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

Dhébora Mozena Dall'Igna[1], Ana Angélica Steil[2], Rosi Zanoni da Silva[3], Valdir Cechinel Filho[2] and Alexandre Bella-Cruz[2]

[1]Department of Environment and Health. University of the Planalto Catarinense (UNIPLAC). Av. Castelo Branco, 170. Universitário. Lages-SC, Brazil (CEP 88509-900). Email: dhe.mozena@gmail.com.

[2]Nucleus of Chemical-Pharmaceutical Research (NIQFAR). University of the Vale do Itajaí (UNIVALI). Rua Uruguai, 458. Centro. Itajaí-SC, Brazil (CEP 88302-901).

[3]Department of Pharmaceutical Sciences. State University of Ponta Grossa. Praça Santos Andrade, 01. Centro. Ponta Grossa-PR, Brazil (CEP 84010-330).

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*.

ORCID

0000-0002-6962-401X
Dhébora Mozena Dall'Igna

0000-0002-9207-7843
Ana Angélica Steil

0000-0001-6734-9520
Rosi Zanoni da Silva

0000-0002-8947-2965
Valdir Cechinel Filho

0000-0002-3944-9265
Alexandre Bella-Cruz

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 commom 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 E_2 synthase-1, as well as antioxidant properties.
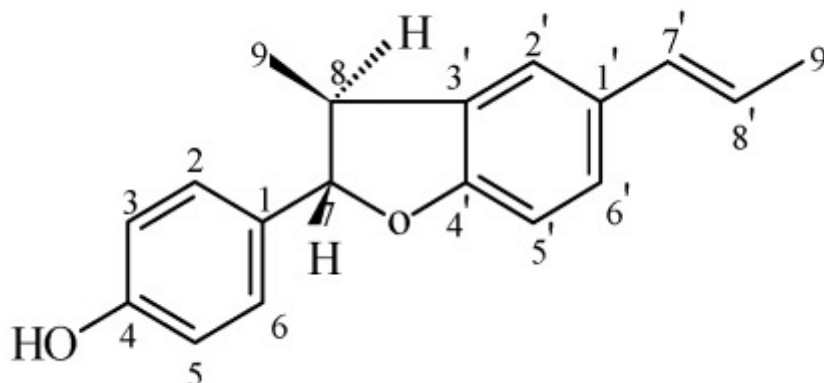
Figure 1. Chemical structure of neolignan conocarpan isolated from *Piper solmsianum* var. *solmsianum*.

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 *Esquerichia coli* K-235), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphe-nyltetrazolium 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 1×10^6 to 5×10^6 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 10×10^5 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% CO_2). 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 $NaNO_2$ (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-propenyl-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 10x10^5 cells/well exhibited the same degree of MTT reduction. MTT reduction by 5 and 10x10^5 cells/well was significantly higher than the MTT reduction by 1 x 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.
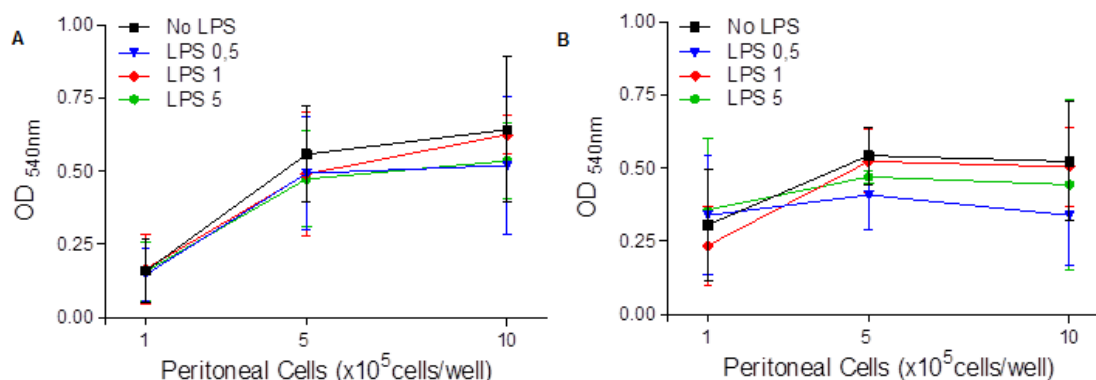
Figure 2. Reduction of MTT by mice peritoneal cells in culture with LPS for 24 h (A) or 48 h (B). Results expressed as mean ± SD of 4 assays.

