

The antimicrobial activity of lapachol and its thiosemicarbazone and semicarbazone derivatives

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Lapachol was chemically modified to obtain its thiosemicarbazone and semicarbazone derivatives. These compounds were tested for antimicrobial activity against several bacteria and fungi by the broth microdilution method. The thiosemicarbazone and semicarbazone derivatives of lapachol exhibited antimicrobial activity against the bacteria Enterococcus faecalis and Staphylococcus aureus with minimal inhibitory concentrations (MICs) of 0.05 and 0.10 µmol/mL, respectively. The thiosemicarbazone and semicarbazone derivatives were also active against the pathogenic yeast Cryptococcus gattii (MICs of 0.10 and 0.20 µmol/mL, respectively). In addition, the lapachol thiosemicarbazone derivative was active against 11 clinical isolates of Paracoccidioides brasiliensis, with MICs ranging from 0.01-0.10 µmol/mL. The lapachol-derived thiosemicarbazone was not cytotoxic to normal cells at the concentrations that were active against fungi and bacteria. We synthesised, for the first time, thiosemicarbazone and semicarbazone derivatives of lapachol. The MICs for the lapachol-derived thiosemicarbazone against S. aureus, E. faecalis, C. gattii and several isolates of P. brasiliensis indicated that this compound has the potential to be developed into novel drugs to treat infections caused these microbes.

Key words: antimicrobial agents - lapachol - *Paracoccidioides*

Lapachol (2-hydroxy-3-(3-methylbut-2-enyl)naphthalene-1,4-dione) is a natural pigment that was originally isolated from species of the Bignoniaceae family, but is also found in other plant families (Araújo et al. 2002, Fonseca et al. 2003). This naphthoquinone exhibits interesting antimicrobial, antitumor, antiplasmodial, antioxidant and trypanocidal properties (Araújo et al. 2002). There are many literature reports on lapachol derivatives. However, the thiosemicarbazone and semicarbazone derivatives of lapachol have never been studied. Thiosemicarbazones and semicarbazones from sources other than lapachol, such as isatin thiosemicarbazone and benzaldehyde semicarbazone, have been widely studied and are known to have extensive pharmacological profiles, e.g., antimicrobial and anticonvulsant activities (Beraldo 2004, Vieira et al. 2010).

In this study, we searched for novel lapachol derivatives with greater activity and lower toxicity for the treatment of infectious diseases. Treating infectious diseases that are caused by bacteria or fungi remains an important and challenging public health problem (Jones et

al. 2008). Although substantial advances in biomedical science and public health have facilitated the control of numerous infectious diseases over the past century, the world has witnessed an increasing incidence and geographical expansion of emerging and re-emerging infectious diseases (Jones et al. 2008, Yang et al. 2012). Discovering new antimicrobial agents is important because several pathogenic microorganisms have acquired resistance. The prevalence of *Staphylococcus aureus* strains that are resistant to conventional antibiotics has greatly increased in some hospitals (Fluit et al. 2001, Hidron et al. 2008, Rosenthal et al. 2010).

There has been an increase in the number of immunocompromised hosts because of advances in medical technology and the human immunodeficiency virus pandemic (Wills et al. 2000) and the risk for opportunistic fungal infections has greatly increased in these patients. Moreover, common pathogens and new pathogenic species that possess intrinsic primary resistance are rapidly developing secondary resistance to the current antifungal agents (White et al. 1998). Other diseases, such as paracoccidioidomycosis (PCM), require long-term therapy to ensure a good clinical response and to avoid a relapse. Importantly, long periods of treatment, which are typically required to control these types of fungal infections, are correlated with drug toxicity, high costs and treatment abandonment by the patients. Relapse is a significant obstacle for patients and those patients who do not respond and severely ill patients pose a problem for conventional therapeutic approaches (Brummer et al. 1993, Coutinho

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et al. 2002, Paniago et al. 2003, Travassos et al. 2008). The increase in antibacterial resistance and the restricted number of commercially available antifungal drugs emphasise the need for the development of novel and more effective antifungal and antibacterial agents.

In this work, we evaluated the antimicrobial activity of the thiosemicarbazone and semicarbazone derivatives of lapachol, which we synthesised for the first time, and tested against several clinically important microorganisms. Lapachol was also tested. Lapachol and its thiosemicarbazone derivative were also tested against human peripheral blood mononuclear cells (PBMCs).

SUBJECTS, MATERIALS AND METHODS

Synthesis - Lapachol was isolated from *Tabebuia ochracea* in a previous study by Zani et al. (1991). Thiosemicarbazide (Sigma-Aldrich, Steinheim, Germany) was purified by crystallisation from water. Acetone (Vetec, RJ, Brazil), methanol (Sigma-Aldrich), semicarbazide hydrochloride (Sigma-Aldrich) and sodium hydroxide (Sigma-Aldrich, Steinheim, Germany) were used without further purification. Column chromatography was performed using silica gel 60G (70-230 mesh). Thin-layer chromatography (TLC) was executed on pre-coated TLC silica gel 60F₂₅₄ plates Merck (Darmstadt, Germany) and the spots were visualised under ultraviolet (UV) light at wavelengths of 254 and 366 nm after the plates had been sprayed with vanillin-H₂SO₄ and heated at 120°C for 10 min. Analytical high-pressure liquid chromatography (Shim-Pak prep SiL, 4 µm, 4.6 x 250 mm, 1 mL/min) was performed on a Shimadzu chromatography system equipped with an LC10AD pump and a UV detector set at λ₂₁₀ nm and λ₂₄₀ nm. Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker DRX 400 spectrometer at 400 MHz using tetramethylsilane as an internal standard. Mass spectrometry (MS) results were acquired on a Thermo Finnigan LCQ-Advantage spectrometer equipped with an electrospray ion (ESI) source. Solutions of the compounds at 200 µg/mL in 1:1 MeOH-H₂O were infused at a rate of 0.025 mL/min and positive and negative mass spectra were acquired with an m/z range of 50-1,000 daltons. The cone voltages were optimised for positive and negative ion analysis between 25-50 V. The capillary voltage was set at 4.5 kV in positive ion mode and 23.1 kV in negative ion mode. For the MS/MS experiments, the parent ion isolation width was 3.8 daltons and the normalised collision energy was set at 30% for both compounds. Fifty scans from 150-600 daltons were collected to generate the averaged spectra.

