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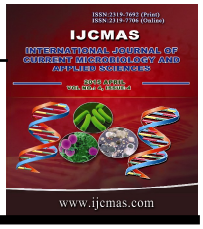

Original Research Article

Effects of Piperonal Nitro Derivatives on *Candida* species: Antifungal Activity against Fluconazole-Resistant Strains is Associated with Oxidative DNA Damage

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Keywords

Nitro derivates, *Candida*, resistance, fluconazole, oxidative DNA damage

A B S T R A C T

Recently, there has been a significant increase in invasive fungal infections, the treatment of which is limited to a quite small number of antifungal drugs. Natural products represent an important source of antifungal agents, mainly because of their natural coexistence with fungi present in each biome. Derivatives or semi-synthetic products can be used to optimize the pharmacological profile of natural products by modulating relevant biological properties. The present study evaluated the antifungal effect of piperonal nitro derivatives (PNDs) in *Candida* spp. strains resistant to fluconazole. The assessment of the antifungal effect was determined both by broth dilution and flow cytometry, as well as by the assessment of a potential mechanism of action of these compounds. All of the tested strains were susceptible to the tested compounds. Treatment with PNDs (1, 2 and 3) led to programmed cell death in *Candida* spp., probably because they play an antifungal role in specific DNA surrounding sites. In addition, ROS production was found to play a role in this process, observed as oxidative damage to DNA purine and pyrimidine bases. The PND compounds (1, 2 and 3) presented antifungal activity *in vitro* against strains of fluconazole-resistant *Candida* spp.

Introduction

Fungal infections have become a major global problem in tertiary hospitals, mainly affecting immunocompromised patients, in particular infections caused by *Candida* spp. Candidemia is a consequence of the advances achieved in health care. Despite the advances in the last two decades, with the introduction of new diagnostic and invasive techniques, as well as the development and commercialization of new antifungal agents and the implementation of candidemia prevention strategies, the incidence of infections has increased (Guinea, 2014)

The mortality rate from candidemia remains unacceptably high (15-47% in adults) with variations across geographical regions. In Latin America, clinical studies have shown that the candidemia rate is higher than in North America and Europe (50-54% versus ~31%, respectively) (Nucci et al., 2014)

The global use of fluconazole is the major cause of the resistance in *Candida* spp., and has driven the development of new antifungal agents (Negri et al., 2014).

Natural products are an important source of anti-infective and anti-tumor agents (Rajeshkumar and Sundararaman, 2012). Due to the structural complexity and sometimes limited availability of pure compounds, semi-synthetic derivatives and analogs can be used to optimize the pharmacological profile of natural products by modulating relevant biological properties (Oliveira, et al., 2012).

Safrole (1,3-benzodioxol-5-il), obtained from sassafras oil (*Ocotea pretiosa Mer., Lauraceae*) and long pepper (*Piper hispidinervum* C. DC), is an abundant natural product in Brazil with a versatile chemical reactivity, allowing for different functionalities (Magalhães Moreira et al, 2007) as well as relevant biophorical features that interact with different therapeutic targets (Romeiro, 2002). Magalhães Moreira et al. (2007) synthesized various analogs of the compounds obtained from piperonal, one of the major constituents of safrole. These nitrocompounds, containing a nitro group at position 6 of the piperonyl ring, showed a significant cytotoxic activity. These data were corroborated by Nascente (2009) in a new series of planned nitrocompounds.

The present study describes the assessment of the antifungal effects of piperonal nitro derivatives (PNDs), using strains of fluconazole-resistant *Candida* spp. The mechanisms of induced cell death were investigated by assessing the effects of these compounds on specific DNA surrounding sites of yeast cells.

Materials and Methods

Materials

Three piperonal nitro derivatives (PNDs) were used in the present study.5-(2-nitrovinyl)benzo[d][1,3]dioxole (PND 1) and 5-(2-nitroprop-1-en-1-yl)benzo[d][1,3]dioxole (PND 2) were synthesized as described by Milhazes et al. (2006). The third compound, 5-chloromethyl-6-nitrobenzo[d][1,3]dioxole (PND 3), was synthesized according to Nascente (2009). Their chemical structures are shown in Figure 1.

Strains

Fluconazole-resistant clinical strains of *C. albicans* (five strains), *C. tropicalis* (seven strains) and *C. parapsilosis* (seven strains) were used. These strains are from the yeast collection of the Laboratory of Bioprospection and Experiments in Yeast (LABEL/FF/UFC). The strains were

inoculated in Sabouraud dextrose agar (Himedia, Mumbai, India) and incubated at 37ºC for 24 h. Next, they were grown in CHROMagar *Candida* (Himedia) to evaluate their purity.

Molecular identification

Genomic DNA was purified using a CTAB-based protocol, as described previously (Warner, 1996). The nuclear DNA region comprising the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene was amplified by polymerase chain reaction (PCR) using the primers ITS4 (5□-TCCTCCGCTTATTGATATGC-3□) and ITS5 (5□-GCAAGTAAAAGTCGTAACA AGA-3□), as suggested by White et al (1996). Once the specificity of the amplifications was confirmed, the PCR products were purified from the remaining reactions using the GFX PCR DNA and Gel Band Purification kit (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). The concentrations of the purified PCR products were determined by measuring the absorbance of a ten-fold dilution at 260 nm. DNA sequencing was performed at Macrogen Inc. (Seoul, South Korea) using Sanger□s dideoxy chain termination method. The determined sequences were compared to those previously deposited in the GenBank database using the BLAST program (Altschul et al., 1990).

***In vitro* antifungal activity**

The broth microdilution (BMD) antifungal susceptibility test was performed according to document M27-A3 (CLSI, 2008). Fluconazole (Sigma-Aldrich) and the piperonal nitro derivatives were dissolved in distilled water and dimethyl sulfoxide (DMSO; Sigma-Aldrich), respectively. Fluconazole and the PNDs were tested at concentrations ranging from 0.125 to 64 mg/L. The yeasts and compounds were incubated in 96-well culture plates at 35°C for 24 h and the results were examined visually, as recommended by CLSI (2012). The minimum inhibitory concentration (MIC) of each compound was determined as the concentration that inhibited 50% of fungal growth. The following cutoff points of the MICs were used to classify the strains as susceptible (S) or resistant (R) to FLC: MIC $\leq$ 2 mg/L (S), MIC $\geq$ 8 mg/L (R) (CLSI, 2012). All the tests were performed in triplicate in three independent experiments.