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 x 10^{5}cells/well produced significantly higher amounts of NO than 1 x 10^5 cells/well.
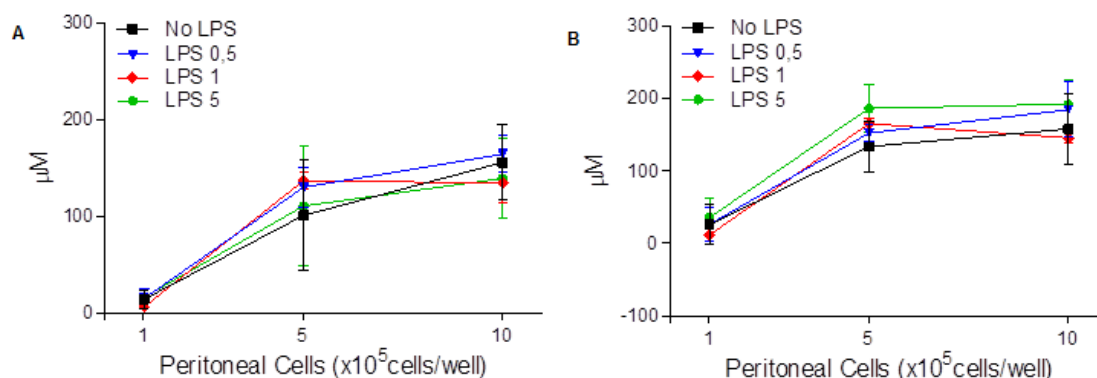
Figure 3. NO production by mice peritoneal cells in culture containing LPS for 24 h (A) or 48 h (B). Results expressed as mean ±SD of 4 assays.

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, 5×10^5 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*.
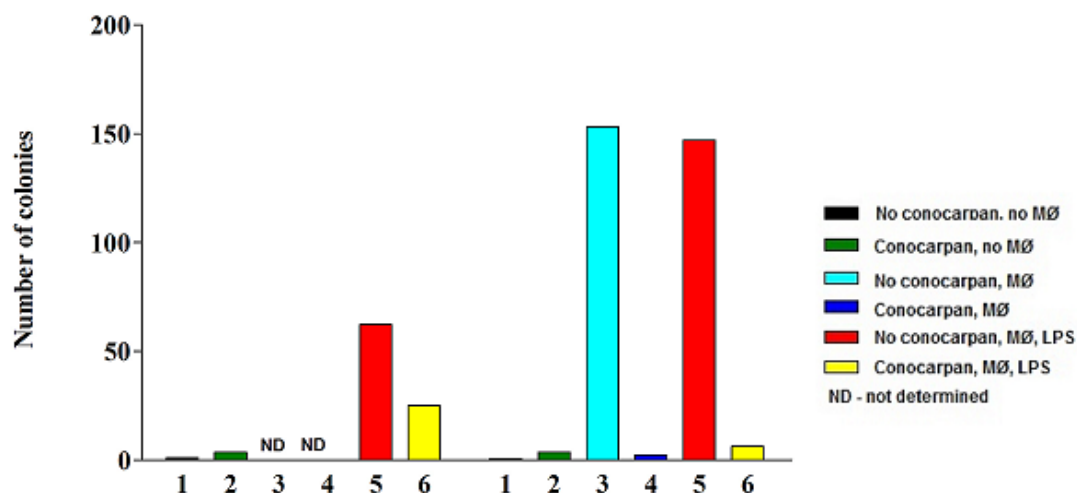
Figure 4. Colony-forming units (CFU) of *Candida albicans*. Macrophages were cultivated with LPS (1 µg/mL) for 48 h and were cultivated for 3 h after receiving *C. albicans* treated and untreated with conocarpan. The wells were washed, cells lysed, and 10µL seeded onto Sabouraud-dextrose broth cultivated 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.

Acknowledgments

Conflicts of interest

Authors declare that they have no conflict of interests.