The X-ray diffraction data were collected on an Oxford-Diffraction GEMINI diffractometer (LabCri) using a graphite-enhanced MoKα radiation source (k = 0.71069 Å) at 293(2) K. The lapachol thiosemicarbazone twin crystal was mounted on a glass fibre and examined by structural X-ray diffraction methods. The data collection, integration and scaling of the reflections were performed with the CrysAlis suite programs (CrysAlis RED, Oxford Diffraction Ltd, version 1.171.32.38 and SCALE3 ABSPACK Scaling Algorithm, CrysAlis RED, Oxford Diffraction Ltd, version 1.171.32.38).

The final unit cell parameters were determined by fitting all the reflections. The structures were solved by direct methods with the program SHELXS-97 (Sheldrick 1997) and were refined by full-matrix least-squares methods against F² using SHELXL-97 (Sheldrick 1997). The positional and anisotropic atomic displacement parameters of the compound were refined for non-hydrogen atoms. Although all the hydrogen atoms could be identified in a Fourier difference map in the final model, the hydrogen atoms of lapachol thiosemicarbazone were included in the molecular model at stereo-chemical positions and were refined with the riding method. The molecular graphic was obtained from ORTEP (Johnson 1965, Farrugia 1997, Farrugia & Win 1999). The studied material was endotoxin free.

Synthesis of lapachol thiosemicarbazone - A suspension of 10 mmol lapachol in 100 mL of water was added to 100 mL of 0.1 M NaOH, yielding a dark red solution. An aqueous-methanolic (50%) solution of thiosemicarbazide (12 mmol) was added dropwise to the above solution with constant stirring. The mixture was stirred for approximately 21 h, after which time the solution was neutralised with 10% HCl. The crude precipitated product was filtered and washed with cold water. The resulting solid was recrystallised from methanol and acetone (1:1), giving an orange crystalline thiosemicarbazone with a yield of 73% (Chikate et al. 2005). The lapachol thiosemicarbazone was analysed by melting point analysis, MS, NMR and X-ray diffraction.

Synthesis of lapachol semicarbazone - A solution of 30 mL of 0.1 M NaOH was added to a suspension of 1 mmol lapachol, yielding a dark red solution. An aqueous-methanolic (50%) solution of semicarbazide (2.5 mmol) was added dropwise to the above solution with constant stirring. The mixture was stirred for approximately 23 h, after which time the solution was neutralised with 10% HCl. The crude product was filtered and washed with cold water. The resulting solid was purified by column chromatography (silica gel 70-230 mesh) using dichloromethane and ethyl acetate as eluents in mixtures with increasing polarity to give the semicarbazone, which was isolated as a yellow solid in a 13% yield (Chikate et al. 2005). The lapachol semicarbazone was analysed by MS and NMR.

Antimicrobial assays - Microorganism targets - The antimicrobial activity was evaluated using the following microorganisms from the American Type Culture Collection (ATCC) (Rockville, MD, USA): *S. aureus* ATCC 25295, *Escherichia coli* ATCC 18804, *Salmonella typhimurium* ATCC 14028, *Pseudomonas aeruginosa* ATCC 49189, *Enterococcus faecalis* ATCC 19433, *Candida albicans* ATCC 18804, *Candida parapsilosis* ATCC 22019, *Candida tropicalis* ATCC 750 and *Cryptococcus gattii* ATCC 32608. In addition, 11 clinical *Paracoccidioides brasiliensis* strains were obtained: Pb-01 ATCC-MYA-826, Pb-18 [Fungi Collection of the Faculty of Medicine of the São Paulo University, São Paulo, state of São Paulo (SP), Brazil], Pb-B339 ATCC 32069, Pb-14 (clinical isolate from an acute PCM patient, SP), Pb-3,

Pb-4 (clinic isolates from chronic PCM patients, SP - MHH Forjaz/TIE Svidzinski), Pb-2 (Epm 60), Pb-1578, Pb-8, Pb-ED01 and Pb-11 (clinic isolates from acute PCM patients, state of Paraná, Brazil, TIE Svidzinski). The bacterial strains were maintained on brain heart infusion agar (Difco, USA). All the fungal strains were maintained on Sabouraud dextrose agar (Oxoid, Basingstoke, UK). The *P. brasiliensis* strains were maintained in yeast-peptone-dextrose (YPD).