Cell treatments

To assess cell density, membrane integrity, mitochondrial transmembrane potential, oxidative stress and DNA damage, fluconazole-resistant strains of *C. albicans* 3, *C. tropicalis* 4 and *C. parapsilosis* 2 were exposed for 24 h to various concentrations (MIC, MIC x2, and MIC x4) of the PNDs (1, 2 and 3).All the tests were performed in triplicate in three independent experiments (Da Silva et al., 2013; Neto et al., 2014).

Preparation of yeast suspensions

Cell suspensions were prepared from cultures in the exponential growth phase. The cells were harvested by centrifugation (1600 *g* for 10 min at 4°C), washed twice with 0.85% saline solution (1200 *g* for 5 min at 4°C) and then resuspended (~10^6 cells/mL) in HEPES buffer (pH 7.2) supplemented with 2% glucose (Da Silva et al., 2013; Neto et al., 2014).

Determination of cell density and membrane integrity

The cell density and membrane integrity of the fungal strains were evaluated by the exclusion of 2 mg/L PI (propidium iodide). Aliquots from yeasts incubated for 24 h with the drugs (PNDs 1-3; fluconazole; Ampho)

were analyzed using flow cytometry. A total of 10,000 events was evaluated per experiment (n=2) and cellular debris was omitted from the analysis. Cellular fluorescence was then determined by flow cytometry using a Guava EasyCyte™ Mini System cytometer (Guava Technologies Inc., Hayward, CA, USA) and analyzed using CytoSoft 4.1 software (Da Silva et al., 2013; Neto et al., 2014).

Measurement of mitochondrial transmembrane potential (Δψm)

The mitochondrial transmembrane potential was determined by measuring the retention of rhodamine 123 dye by the mitochondria of the yeast cells after exposure for 24 h. The cells were washed with PBS, incubated with 5 mg/L rhodamine 123 at 37°C for 30 min in the dark, and then washed twice with PBS. Their fluorescence was measured by flow cytometry (Guava EasyCyte™ Mini System). A total of 10,000 events was evaluated per experiment (n=2) and cellular debris was omitted from the analysis (Da Silva et al., 2013; Neto et al., 2014).

Detection of reactive oxygen species (ROS) produced by yeast cells

ROS produced over a 24-h culture period were detected by incubating the cells with 20 μM CM-H_2DCFDA [5-(and-6)-chloromethyl-2□,7□-dichloro-dihydro-fluorescein diacetate acetyl ester] for 30 min in the dark at 35°C. Next, the cells were harvested by centrifugation, washed with PBS, resuspended in the same buffer and immediately analyzed by flow cytometry (Guava EasyCyte Mini System) (Da Silva et al., 2013; Neto et al., 2014).

Alkaline comet assay

The alkaline comet assay was performed essentially as described by Miloshev et al (2002). The cells were visually inspected and scores in five classes (0 to 4) were assigned according to their tail sizes (from 0 = no damage to 4 = extensive DNA damage) and a damage index value was calculated for each sample of cells. The damage index values ranged from 0 (100 cells with no damaged DNA: 100 x 0) to 400 (100 cells displaying extensive DNA damage: 100 x 4) (Da Silva et al., 2013).

Analysis of oxidized purine and pyrimidine bases in yeast DNA

The analysis of oxidized purine and pyrimidine bases was performed essentially as described by Neto et al (2014). Images of 100 randomly selected cells (50 cells from each of two replicate slides) were visually analyzed per group. The number of oxidized purines (FPG-sensitive sites) and pyrimidines (ENDO III-sensitive sites) was then determined by subtracting the amount of strand breaks observed in the control (samples incubated with buffer alone) from the total amount of breaks obtained after incubation with FPG or ENDO.

Annexin V staining

The analysis of annexin v staining was performed essentially as described by Neto et al (2014). For each experiment (n=2), 10,000 events were evaluated and cell debris was omitted from the analysis.

Statistical analysis

In vitro susceptibility experiments were repeated at least three times on different days. Geometric means were used to compare the MIC results. The data obtained from the flow cytometry and alkaline comet assays were compared using a one-way analysis of variance (ANOVA) followed by the Newman-Keuls test ($p<0.05$).

Result and Discussion

Molecular identification

The complete ITS/5.8S region (ITS1, 5.8S, and ITS2) of the nuclear ribosomal DNA from *Candida* strains was amplified, sequenced and compared to the sequences deposited in the GenBank database (data not shown). The BLAST searches revealed that the sequences from the isolates were identical to the ITS/5.8S sequences from different isolates and strains of *C. albicans*, *C. tropicalis* and *C. parapsilosis*, as shown in Table 1.

Piperonal nitro derivatives inhibit the growth of FLC-resistant strains of *Candida* spp.

The three piperonal nitro derivatives (PNDs; Figure 1) were able to inhibit the growth of all FLC-resistant strains used in the present work (Table 1). The MICs of the PNDs 1 and 2 ranged from 0.5 to 1.3 μg/mL (PND 1) and from 0.5 to 2.6 μg/mL (PND 2), respectively. PND 3 was less effective, with MICs ranging from 16 to 32 μg/mL. Based on these results, one representative strain of each species (*C. albicans* 3, *C. tropicalis* 4 and *C. parapsilosis* 2) was selected to further investigate the mechanism of action of the PNDs.

Loss of cell viability and plasma membrane damage in *Candida* species are induced by the PNDs

The PNDs reduced the number of viable cells of the *Candida* species at all tested concentrations; this effect was concentration-dependent (Figure 2). Moreover, the three compounds also promoted cell membrane instability in the FLC-resistant yeast strains (Figure 3).

PNDs increase the intracellular levels of ROS in FLC-resistant strains of *Candida* species

When the yeast strains were exposed to different PND concentrations, we detected an increase in the green fluorescence of the cells (Figure 4). The ROS production induced by the PNDs was similar among exposed cells.

Changes in the yeast mitochondrial transmembrane potential (Δψm) are induced by PNDs

Significant ($p<0.05$) changes in the mitochondrial transmembrane potential were observed when the yeast cells were exposed to increasing concentrations of the PNDs (Figure 5).