References

Amulic, B.; Cazalet, C.; Hayes, G. L., Metzler, K. D.; Zychlinsky, A. Neutrophil function: From mechanisms to disease. **Annual Review of Immunology**, v. 30, p. 459-489, 2012. https://doi.org/10.1146/annurev-immunol-020711-074942

Baumgartner, L.; Sosa, S.; Atanasov, A. G.; Bodensieck, A.; Fakhrudin, N.; Bauer, J.; Favero, G. D.; Ponti, C.; Heiss, E. H.; Schwaiger, S.; Ladurner, A.; Widowitz, U.; Loggia, R. D.; Rollinger, J. M.; Werz, O.; Bauer, R.; Dirsch, V. M.; Tubaro, A.; Stuppner, H. Lignan derivatives from *Krameria lappacea* roots inhibit acute inflammation *in vivo* and pro-inflammatory mediators *in vitro*. **Journal of Natural Products**, v. 74, no. 8, p. 1779-1786, 2011. https://doi.org/10.1021/np200343t

Bedard, K.; Krause, K.-H. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. **Physiological Reviews**, v. 87, no. 1, p. 245-313, 2007. https://doi.org/10.1152/physrev.00044.2005

Campos, M. P.; Cechinel Filho, V.; Silva, R. Z.; Yunes, R. A.; Zacchino, S.; Juarez, S.; Bella Cruz, R. C.; Bella Cruz, A. Evaluation of antifungal activity of *Piper solmsianum* C. DC. var. *solmsianum* (Piperaceae). **Biological and Pharmaceutical Bulletin**, v. 28, no. 8, p. 1527-1530, 2005. https://doi.org/10.1248/bpb.28.1527

Cheng, S.-C.; Joosten, L. A. B.; Kullberg, B.-J.; Netea, M. G. Interplay between *Candida*

albicans and the mammalian innate host defense. **Infection and Immunity**, v. 80, no. 4, p. 1304-1313, 2012. https://doi.org/10.1128/IAI.06146-11

Colombo, A. L.; Nucci, M.; Park, B. J.; Nouér, S. A.; Arthington-Skaggs, B.; Da Matta, D. A.; Warnock, D.; Morgan, J. Epidemiology of candidemia in Brazil: A nationwide sentinel surveillance of candidemia in eleven medical centers. **Journal of Clinical Microbiology**, v. 44, no. 8, p. 2816-2823, 2006. https://doi.org/10.1128/JCM.00773-06

Cruz, A. H., Mendonça, R. Z.; Petricevich, V. L. *Crotalus durissus terrificus* venom interferes with morphological, functional and biochemical changes in murine macrophage. **Mediators of Inflammation**, v. 6, p. 349-359, 2005. https://doi.org/10.1155/MI.2005.349

Espinel-Ingroff, A.; Dawson, K.; Pfaller, M.; Anaissie, E.; Breslin, B.; Dixon, D.; Fothergill, A.; Paetznick, V.; Peter, J.; Rinaldi, M.; Walsh, T. Comparative and collaborative evaluation of standardization of antifungal susceptibility testing for filamentous fungi. **Antimicrobial Agents and Chemotherapy**, v. 39, no. 2, p. 314-319, 1995. https://doi.org/10.1128/AAC.39.2.314

Felk, A.; Kretschmar, M.; Albrecht, A.; Schaller, M.; Beinhauer, S.; Nichterlein, T.; Sanglard, D.; Korting, H. C.; Schäfer, W.; Hube, B. *Candida albicans* hyphal formation and the expression of the Efg1-regulated proteinases Sap4 to Sap6 are required for the invasion of parenchymal organs. **Infection and Immunity**, v. 70, no. 7, p. 3689-3700, 2002. https://doi.org/10.1128/IAI.70.7.3689-3700.2002

Filler, S. G.; Sheppard, D. C. Fungal invasion of normally non-phagocytic host cells. **PLoS Pathogens**, v. 2, no. 12, 2006. https://doi.org/10.1371/journal.ppat.0020129

Freixa, B.; Vila, R.; Ferro, E. A.; Adzet, T.; Cañigueral, S. Antifungal principles from *Piper fulvescens*. **Planta Medica**, v. 67, no. 9, p. 873-875, 2001. https://doi.org/10.1055/s-2001-18838

Hamada, E.; Nishida, T.; Uchiyama, Y.; Nakamura, J.; Isahara, K.; Kazuo, H.; Huang, T. P.; Momoi, T.; Ito, T.; Matsuda, H. Activation of Kupffer cells and caspase-3 involved in rat hepatocyte apoptosis induced by endotoxin. **Journal of Hepatology**, v. 30, no. 5, p. 807-818, 1999. https://doi.org/10.1016/S0168-8278(99)80133-0

Horn, D. L.; Neofytos, D.; Anaissie, E. J.; Fishman, J. A.; Steinbach, W. J.; Olyaei, A. J.; Marr, K. A.; Pfaller, M. A.; Chang, C.-H.; Webster, K. M. Epidemiology and outcomes of candidemia in 2019 patients: Data from the prospective antifungal therapy alliance registry. **Clinical Infectious Diseases**, v. 48, no. 12, p. 1695-1703, 2009. https://doi.org/10.1086/599039