Culture media and inocula - Mueller-Hinton broth (HiMedia, India) was prepared in accordance with the Clinical and Laboratory Standards Institute (CLSI) document M7-A6 (NCCLS 2003) for minimal inhibitory concentration (MIC) bacterial assays. Inocula of all the bacteria were prepared using the spectrophotometric method according to CLSI M7-A6 (NCCLS 2003) at a final concentration of 5×10^5 colony-forming unit/mL. The fungal cultures of the *Candida* species and *C. gattii* were freshly grown at 35°C and the inoculum suspensions were prepared by the spectrophotometric method according to the CLSI document M27-A3 (CLSI 2008) with a final concentration of $1.5 \pm 1.0 \times 10^3$ cells/mL for susceptibility tests. A weekly passage on solid YPD medium at 37°C was performed to grow *P. brasiliensis*. Yeast cells in the exponential phase were collected aseptically with a sterile loop and resuspended in a tube containing 5 mL of sterile saline. Large aggregates were allowed to settle for several minutes and the supernatants were collected. The suspensions were diluted in synthetic RPMI medium (Sigma-Aldrich, St. Louis, MO, USA) containing L-glutamine and buffered to pH 7.0 with 0.165 M morpholine propanesulfonic acid (Sigma-Aldrich). The suspensions were prepared according to the CLSI document M27-A3 to obtain a final inoculum size suitable for each strain (CLSI 2008). After homogenisation of the inocula by vortexing, the transmittance was measured at 520 nm and adjusted to 69-70% (Hahn & Hamdan 2000).

Susceptibility test - Broth microdilution testing was performed in accordance with the guidelines presented in the CLSI documents M7-A6 (NCCLS 2003) for bacteria and M27-A3 for fungi (CLSI 2008). The susceptibility to antimicrobial agents was determined by the microbroth dilution method and was performed in sterile flat-bottom 96-well microplates (Difco, Detroit, MI, USA).

The compounds were dissolved in dimethyl sulfoxide (DMSO) after the addition of Mueller-Hinton broth for the bacterial assays or RPMI for the fungal assays. Subsequently, serial dilutions were prepared using the corresponding media as the diluent, maintaining a constant volume of 1 mL in each tube. The compounds were tested at eight concentrations from 0.006-0.84 µmol/mL. For each dilution, aliquots of 0.1 mL were added to the wells of the microplates.

For a growth and sterility control, media was used without the addition of extract or solvent. As a control for the toxicity of the solvent, a culture was inoculated with DMSO. Chloramphenicol (2.41×10^{-3} to 0.31 µmol/mL; Sigma-Aldrich) was used as a positive antibacterial control. Amphotericin B (3.25×10^{-5} to 0.03 µmol/mL;

Sigma-Aldrich) and trimethoprim/sulphamethoxazole (4.49×10^{-3} to 2.3 µmol/mL; Roche, state of Rio de Janeiro, Brazil) were utilised as positive antifungal controls.

After the plates were prepared, the inocula of each bacterial and fungal strain were added and the plates were incubated at 37°C for 24 h for the bacteria, 48 h for *Candida* spp, 72 h for *C. gattii* and 10 days for *P. brasiliensis*. Each test was performed in triplicate. The endpoints were determined visually by comparison of the samples with the drug-free control well. The MIC was defined as the lowest compound concentration at which the well was optically clear and was expressed in µmol/mL.

Sorbitol protection assays - The MICs were determined using *P. brasiliensis* strain Pb18 by the standard broth microdilution procedure described above. Duplicate plates were prepared: one contained lapachol thiosemicarbazone and 0.8 M sorbitol as an osmotic support and the other contained lapachol thiosemicarbazone alone. The MICs were determined after 10 days (Escalante et al. 2008).

Human PBMCs - PBMCs were prepared using a modified version of the protocol previously described by Gazzinelli et al. (1983). Briefly, PBMC samples were obtained via an agreement with the Minas Gerais Haematology and Haemotherapy Centre Foundation (protocol 105/2004). The PBMCs were obtained from healthy adult volunteers of both sexes by the centrifugation of heparinised venous blood over a Ficoll cushion (Sigma-Aldrich, St. Louis, MO). The mononuclear cells were collected from the interphase after Ficoll separation and were washed three times in RPMI-1640 medium before further processing. All the cultures were grown in RPMI-1640 medium (Sigma-Aldrich) supplemented with 5% (v/v) heat-inactivated, pooled AB (GIBCO/BRL, Grand Island, NY) sera and 2 mM L-glutamine. An antibiotic/antimycotic solution containing 1,000 U/mL penicillin, 1000 µg/mL streptomycin and 25 µg/mL fungizone (GIBCO/BRL, Grand Island, NY) was added to prevent fungal and bacterial contamination.

Analysis of cell viability - The PBMCs were cultured in 96-well plates at a density of 200,000 cells/well in a final volume of 0.2 mL/well. The plates were pre-incubated in a humidified 5% CO₂/95% air atmosphere at 37°C for 24 h to allow the cells to adapt prior to the addition of the test compounds. All the compounds were dissolved in DMSO prior to dilution. The toxicity was determined over a concentration range of 0.3-200 µg/mL. All the cell cultures were incubated in a humidified 5% CO₂/95% air atmosphere at 37°C for 48 h. The cell viability was estimated by measuring the rate of the mitochondrial reduction of a yellow tetrazolium salt, 2-(3,5-diphenyltetrazol-2-ium-2-yl)-4,5-dimethyl-1,3-thiazole bromide (MTT) (Sigma-Aldrich, St. Louis, MO), to insoluble purple formazan crystals (Mosmann 1983). After incubation of the cultures with the test compounds, the MTT solution (0.020 mL at 5 mg/mL) was added to each well and the plates were incubated for 4 h. At the end of this incubation, the supernatant was removed and 0.04 M HCl in isopropyl alcohol (200 µL)

was added to dissolve the formazan crystals. The optical densities were measured with a spectrophotometer at a wavelength of 590 nm. The results were normalised to the DMSO control (0.01%) and were expressed as the per cent inhibition of cell viability. The interactions between the compounds and the media were estimated based on the variations between the drug-containing medium and the drug-free medium to control for false-positive and false-negative results. The data were analysed using Prism 5.0 (GraphPad Software, Inc).