DNA damage

The tested compounds induced significant ($p<0.05$) DNA damage in the yeast cells as compared to untreated cells. A similar level of DNA damage induced by the PNDs was observed in the three *Candida* species. Furthermore, the compounds also promoted significant ($p<0.05$) increases in the amounts of oxidized purines and pyrimidines (Table 2, Table 3, Table 4).

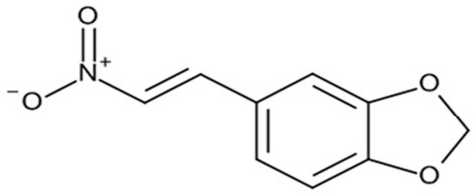
Externalization of phosphatidylserine in yeast cells

The populations of cells in the lower and upper right quadrants, respectively, correspond to early (Annexin V-positive, 7AAD-negative) and late (Annexin V-positive, 7AAD-positive) apoptotic cells with externalized phosphatidylserine.

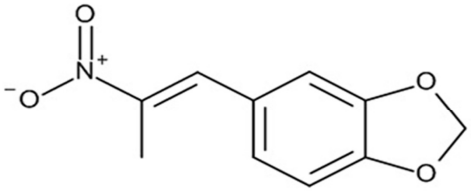
Table 1. The effects of piperonal nitro-derivatives (PND 1, PND 2 and PND 3) against FLC-resistant strains of *Candida* spp. isolated in Ceará

Strain				MIC Values of MIC (µg/mL)	
	Origin	**FLC**	**PND 1**	**PND 2**	**PND 3**
C. albicans 1	Sangue	≥ 8	0.5	1.0	16
*C. albicans 2**	Sangue	≥ 8	0.5	1.0	16
C. albicans 3	Sangue	≥ 8	0.5	1.0	16
C. albicans 4	Sangue	≥ 8	0.5	1.0	16
C. albicans 5	Sangue	≥ 8	0.5	1.0	16
C. tropicalis 1	Sangue	≥ 8	1.0	2.0	32
C. tropicalis 2	Sangue	≥ 8	1.0	2.0	32
C. tropicalis 3	Sangue	≥ 8	0.5	0.5	32
*C. tropicalis 4**	Sangue	≥ 8	1.0	2.0	32
C. tropicalis 5	Sangue	≥ 8	1.0	2.0	32
C.tropicalis 7	Urina	≥ 8	1.3	2.6	32
C.parapsilosis 1	Sangue	≥ 8	0.8	0.75	24
*C.parapsilosis 2**	Sangue	≥ 8	0.8	0.75	24
C.parapsilosis 3	Sangue	≥ 8	0.8	1.0	24
C.parapsilosis 4	Sangue	≥ 8	0.8	0.75	24

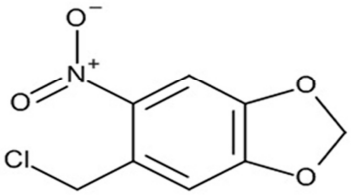
Minimum inhibitory concentrations (MIC) of FLC and piperonal nitro-derivatives (PND 1, PND 2 and PND 3) against clinical strains of *Candida* species. The MIC was defined as the lowest concentration that produced a 90% reduction in the growth of fungal cells after 24h of incubation. The microdilution in broth was performed according to CLSI protocol M27-S4. The FLC concentrations ranged from 0.125-64 µg/mL and the PNDs (1, 2 and 3) concentrations varied from 0.25-128 µg/mL. The MICs represent the geometric means of at least three MICs determined on different days.
*The strains were used for determination of action mechanism.



PND 1



PND 2



PND 3

Figure 1.Chemical structures of the piperonal nitro derivatives (PNDs) used in the present study.

Table.2 Effects of piperonal nitro-derivatives (PND 1, PND 2 and PND 3) on DNA damage index for 24 h using standard and modified version of alkaline comet assay in fluconazole-resistant *C. albicans*

Compounds	Treatments	Without endonucleases	FPG-sensitive sites[b]	Endo III-sensitive sites[c]
		Damage index ± S.E.M.	Damage index ± S.E.M.	Damage index ± S.E.M.
NC[a]	-	11.61 ± 1.13	8.55 ± 1.17	12.49 ± 2.15
Fluconazole	64 µg/mL	9.25 ± 2.25	45.71 ± 0.10$^{*,\#}$	30.24 ± 1.10$^{*,\#}$
Amphotericin B	4 µg/mL	92.52 ± 0.17*	152.25 ± 7.25$^{*,\#}$	136 ± 4.25$^{*,\#}$
PND 1	MIC_{50}	64.07 ± 2.10*	104.23 ± 4.15$^{*,\#}$	108.38 ± 0.56$^{*,\#}$
	2 x MIC_{50}	83.39 ± 3.45*	136.83 ± 3.17$^{*,\#}$	145± 1.10$^{*,\#}$
	4 x MIC_{50}	88.39 ± 2.15*	149.41 ± 2.10$^{*,\#}$	136 ± 0.11$^{*,\#}$
PND 2	MIC_{50}	71.55 ± 1.08*	128.33 ± 1.06$^{*,\#}$	115 ± 2.20$^{*,\#}$
	2 x MIC_{50}	87.25 ± 0.55*	148 ± 5.16$^{*,\#}$	129 ± 3.15$^{*,\#}$
	4 x MIC_{50}	91.47 ± 4.10*	164 ± 3.08$^{*,\#}$	133.72 ± 1.15$^{*,\#}$
PND 3	MIC_{50}	73.93 ± 2.50*	105.78 ± 1.05$^{*,\#}$	111.43 ± 0.21$^{*,\#}$
	2 x MIC_{50}	93.48 ± 1.10*	127 ± 2.03$^{*,\#}$	134 ± 1.11$^{*,\#}$
	4 x MIC_{50}	102.61 ± 1.15*	158 ± 9.15$^{*,\#}$	130 ± 2.25$^{*,\#}$

$^{*}p < 0.05$ compared to control by ANOVA followed by Tukey's test. Data are presented as means ± S.E.M. for three independent experiments in triplicate.
$^{\#}p < 0.05$ compared to set of experiments carried out without endonucleases by ANOVA followed by Tukey'stest. Data are presented as means ± S.E.M. for three independent experiments in triplicate.
[a]Negative control was treated with the vehicle (DMSO, 0.1%) used for diluting the test substances.
[b]FPG: formamidopyrimidine DNA glycosylase.
[c]Endo III: endonuclease III.