Kato, M. J.; Furlan, M. Chemistry and evolution of Piperaceae. **Pure and Applied Chemistry**, v. 79, no. 4, p.529-538, 2007. https://doi.org/10.1351/pac200779040529

Knowles, R. G.; Moncada, S. Nitric oxide synthases in mammals. **Biochemical Journal**, v. 298, no. 2, p. 249-258, 1994. https://doi.org/10.1042/bj2980249

Lago, J. H. G.; Ramos, C. S.; Casanova, D. C. C.; Morandim, A. A.; Bergamo, D. C. B.; Cavalheiro, A. J.; Bolzani, V. S.; Furlan, M.; Guimarães, E. F.; Young, M. C. M.; Kato, M. J. Benzoic acid derivatives from *Piper* species and their fungitoxic activity against *Cladosporium cladosporioides* and *C. sphaerospermum*. **Journal of Natural Products**, v. 67, no. 11, p. 1783-1788, 2004. https://doi.org/10.1021/np030530j

Miranda, K. M.; Espey, M. G.; Wink, D. A. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. **Nitric Oxide**, v. 5, no. 1, p.62-71, 2001. https://doi.org/10.1006/niox.2000.0319

Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Applications to proliferation and cytotoxicity assays. **Journal of Immunological Methods**, v. 65, no. 1/2, p. 55-63, 1983. https://doi.org/10.1016/0022-1759(83)90303-4

National Committee for Clinical Laboratory Standards. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts. Fourth Informational Supplement. Approved Standard M27-S4. National Committee for Clinical Laboratory Standards, Ghannoum, M.A.: NCCLS, 2012.

Netea, M. G.; Maródi, L. Innate immune mechanisms for recognition and uptake of *Candida* species. **Trends in Immunology**, v. 31, no. 9, p. 346-353, 2010. https://doi.org/10.1016/j.it.2010.06.007

Parmar, V. S.; Jain, S. C.; Bisht, K. S.; Jain, R.; Taneja, P.; Jha, A.; Tyagi, O. D.; Prasad, A. K.; Wengel, J.; Olsen, C. E.; Boll, P. M. Phytochemistry of the genus Piper. **Phytochemistry**, v. 46, no. 4, p. 597-673, 1997. https://doi.org/10.1016/S0031-9422(97)00328-2

Pessini, G. L.; Dias Filho, B. P.; Nakamura, C. V.; Cortez, D. A. G. Antifungal activity of the extracts and neolignans from *Piper regnellii* (Miq.) C. DC. var. *pallescens* (C. DC.) Yunck. **Journal of the Brazilian Chemical Society**, v. 16, no. 6a, p. 1130-1133, 2005. https://doi.org/10.1590/S0103-50532005000700007

Rao, V. S.; Paiva, L. A.; Souza, M. F.; Campos, A. R; Ribeiro, R. A.; Brito, G. A.; Teixeira, M. J.; Silveira, E. R. Ternatin, an anti-inflammatory flavonoid, inhibits thioglycolate-elicited rat peritoneal neutrophil accumulation and LPS-activated nitric oxide production in murine macrophages. **Planta Medica**, v. 69, no. 9. p.851-853, 2003. https://doi.org/10.1055/s-2003-43213

Steil, A. A. **Inflamação induzida por imunocomplexos no peritôneo e pulmão**: papel do fator ativador de plaquetas, eicosanóides e óxido nítrico. São Paulo: Universidade de São Paulo, 1996. (Thesis).

Stuehr, D. J.; Nathan, C. F. Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. **Journal of Experimental Medicine**, v. 169, no. 5, p. 1543-1555, 1989. https://doi.org/10.1084/jem.169.5.1543

Terreaux, C.; Gupta, M. P.; Hostettmann, K. Antifungal benzoic acid derivatives from *Piper dilatatum*. **Phytochemistry**, v. 49, no. 2, p. 461-464, 2010. https://doi.org/10.1002/chin.199902259

Xu, Y.; Jagannath, C., Liu, X. D.; Sharafkhaneh, A.; Kolodziejska, K. E.; Eissa, N. T. Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. **Immunity**, v. 27, no. 1, p.135-144, 2007. https://doi.org/10.1016/j.immuni.2007.05.022