Statistical analyses - All the PBMC results were expressed as the mean \pm standard deviation of three independent experiments that were performed at least in triplicate. These data were analysed using Student's *t* test for paired comparisons. A *p* value of less than 0.05 indicated statistical significance.

RESULTS

The reactions of lapachol (compound 1) with thiosemicarbazide and semicarbazide gave a thiosemicarbazone (compound 2) and a semicarbazone (compound 3) with yields of 73% and 13%, respectively (Fig. 1). The ESI-MS data for compound 2 showed a positive ion peak at *m/z* 316, which was attributed to the quasi-molecular ion peak $[M + H]^+$ and the ESI-MS of compound 3 exhibited a negative ion peak at *m/z* 298, which was attributed to the quasi-molecular ion peak $[M - H]^-$; these quasi-molecular ion peaks correspond with the expected molecular weights of 315 and 299 g/mol for the compound 2 and the compound 3, respectively. The compound 2 had a melting point of 174.3-175.3°C.

The comparison of the ^1H spectra (Table I) between lapachol and its derivatives (compounds 2 and 3) indicat-

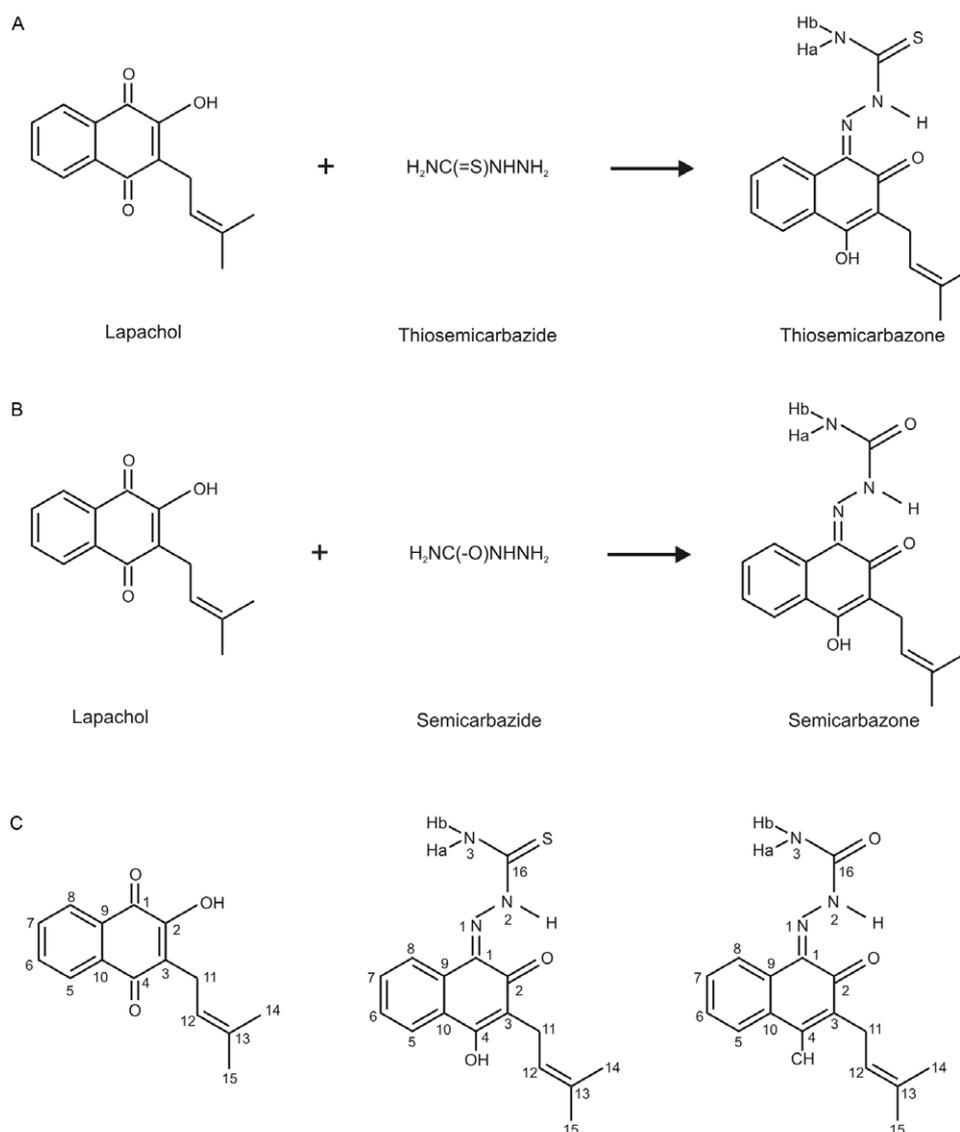


Fig. 1: synthesis of lapachol thiosemicarbazone and semicarbazone and structures of lapachol (A), lapachol derivatives thiosemicarbazone (B) and semicarbazone (C).