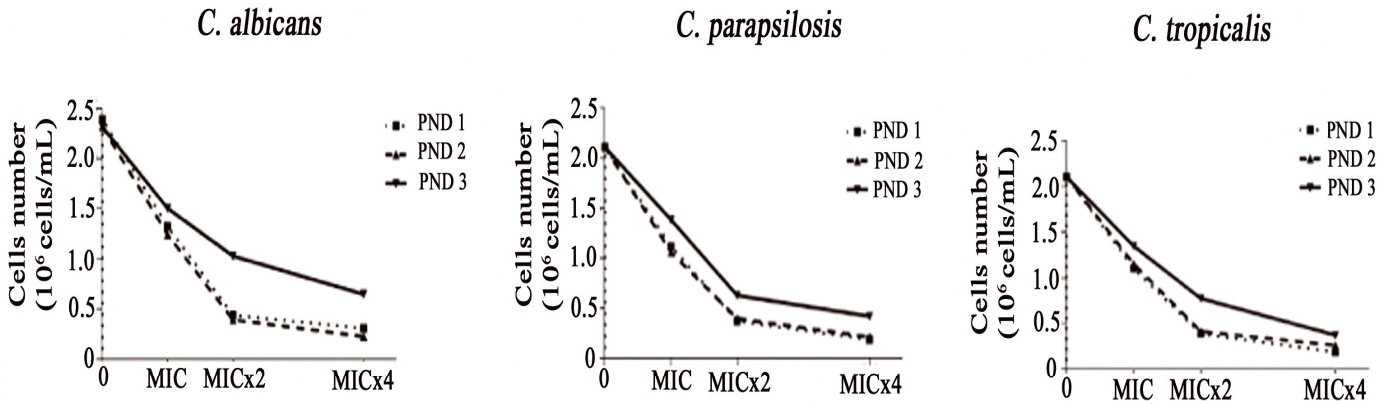


Figure 2. The cell number viable. The effect of the piperonal nitro derivatives (PNDs) on the cell number viable of representative FLC-resistant strains of *Candida* sp.

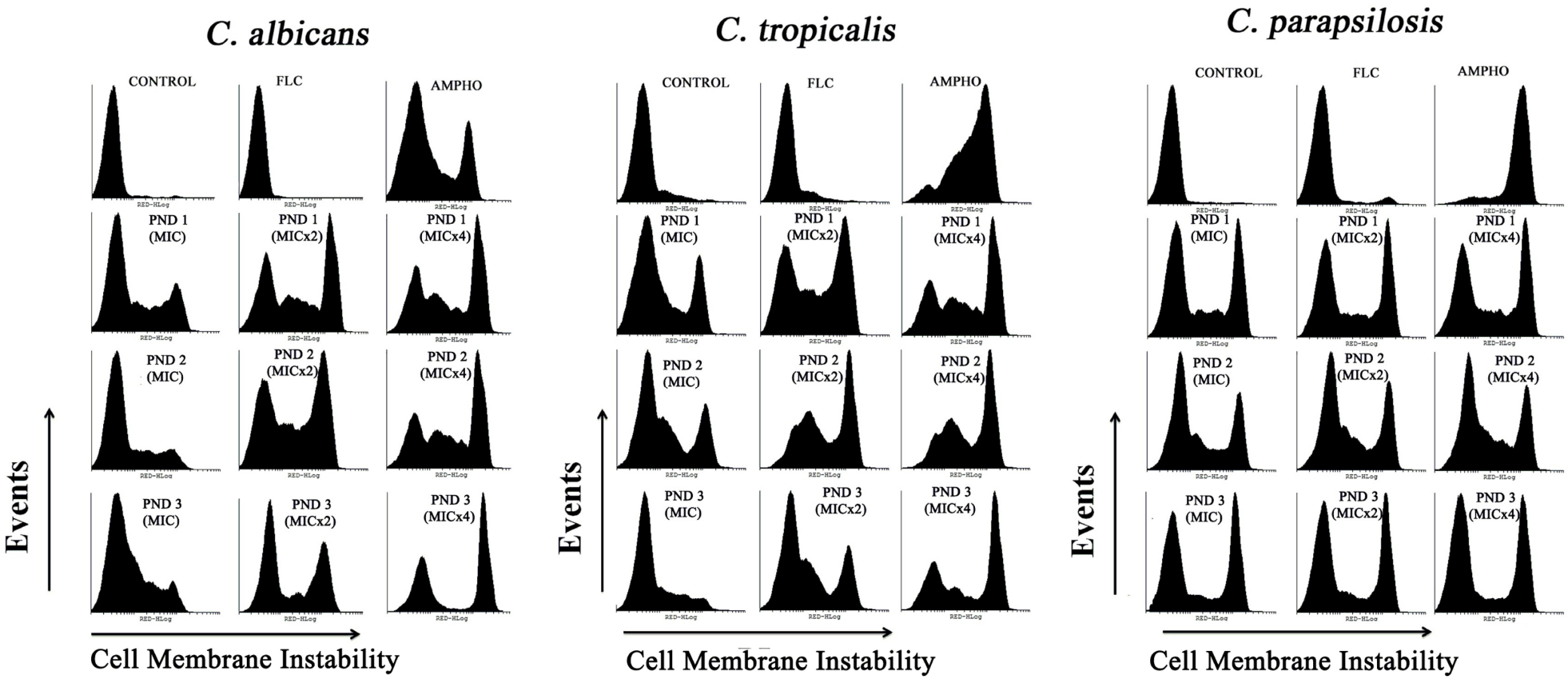


Figure 3. The effect of the piperonal nitro derivatives on the cell membrane stability of FLC-resistant strains of *Candida* spp. PNDs are identified as 1, 2 and 3, and they were tested at MIC, MIC x2 and MIC x4 values.

