced by the low colony growth in Groups 1 and 2. The presence of macrophages in the well led to growth of around 150 colonies in both the macrophages untreated (Group 3) and treated (Group 5) with LPS. For *C. albicans* cultured in the presence of conocarpan before exposure to macrophages, the macrophages were able to kill the yeast (Groups 4 and 6), having become more susceptible to killing by macrophages. These results show that conocarpan weakened the *C. albicans*. 

**Figure 3.** NO production by mice peritoneal cells in culture containing LPS for 24 h (A) or 48 h (B). Results expressed as mean ±SD of 4 assays.
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Figure 4. Colony-forming units (CFU) of Candida albicans. Macrophages were cultivated with LPS (1 µg/mL) for 48 h and were cultivated for 3 h after receiving C. albicans treated and untreated with conocarpan. The wells were washed, cells lysed, and 10 µL seeded onto Sabouraud-dextrose broth cultivated for a further 18 h to check for yeast growth. The data represent mean CFU from the results of one experiment, but are representative of two experiments.

Conclusion

The results showed that the neolignan conocarpan exhibited antifungal activity against C. albicans and that yeast grown in the presence of the compound had greater susceptibility to death by macrophagic action.

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Conflicts of interest

Authors declare that they have no conflict of interests.

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