TABLE I
¹H nuclear magnetic resonance (NMR) [400 MHz, dimethyl sulfoxide (DMSO), δ , J (Hz)]
 and ¹³C NMR (100 MHz, DMSO, δ) data for lapachol, thiosemicarbazone and semicarbazone

	¹ H NMR			¹³ C NMR		
	Lapachol	Thiosemicarbazone	Semicarbazone	Lapachol	Thiosemicarbazone	Semicarbazone
1	-	-	-	181.00	129.53	129.21
2	-	-	-	154.98	180.44	181.00
3	-	-	-	122.87	117.39	117.23
4	-	-	-	184.10	163.62	163.00
5	7.95 m	8.00 d (7.2)	7.98 d (6.4)	125.52	123.25	123.55
6	7.76 t (7.2)	7.55 m	7.50 m	134.37	128.82	128.09
7	7.81 t (7.0)	7.57 m	7.53 m	133.16	129.93	129.74
8	7.97 m	8.64 d (7.2)	8.50 d (6.4)	125.60	123.78	123.49
9	-	-	-	129.85	131.28	131.61
10	-	-	-	131.83	127.08	126.41
11	3.15 d (7.2)	3.27 d (6.8)	3.26 d (6.6)	21.95	21.15	21.16
12	5.11 t (7.2)	5.10 t (6.8)	5.09 t (6.6)	120.60	122.00	122.16
13	-	-	-	131.86	131.14	131.03
14	1.71 s	1.65 s	1.75 s	17.66	17.88	17.88
15	1.62 s	1.53 s	1.63 s	25.35	25.45	25.47
16	-	-	-	-	179.51	155.45
2 (NH)	-	14.94 s	14.26 s	-	-	-
3 (NH _a)	-	9.22 s	7.32 b	-	-	-
3 (NH _b)	-	8.96 s	7.32 b	-	-	-

ed that the signals from the side-chain at C-3 were virtually unchanged. In addition, the N2-H chemical shifts at 14.94 and 14.26 in the spectra of 2 and 3 were indicative of conformations in which N2-H is hydrogen bonded to O-2. The N3-H_a and N3-H_b hydrogens of 2 were diastereotopic. The different chemical shifts likely result from a hydrogen bond between N3-H_a and N1, indicating that the same conformation that has been suggested in solution is found in the solid state.

Changes were predominantly observed in the ¹³C NMR spectrum (Table I). Shielding effects were observed at C-1 [δ_c 129.53 (2) and 129.21 (3)], C-4 [δ_c 163.62 (2) and 163.00 (3)] and C-3 [δ_c 117.39 (2) and 117.23 (3)] with the carbonyl group of lapachol being replaced with an imine group (C-1), a hydroxyl group (C-4) or a C3-C4 double bond in the obtained products. Changes in the values of the aromatic ring and a deshielding effect at C-2 [δ_c 180.44 (2) and 181.00 (3)] compared with lapachol (δ_c 154.98) suggested the formation of a carbonyl group at C-2. The ¹³C signals related to the thiosemicarbazone (compound 2) and semicarbazone (compound 3) moieties were observed at δ_c 179.51 (C = S) for 2 and at δ_c 155.45 (C = O) for 3 (Table I). The COSY, HMQC and HMBC spectra were used to confirm the structures and to unambiguously assign the chemical shifts for all the hydrogen and carbon atoms of 2 and 3 (Table I).

The presence of the thiosemicarbazone and semicarbazone moieties at C-1 was confirmed by the observation of N2-H...O1 hydrogen bonds in (2) and (3) and by the heteronuclear long-range couplings of the hydrogen

and carbon atoms that were revealed by the cross-peaks in the HMBC spectra. These cross-peaks corresponded to the spin-spin interactions of H-11 [δ_H 3.27 (2) and 3.26 (3)] with C-2 [δ_c 181.00 (3)] and C-4 [δ_c 163.62 (2) and 163.00 (3)] and of H-5 with C-4 [δ_c 163.62 (2)].

Fig. 2 presents a perspective view of the lapachol thiosemicarbazone. The crystal data and refinement results are listed in Table II. Selected intramolecular bond distances and angles in the structure are presented in Table III. The bond lengths and angles for the lapachol moiety are in good agreement with the values that were reported for Hooker's "lapachol peroxide" (Júnior et al. 2009). The most significant differences are the C-O bonds. The C-O distance varies from 1.364-1.431 Å for the single bonds and from 1.209-1.227 Å for the double bonds in Hooker's lapachol structure. In lapachol thiosemicarbazone, the C4-O2 (1.350(2) Å) single bond is shorter and the C2-O1 [1.236(2) Å] double bond is longer. The bond distances and angles in the thiosemicarbazone framework are similar to the bonds and angles in other thiosemicarbazones (Beraldo et al. 2001, Lessa et al. 2010).

Lapachol thiosemicarbazone adopts the *E* conformation with respect to the N2-C16 bond in the solid state. A weak intramolecular N2-H...O1 hydrogen bond (Table IV) is present in the structure, but is not likely to facilitate rotation around the N2-C16 bond. The same conformation occurs in 2 in a DMSO solution, as was discussed earlier. In the compound packing, weak intermolecular O2-H...S1 and N3-H...O2 hydrogen bonds result in the formation of linear arrangements in the solid state (Fig. 3).

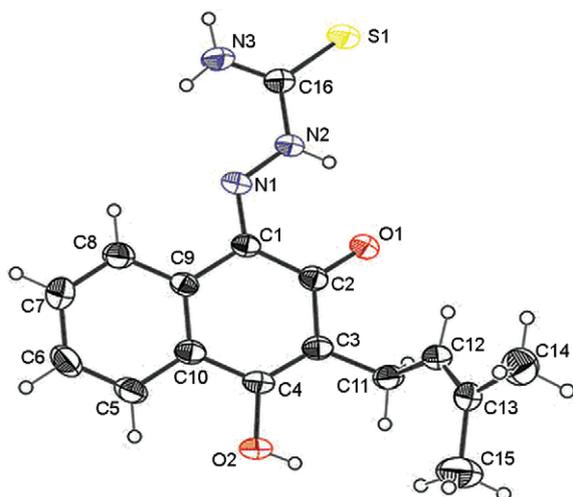


Fig. 2: molecular plot of lapachol thiosemicarbazone showing the labelling scheme of the non-H atoms and their displacement ellipsoids at the 50% probability level.