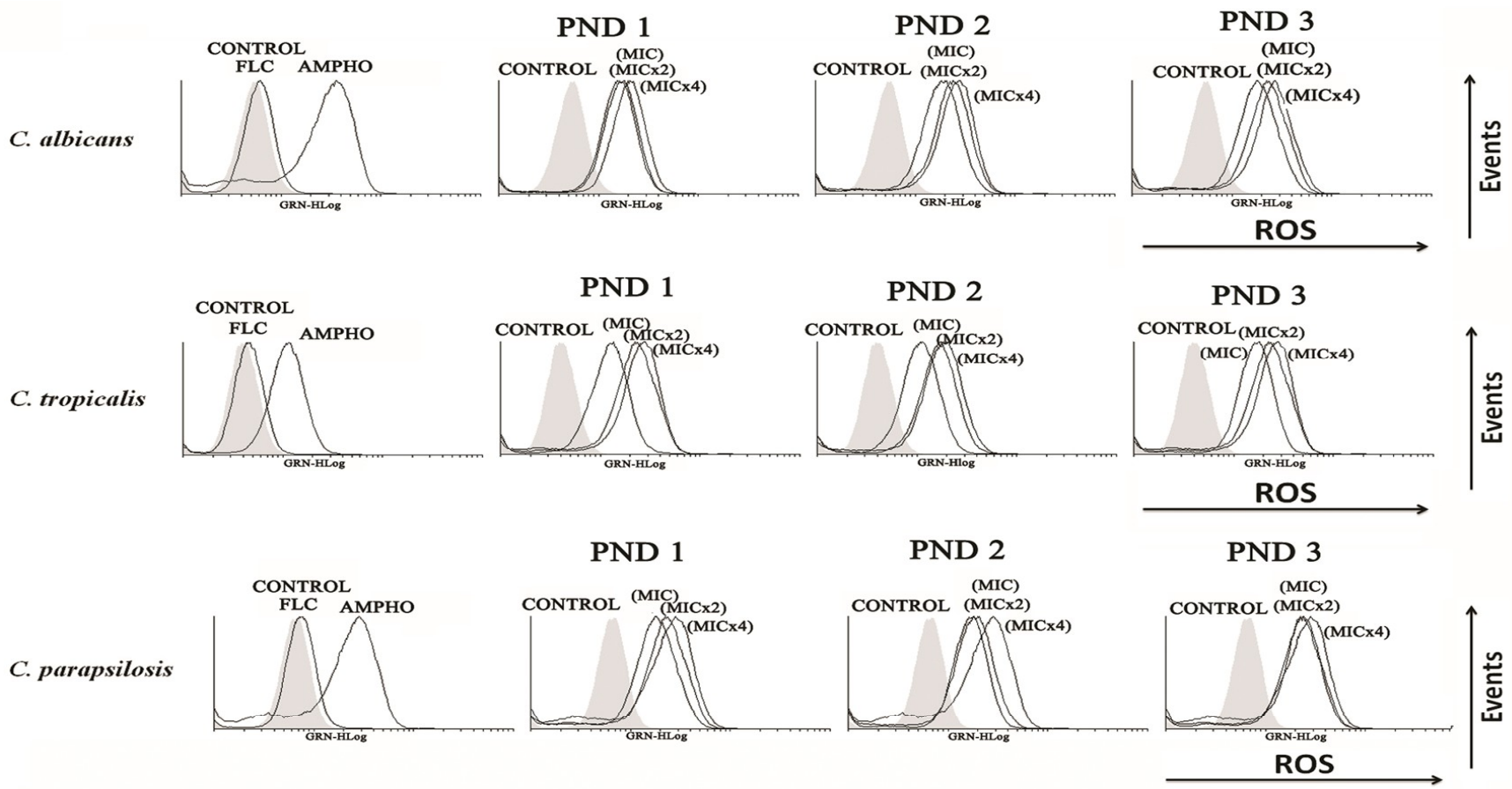


Figure 4. ROS formation. An evaluation of ROS formation in fluconazole-resistant *Candida* spp. strains after treatment with piperonal nitro derivatives (PNDs) using the concentrations MIC, MIC x2 and MIC x4. The percentage of ROS formation in therepresentative strains of FLC-resistant of Candida spp. was evaluated for 24 hours.

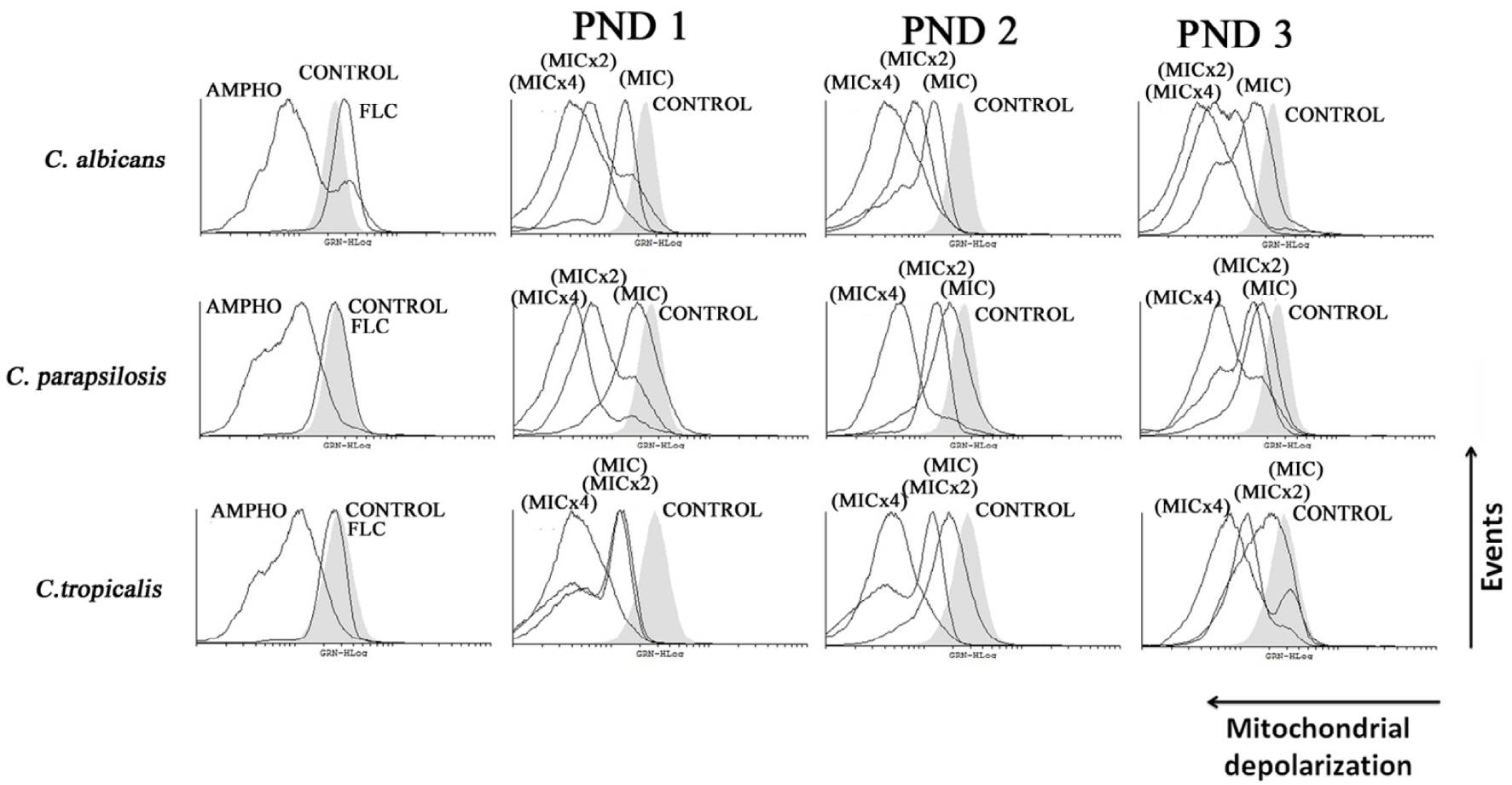


Figure 5. The mitochondrial membrane potential. An assessment of the mitochondrial membrane potential (Δψm) of fluconazole-resistant *Candida* spp. strains. The cells were labeled with Rh123 (50 nM). The graph shows strains incubated for 24 hours with RPMI-1640 (control), with FLC (64 µg/mL) and Ampho (4 µg/mL) and with piperonal nitro derivatives (PNDs) at concentrations of MIC, MIC x2 and MIC x4. The percentage of cells with mitochondrial dysfunction in the representative strains FLC-resistant of *Candida* spp. was evaluated for 24 hours.

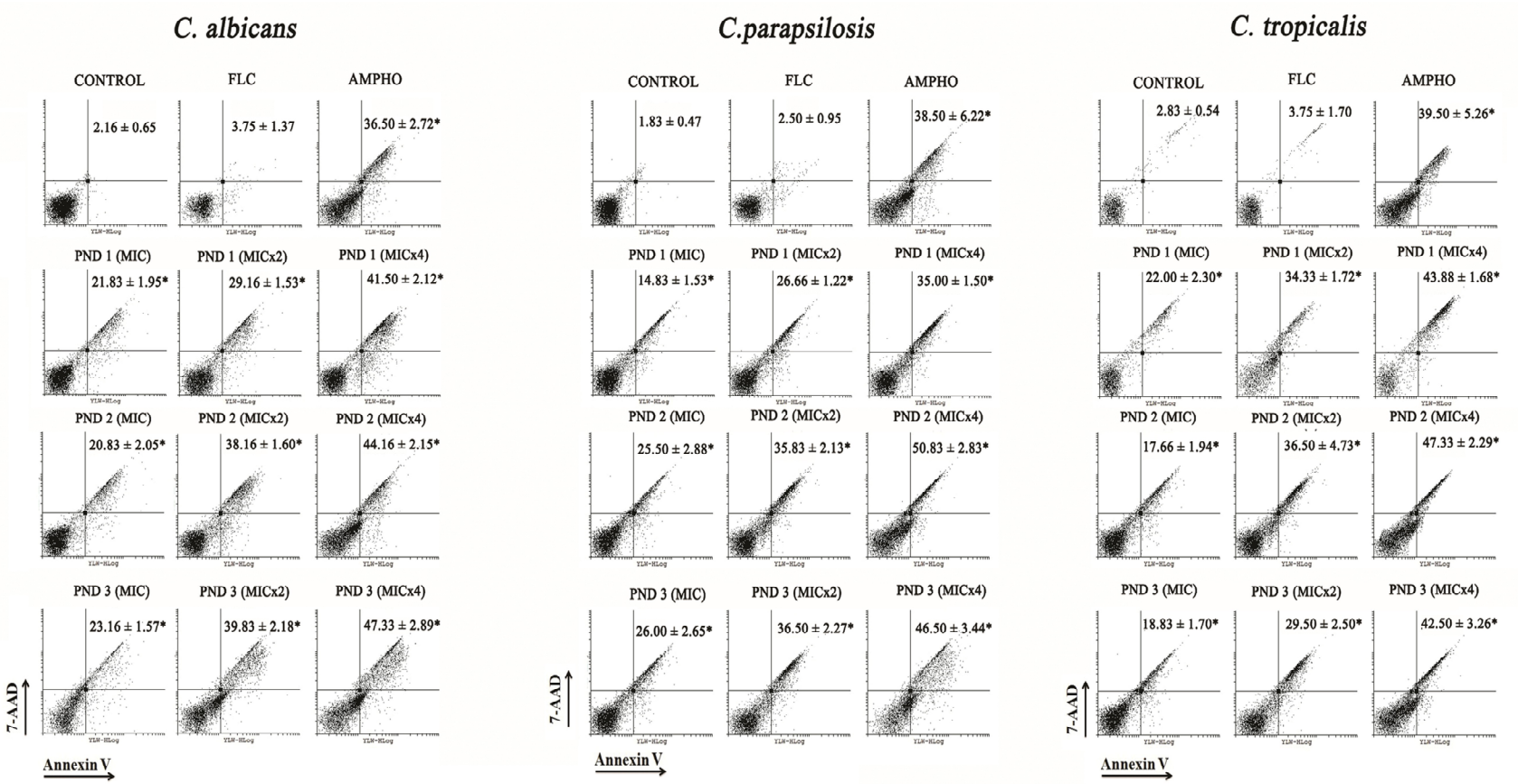


Figure 6. Phosphatidylserine externalization. Phosphatidylserine externalization, which is observed at an early stage of apoptosis, was shown by annexin V staining. This probe enabled us to detect alterations in phosphatidylserine localization from the inner membrane to the outer membrane.The intensity of fluorescence indicates the quantity of exposed phosphatidylserine on cells treated with piperonal nitro derivatives (PNDs) at concentrations of MIC, MIC x2 and MIC x4. The percentage of Annexin V+ cells in the in the representative strains FLC-resistant of Candida spp. was evaluated for 24 hours.*P<0.05 compared to control by ANOVA followed by the Newman-Keuls test