The lapachol thiosemicarbazone and semicarbazone derivatives were tested against five pathogenic bacteria (*E. coli*, *S. aureus*, *P. aeruginosa*, *S. typhimurium* and *E. faecalis*). The most effective antimicrobial activities were observed against two pathogenic Gram-positive bacteria, *S. aureus* and *E. faecalis* (Table V). These compounds were inactive against Gram-negative bacteria. The lapachol thiosemicarbazone and semicarbazone derivatives had MICs of 0.10 $\mu\text{mol/mL}$ against *S. aureus* and 0.05 $\mu\text{mol/mL}$ against *E. faecalis*. Lapachol inhibited the growth of *S. aureus* and *E. faecalis* at a concentration of 0.52 $\mu\text{mol/mL}$. The semicarbazide and thiosemicarbazide reagents were tested against five pathogenic bacteria and were active against *E. faecalis*, inhibiting its growth at a concentration of 0.83 and 0.69 $\mu\text{mol/mL}$, respectively (Table V).

Lapachol was tested against opportunistic *Candida* sp., *C. gattii* and *P. brasiliensis*. It had superior activity only against *P. brasiliensis*; the MIC was 0.13 $\mu\text{mol/mL}$ for most of the isolates and 0.26 $\mu\text{mol/mL}$ for *P. brasiliensis* 01.

The lapachol thiosemicarbazone derivative exhibited the best antifungal activity, with MICs ranging from 0.01–0.10 $\mu\text{mol/mL}$ for the isolates of *P. brasiliensis*. The lapachol semicarbazone derivative was less active than the thiosemicarbazone derivative against the *P. brasiliensis* isolates (MICs 0.42 vs. 0.84 $\mu\text{mol/mL}$, respectively). *C. albicans* and *C. tropicalis* were resistant to the thiosemicarbazone and semicarbazone derivatives at the tested concentrations. *C. gattii* was susceptible to the thiosemicarbazone and semicarbazone derivatives, with MICs of 0.10 and 0.21 $\mu\text{mol/mL}$, respectively. The semicarbazide and thiosemicarbazide reagents were inactive against all fungi tested except *P. brasiliensis* isolates, the growth of which was inhibited by the thiosemicarbazide reagent at a concentration of 1.37 $\mu\text{mol/mL}$.

In this study, the lapachol thiosemicarbazone derivative had the greatest activity against *P. brasiliensis*;

TABLE II
Crystal data and refinement results
for lapachol thiosemicarbazone

Empirical formula	$\text{C}_{16}\text{H}_{17}\text{N}_3\text{O}_2\text{S}$
Formula weight	315.39
Crystal system	Triclinic
Space group	P-1
Wavelength, Å	0.71073
Unit cell dimensions	
a, Å	6.9260(6)
b, Å	10.2676(18)
c, Å	11.0379(8)
α , °	84.659(10)
β , °	73.210(7)
γ , °	85.264(12)
Volume, Å ³	746.94(16)
Z, density calc, mg/m ³	2, 1.402
Absorption coefficient, mm ⁻¹	0.228
F(000)	332
Crystal size, mm	0.31 x 0.28 x 0.20
θ range for data coll., °	2.88–26.37
Index range, θ	-8 $\leq h \leq$ 8
	-12 $\leq k \leq$ 12
	-13 $\leq l \leq$ 13
Completeness	$\theta = 26.37^\circ$ 99.9%
Goodness-of-fit on F^2	0.960
Reflec. collect./unique	8729/3049
Data/restraints/parameters	8729/0/202
Observed reflections, $I > 2\sigma(I)$	5780
Final R indices [$I > 2\sigma(I)$]	$R_1 = 0.0466$, $wR_2 = 0.1127$
R indices (all data)	$R_1 = 0.0800$, $wR_2 = 0.1289$
Larg. peak and hole, e Å ⁻³	0.265/-0.255

therefore, its effect on the fungal cell wall was determined using the sorbitol assay. A distinctive feature of specific inhibitors of fungal cell wall synthesis is that the antifungal effect is reversed in media containing an osmotic stabiliser such as sorbitol (Frost et al. 1995). However, the lapachol thiosemicarbazone derivative did not affect the fungal cell wall because the MIC for *P. brasiliensis* did not change upon the addition of sorbitol to the culture medium.

Compared with the control (0.01% DMSO), the lapachol-derived thiosemicarbazone did not reduce cell viability over the concentration range of 9.52×10^{-4} to 0.32 $\mu\text{mol/mL}$ (0.3–100 $\mu\text{g/mL}$), but at 0.64 $\mu\text{mol/mL}$ (200 $\mu\text{g/mL}$), this compound significantly reduced cell viability, indicating cytotoxicity (Fig. 4). The MICs for this compound against the isolates of *P. brasiliensis* were in the range of 0.10–5.40 $\times 10^{-3}$ $\mu\text{mol/mL}$ (31.2–1.7 $\mu\text{g/mL}$). For

E. faecalis and *S. aureus*, the MICs were between 0.10-0.05 $\mu\text{mol/mL}$ (between 31.2-15.6 $\mu\text{g/mL}$). These results indicate that the lapachol-derived thiosemicarbazone is not cytotoxic against normal cells at the concentrations that were active against fungi and bacteria. Conversely, lapachol had a biphasic dose-response curve, exhibiting toxicity at both the maximal concentration of 0.83 $\mu\text{mol/mL}$ (200 $\mu\text{g/mL}$) and the intermediate concentration of 0.01 $\mu\text{mol/mL}$ (3 $\mu\text{g/mL}$). At 0.01 mmol/mL (3 mg/mL), there was a significant reduction in cell viability compared with the control (0.01% DMSO). At the other tested concentrations, this compound was not toxic. The MICs for lapachol against *P. brasiliensis* and the two tested bacterial strains were in the range of 0.52-0.13 $\mu\text{mol/mL}$ (124-31.2 $\mu\text{g/mL}$) and 0.52-0.06 $\mu\text{mol/mL}$ (125-15.6 $\mu\text{g/mL}$), respectively, and no toxicity was observed against the PBMCs at these concentrations.