Table 3. Effects of piperonal nitro-derivatives (PND 1, PND 2 and PND 3) on DNA damage index for 24 h using standard and modified version of alkaline comet assay in fluconazole-resistant *C. parapsilosis.*

Compounds	Treatments	Without endonucleases	FPG-sensitive sites[b]	Endo III-sensitive sites[c]
		Damage index ± S.E.M.	Damage index ± S.E.M.	Damage index ± S.E.M.
NC[a]	-	7.81 ± 0.10	8.38 ± 0.15	9.20 ± 1.10
Fluconazole	64 µg/mL	11.38 ± 0.10	42.81 ± 2.20$^{*,\#}$	36.43 ± 0.11$^{*,\#}$
Amphotericin B	4 µg/mL	86.25 ± 0.21*	122.83 ± 2.07$^{*,\#}$	121.50 ± 1.15$^{*,\#}$
PND 1	MIC_{50}	47.29 ± 2.05*	81.33 ± 4.25$^{*,\#}$	77.38 ± 1.10$^{*,\#}$
	2 x MIC_{50}	88.92 ± 3.10*	121.44 ± 2.15$^{*,\#}$	116 ± 0.21$^{*,\#}$
	4 x MIC_{50}	94.61 ± 1.17*	137.39 ± 1.15$^{*,\#}$	125.84 ± 1.07$^{*,\#}$
PND 2	MIC_{50}	75.82 ± 0.15*	121 ± 1.05$^{*,\#}$	109 ± 0.17$^{*,\#}$
	2 x MIC_{50}	102.63 ± 1.21*	147.38 ± 4.25$^{*,\#}$	152 ± 1.11$^{*,\#}$
	4 x MIC_{50}	95.16 ± 2.15*	144 ± 0.22$^{*,\#}$	1.37.25 ± 3.25$^{*,\#}$
PND 3	MIC_{50}	79.34 ± 2.20*	107 ± 1.11$^{*,\#}$	112 ± 0.81$^{*,\#}$
	2 x MIC_{50}	98.51 ± 0.15*	139.64 ± 3.05$^{*,\#}$	134.08 ± 1.10$^{*,\#}$
	4 x MIC_{50}	117 ± 0.75*	148 ± 2.11$^{*,\#}$	155.16 ± 2.15$^{*,\#}$

$^{*}p < 0.05$ compared to control by ANOVA followed by Tukey's test. Data are presented as means ± S.E.M. for three independent experiments in triplicate.

$^{\#}p < 0.05$ compared to set of experiments carried out without endonucleases by ANOVA followed by Tukey'stest. Data are presented as means ± S.E.M. for three independent experiments in triplicate.

[a]Negative control was treated with the vehicle (DMSO, 0.1%) used for diluting the test substances.

[b]FPG: formamidopyrimidine DNA glycosylase; [c]Endo III: endonuclease III.

Table 4. Effects of piperonal nitro-derivatives (PND 1, PND 2 and PND 3) on DNA damage index for 24 h using standard and modified version of alkaline comet assay in fluconazole-resistant *C. tropicalis.*

Compounds	Treatments	Without endonucleases	FPG-sensitive sites[b]	Endo III-sensitive sites[c]
		Damage index ± S.E.M.	Damage index ± S.E.M.	Damage index ± S.E.M.
NC[a]	-	14.27 ± 0.15	13.25 ± 0.10	9.18 ± 1.17
Fluconazole	64 µg/mL	12.92 ± 1.11*	53.17 ± 2.10$^{*,\#}$	43.71 ± 0.25$^{*,\#}$
Amphotericin B	4 µg/mL	79.34 ± 3.21*	126.48 ± 1.15$^{*,\#}$	113 ± 0.11$^{*,\#}$
PND 1	MIC_{50}	58.16 ± 0.56*	91.33 ± 4.17$^{*,\#}$	107.63 ± 2.11$^{*,\#}$
	2 x MIC_{50}	91.45 ± 0.22*	142 ± 6.15$^{*,\#}$	126.82 ± 3.25$^{*,\#}$
	4 x MIC_{50}	87.69 ± 2.15*	167.16 ± 3.25$^{*,\#}$	138.95 ± 4.10$^{*,\#}$
PND 2	MIC_{50}	78.34 ± 0.11*	127.83 ± 1.15$^{*,\#}$	116 ± 0.33$^{*,\#}$
	2 x MIC_{50}	91.40 ± 1.05*	141.51 ± 1.15$^{*,\#}$	138 ± 0.55$^{*,\#}$
	4 x MIC_{50}	106.24 ± 2.07*	166.53 ± 3.10$^{*,\#}$	144.17 ± 2.07$^{*,\#}$
PND 3	MIC_{50}	84.91 ± 2.25*	134 ± 2.01$^{*,\#}$	128.21 ± 0.10$^{*,\#}$
	2 x MIC_{50}	103.17 ± 1.10*	147 ± 6.15$^{*,\#}$	141.22 ± 1.11 $^{*,\#}$
	4 x MIC_{50}	109.45 ± 3.17*	152 ± 2.11$^{*,\#}$	147.63 ± 1.05$^{*,\#}$

$^{*}p < 0.05$ compared to control by ANOVA followed by Tukey's test. Data are presented as means ± S.E.M. for three independent experiments in triplicate.

$^{\#}p < 0.05$ compared to set of experiments carried out without endonucleases by ANOVA followed by Tukey'stest. Data are presented as means ± S.E.M. for three independent experiments in triplicate.

[a]Negative control was treated with the vehicle (DMSO, 0.1%) used for diluting the test substances.

[b]FPG: formamidopyrimidine DNA glycosylase; [c]Endo III: endonuclease III.

When the yeast cells were treated with the PNDs, a significant ($p<0.05$) increase in the number of apoptotic cells was found. Each compound induced a similar number of apoptotic cells in the *Candida* species (Figure 6).