Supplementary material - CCDC reference 844492 for 2 contains the supplementary crystallographic data. These data can be obtained free of charge from the CCDC via ccdc.cam.ac.uk/data_request/cif.

DISCUSSION

In this study, we determined the MICs for lapachol and its derivatives against clinical pathogens and the results indicate that these compounds were active against *C. gattii*, several isolates of *P. brasiliensis* and Gram-positive bacteria, including *S. aureus* and *E. faecalis*.

The lapachol semicarbazone derivative had weaker antimicrobial activity than the lapachol thiosemicarbazone. Although semicarbazones have extensive pharmacological profiles, many activities are lost or diminished by the substitution of the sulphur for oxygen (Beraldo 2004).

The activity against *E. faecalis* is promising because this bacterium has adapted such that it can survive and prevail in the bacterial flora that colonise the gastrointestinal tract of critically ill, immunocompromised and/or neutropenic patients. Consequently, the number of severe enterococci infections is increasing, especially in tertiary hospitals (McBride et al. 2007). The thiosemicarbazone and semicarbazone derivatives of lapachol

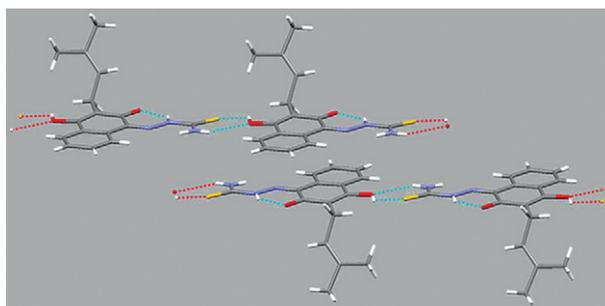


Fig. 3: molecular packing of lapachol thiosemicarbazone showing the scheme of hydrogen bonding.

TABLE III

Selected bond distances (\AA) for lapachol thiosemicarbazone

Atoms	Bond distance (\AA)	Atoms	Angle ($^\circ$)
C1-N1	1.307(2)	C1N1N2	119.36(15)
N1-N2	1.342(2)	N1N2C16	119.01(15)
N2-C16	1.365(2)	N2C16N3	115.96(15)
C16-N3	1.312(2)	N2C16S1	118.81(13)
C16-S1	1.668(2)	N3C16S1	125.23(14)
C2-O1	1.236(2)	C1C2O1	120.47(15)
C4-O2	1.350(2)	C3C2O1	120.79(16)
C3-C11	1.510(2)	C10C4O2	112.30(15)
C12-C13	1.317(2)	C3C4O2	123.81(17)

Viability (%)

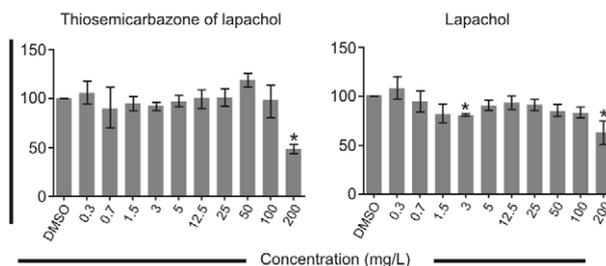


Fig. 4: impact of thiosemicarbazone of lapachol and lapachol on viability of human peripheral blood mononuclear cells. The cells were incubated with different concentrations of compounds for 48 h and viability was determined by 2-(3,5-diphenyltetrazol-2-ium-2-yl)-4-,5-dimethyl-1,3-thiazole bromide assay. The values represent means \pm standard deviation from six independent experiments (asterisk means $p < 0.05$, Student *t* test). DMSO: dimethyl sulfoxide.

TABLE IV

Hydrogen bonds distances (\AA) and angles ($^\circ$) for lapachol thiosemicarbazone with $d(\text{H}\cdots\text{A}) < r(\text{\AA}) + 2.00 \text{\AA}$ and $\angle \text{D-H}\cdots\text{A} > 110^\circ$

D-H \cdots A	d(D-H)	d(H \cdots A)	d(D \cdots A)	(D-H \cdots A)	Symmetry operation
N3-H3B \cdots O2	0.86	2.32	3.0805(18)	147.4	[x,y,z-1]
O2-H2 \cdots S1	0.82	2.49	3.1556(14)	139.3	[x,y,z+1]
N2-H2A \cdots O1	0.86	1.90	2.5682(18)	133.2	intramolecular

TABLE V
Antimicrobial activity ($\mu\text{mol/mL}$) of lapachol and its derivatives thiosemicarbazone and semicarbazone against several opportunistic microorganisms