The present study demonstrates the antifungal effect of PND compounds (1, 2 and 3) on strains of fluconazole-resistant *Candida* spp. Data published on the antifungal activity of PND compounds against *Candida* species, including *C. albicans*, demonstrate different profiles of activities. However, when assessing the treatment of both species, no significant differences were found between the compounds.

The bioactivity of nitro derivatives has been reported in several studies (Paula et al., 2009; Tebbets et al., 2012), demonstrating their significant antifungal activity against various pathogenic yeasts. After exposure of *Candida* spp. strains to these compounds, there was a decrease in the number of viable cells, indicating damage to cell membranes, the functions of which may have been compromised. Microbial membrane lesions can directly lead to cell lysis or to increased permeabilization of the membrane, thereby allowing nitrocompounds to reach intracellular targets (Di Marino et al., 2012).

The increased propidium iodide (PI) absorption in to cells of fluconazole-resistant *Candida* spp. indicates that these compounds promote cell death, considering this marker only gets connected to nuclear DNA of dead cells (Xu et al., 2010).

Several papers on mitochondrial functions and dynamics have shown the crucial role of this organelle in biological processes such as aging and programmed cell death (PCD) (Mazzoni, et al., 2013). In this study, the mitochondrial function of *Candida* spp. cells appeared to be affected after exposure to compounds 1-3. The collapse of $\Delta\psi m$ can lead to transient pores in the mitochondrial membrane and to the release of pro-apoptotic factors into the cytosol (Hwang et al., 2012). Papers recently published by our group have shown that damage to mitochondria is an irreversible precursor of cell death and leads to the formation of ROS (Mazzoni, et al., 2013).

Cell death in yeast correlates with $\Delta\psi m$ dysfunction resulting from oxidative damage by ROS accumulation (Xu et al., 2010). Therefore, the formation of free radicals seems to be an important mechanism of the cytotoxicity of the nitro derivative compounds 1-3 against strains of fluconazole-resistant *Candida* spp. The transmembrane passage of nitrocompounds occurs by passive diffusion and increases as free radicals, originating from the bioreduction process, destabilize the cell membrane. Thus, due to the increase in the intracellular concentration of nitrocompounds, a greater quantity of free radicals is produced and hence greater damage is caused by oxidative stress (Paula et al., 2009). ROS are essential regulators of aging and are referred to as key players in cell death (Cho and Lee, 2011).

Thus, in relation to the various mechanisms of action described for nitrocompounds, there is evidence that DNA is the target of intermediate molecules (nitro radicalsor generation of hydroxyl radicals) formed from the bioreduction of nitrocompounds (Paula et al., 2009). Our data suggest that, after exposure to nitroderivative compounds (1-3), cells of *Candida* spp. presented total breaks in DNA chains. These results corroborate with those of Tebbets et al., (2012), who demonstrated deficient DNA repair in yeast treated with compounds containing a nitro group. It is also worth pointing out that nitro groups can play

a role as electron acceptors, thereby acting as alkylating agents at specific sites and thus allowing bioreduction in nearby DNA regions of eukaryotic cells (Paula et al., 2009).

Structural changes to nucleotide bases may occur as a result of oxidative stress. The oxidation of a nucleotide base is highly important, just like breakage in the DNA chain, for cellular homeostasis and survival (Bjelland et al., 2003). The endonucleases most commonly used in the modified comet assay are formamidopyrimidine DNA-glycosylase (FPG, also known as MutM) and endonuclease III (ENDOIII, also known as NTH). FPG is specific for oxidized purines, especially for 8-oxo-7,8-dihydroguanine (8-oxoGua), while ENDOIII recognizes oxidized pyrimidines, including thymineglycol and uracilglycol (Silva et al., 2011).

The current study demonstrated DNA damage caused by exposure to the compounds (1-3). It was found that, after incubation with the ENDOIII and FPG enzymes, there was a clear increase in DNA migration of cells treated with the nitroderivative compounds. The results revealed an extension of DNA oxidative damage in both purine and pyrimidine bases through ROS, thus leading to filament rupture.

Similar to the findings of Silva Jr et al. (2011), our molecules appear to facilitate DNA oxidative damage through the formation of oxygen reactive species. According to the literature, the main ROS described are superoxide (O_2•-) and hydrogen peroxide (H_2O_2). However, hydrogen peroxide is not toxic, but,*in vivo*, this molecule may react with partially reduced metal ions (Fenton reaction), leading to the formation of hydroxyl radicals(HO•), the main radical that causes DNA damage (Valko et al., 2007). As for the tested PND compounds (1, 2 and 3), the lesion produced was probably 8-oxoGua, the preferred substrate for FPG, and modified thymineglycol bases, the most usual injury distinguished by ENDOIII (Silva et al., 2011).

Programmed cell death (PCD) is a specific cell suicide program characterized by the externalization of phosphatidylserine on the plasma membrane, chromatin condensation and DNA fragmentation, increased ROS generation, mitochondrial damage and cytochrome C release from the mitochondria to the cytosol (Sukhanova et al., 2012). The condensation and fragmentation of the DNA represents an irreversible step in cell death. The detection of apoptosis at an early stage can be determined using Annexin V. This marker, in the presence of Ca^{2+} ions, binds with high affinity to phosphatidylserine on the membrane of apoptotic cells (Hwang et al., 2012). However, the co-staining of FITC-conjugated Annexin V and PI allows for discrimination between early apoptosis and necrosis (Eisenberg et al., 2010). The results of our experiment show that the nitro derivative compounds (1-3) induced cell death by apoptosis in strains of fluconazole-resistant *Candida* spp. These data corroborate the results of Neto et al., (2014) who found similar characteristics in cell death in yeast treated with naphthoquinone-derived molecules. Based on the cell death characteristics, it is suggested that these compounds may play an antifungal role at specific DNA surrounding sites. However, the production of ROS also seems to play a role in this process, because it leads to oxidative damage to purine and pyrimidine bases of DNA.

The present work established the biological activity of semi-synthetic molecules; structural changes to these compounds may

improve the safety and efficiency of thedrug usedfor treating antimicrobial-resistant microorganisms. The PND compounds (1, 2 and 3) presented antifungal activity *in vitro* against strains of fluconazole-resistant *Candida* spp. besides promoting changes to the integrity of the mitochondrial and plasma membranes, the respective compounds seemed to act on specific DNA surrounding sites of yeast cells, leading to cell death by apoptosis.

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