	Lapachol	Thiosemicarbazone lapachol	Semicarbazone lapachol	Sulfamethoxazole + trimethoprim	Anphoterin B	Chloramphenicol
<i>Escherichia coli</i>	> 1.03	> 0.80	> 0.84	-	-	0.05
<i>Pseudomonas aeruginosa</i>	> 1.03	> 0.80	> 0.84	-	-	0.10
<i>Salmonella typhimurium</i>	> 1.03	> 0.80	> 0.84	-	-	0.10
<i>Staphylococcus aureus</i>	0.52	0.10	0.10	-	-	0.10
<i>Enterococcus faecalis</i>	0.52	0.05	0.05	-	-	0.10
<i>Candida albicans</i>	> 1.03	> 0.80	> 0.84	-	1.08×10^{-3}	-
<i>Candida tropicalis</i>	> 1.03	> 0.80	> 0.84	-	2.70×10^{-4}	-
<i>Cryptococcus gattii</i>	> 1.03	0.10	0.2-	-	1.30×10^{-3}	-
<i>Paracoccidioides brasiliensis</i> Pb18	0.13	0.04	0.84	1.15	6.71×10^{-5}	-
<i>P. brasiliensis</i> ED01	0.13	0.10	0.84	0.29	3.35×10^{-5}	-
<i>P. brasiliensis</i> 2	0.13	0.02	0.84	0.58	3.35×10^{-5}	-
<i>P. brasiliensis</i> 11	0.13	0.04	0.84	0.58	3.35×10^{-5}	-
<i>P. brasiliensis</i> 01	0.26	0.04	0.84	1.15	1.30×10^{-4}	-
<i>P. brasiliensis</i> B339	0.13	0.02	0.42	0.29	6.71×10^{-5}	-
<i>P. brasiliensis</i> 3	0.13	0.04	1.03	1.15	1.62×10^{-5}	-
<i>P. brasiliensis</i> 14	0.13	0.04	0.42	0.29	1.35×10^{-4}	-
<i>P. brasiliensis</i> 8	0.13	0.10	0.84	1.15	6.71×10^{-5}	-
<i>P. brasiliensis</i> 1578	0.13	0.01	0.84	0.29	6.71×10^{-5}	-
<i>P. brasiliensis</i> 4	0.13	0.04	0.84	0.58	6.71×10^{-5}	-

-: not tested.

were two-fold more active than chloramphenicol against *E. faecalis* and had MICs similar to that of chloramphenicol when tested against *S. aureus*. *S. aureus* causes staphylococcal infections and following the introduction of methicillin, there has been a steady increase in methicillin-resistant *S. aureus* isolates that are resistant to vancomycin, even in Brazil (Palazzo et al. 2005).

The initial studies, conducted at the Department of Antibiotics at the Federal University of Pernambuco in 1973, demonstrated the robust activity of lapachol against Gram-positive bacteria (Lima & Weigert 1972, Nagata et al. 1998). These studies revealed that lapachol was active against *Helicobacter pylori*, *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Bacillus* and *Clostridium*, with MICs ranging from 1,560-25,000 µg/mL (Almeida 2009). In general, thiosemicarbazones obtained from other sources, in particular, α-(N)-heterocyclic thiosemicarbazones and their metal complexes, inhibit the growth of Gram-positive bacteria, such as *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Staphylococcus faecalis*, *Streptococcus faecalis* and *Enterococcus*, but do not effectively inhibit Gram-negative bacteria, such as *Pseudomonas*, *Klebsiella*, *Enterobacter*, *Shigella*, *E. coli* and *Proteus* (Beraldo 2004).

In this study, the lapachol-derived thiosemicarbazone had superior antimicrobial activity against clinical isolates of *P. brasiliensis*. Our data indicated that the lapachol thiosemicarbazone derivative was more active than sulphonamides, the first class of drugs available for treating patients with PCM. This finding is important because the use of sulphonamides for more than two years may be required to treat PCM. Moreover, there is increasing concern about drug toxicity and treatment cost (Brummer et al. 1993, Paniago et al. 2003, Travassos et al. 2008).

The lapachol-derived semicarbazone and thiosemicarbazone also exhibited antimicrobial activity against *C. gattii* (Table V). These results are significant because this pathogenic yeast primarily infects healthy individuals and has a high mortality rate (Chaturvedi et al. 2005). Amphotericin B, alone or in combination with flucytosine, remains the standard antifungal therapy for these infections, despite the toxicity of both drugs (Lima & Weigert 1972). Other drugs, such as fluconazole and itraconazole, are used as oral maintenance or consolidation therapy for cryptococcosis (Perfect & Casadevall 2002). However, resistance to fluconazole has arisen in recent years (Sabbatani et al. 2004).

Lapachol was previously tested against *C. albicans* and *Cladosporium cucumerinum* on silica gel plates and it exhibited antimicrobial activity at minimal concentrations of 0.04 and 2.48 x 10⁻³ µmol/mL, respectively (Gafner et al. 1996). However, the MICs for lapachol were not determined. In addition, it has been reported that analogues of furanonaphthoquinone from *Tecoma ipe* have MICs of 1-8 µg/mL against several pathogenic fungi (Nagata et al. 1998), indicating a therapeutic potential for naphthoquinones against fungi.

Lapachol and its thiosemicarbazone derivative had dramatic antifungal activity. Lapachol is known for its antimicrobial activity, but its thiosemicarbazone de-

rivative has never been described. In this study, this derivative was found to be superior to lapachol in terms of antifungal activity.

Our data demonstrate that these derivatives were less toxic than lapachol, which exhibited a biphasic response with toxicity at higher concentrations and hormesis at 0.01 µmol/mL (3 µg/mL). Hormesis describes a dose-response curve with opposite effects at low doses vs. high doses (Hoffman 2009). Therefore, the in vitro cytotoxicity assays performed using immune system cells revealed a potentially greater immunotoxicity for lapachol than for its derivatives.

The MICs for the lapachol-derived thiosemicarbazone against pathogenic bacteria (*S. aureus* and *E. faecalis*) and fungi (mainly *P. brasiliensis*) indicate that these compounds are excellent choices for the development of novel drugs to treat microbial infections. Further studies are necessary to verify the effectiveness of these compounds in treating infections caused by microorganisms.

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