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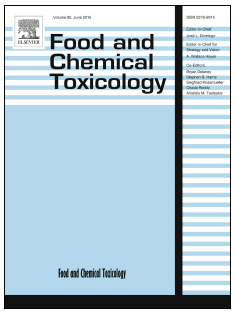

Antifungal toxicity of linear geranylphenol. Influence of oxigenate substituents

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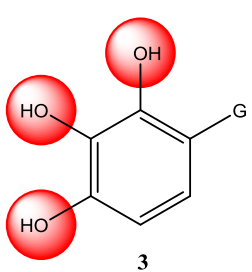

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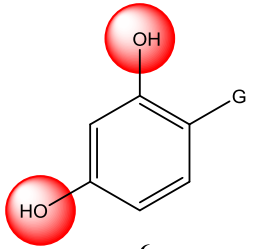

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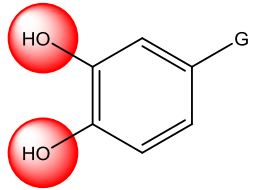

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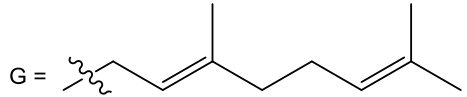

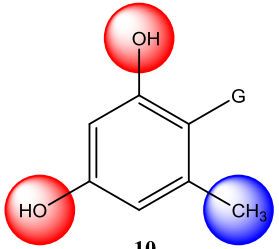

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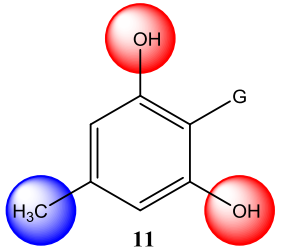

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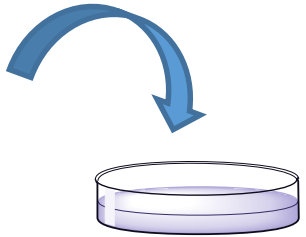

"Antifungal toxicity of Linear Geranylphenol. Influence of Oxigenate Substituents"

Authors:

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Abstract:

Twenty four linear geranylphenols were evaluated for their antifungal properties against ATCC and clinical isolates of *Candida albicans* and *Cryptococcus neoformans*. For the analysis of their antifungal behavior the compounds were grouped into two series: (i) compounds with only one geranyl substituent on the benzene ring and (ii) compounds with two geranyl moieties on the benzene ring. Results showed that compounds of series (i) present better antifungal activities than those of series (ii). In addition, within group (i) all compounds showed better activities against *C. neoformans* than against *C. albicans* which can be easily verified by comparing MIC_{100} or MIC_{50} of each compound against both fungi. Di- (**10** and **11**) and tri- hydroxy (**3** and **4**) compounds showed significant anti-cryptoccocal activity, being **3**, **10** and **11** highly active with MIC_{100} or MIC_{50} = 3.9 μg/mL similar to the standard drug amphotericin B. Moreover, when evaluating the toxicity of compounds **6**, **10** and **11** on the HDF cell line (human dermal fibroblasts), results were obtained with IC_{50} values $> 100\ \mu M$, considered as non-toxic for the cell. This indicates that the toxicity of the analyzed compounds is selective towards fungi, which makes them a very attractive family for the development of future drugs.

Keywords:

linear geranylphenols; antifungal activity; *Candida albicans*; *Cryptococcus neoformans.*

1. Introduction:

In the last years, fungi have emerged as major cause of human infections especially among immunocompromised hosts having an enormous impact on morbidity and mortality (Mathew and Nath, 2009). Although there are several available drugs for the treatment of systemic and superficial mycoses, any of them is completely effective (Brown and Wright, 2005; Chen et al., 2010) and therefore quickly develops resistance due to the large-scale use (Mukherjee et al., 2003) and all possess a certain degree of toxicity (Kontoyiannis, 2012). There is, therefore, an urgent need for new antifungal chemical structures, alternatives to the existing ones (Vicente et al., 2003).

On the other hand, linear geranylphenols are an interesting sub-class of secondary metabolites whose terpene portion may have a length of up to eight isoprene units (De Rosa et al., 1995). They were found mainly in marine organisms such as brown algae (Capon et al., 1981; Faulkner, 1986; Gerwick and Fenical, 1981; Ochi et al., 1979), sponges (Bifulco et al., 1995; De Rosa et al., 1995), alcyonaceas (Bowden and Coll, 1981), gorgonians (Ravi and Wells, 1982) and ascidians (Faulkner, 1993; Fu et al., 1997, 1994; Guella et al., 1987; Howard et al., 1979; Targett and Keeran, 1984). Among them, geranylphenols with mono-, di- and sesquiterpene (Capon et al., 1981; Faulkner, 1986; Gerwick and Fenical, 1981; Ochi et al., 1979) chains were isolated from brown algae while structures with longer linear chains were isolated from sponges (Bifulco et al., 1995; De Rosa et al., 1995).

Potent biological activities including anti-inflammatory (Bauer et al., 2011; Quang et al., 2006), antifungal (Danelutte et al., 2003) anti-HIV (Manfredi et al., 2001), antioxidant (Yamaguchi et al., 2006) as well as antineoplastic (Han et al., 2007; Liu et al., 2008) have been reported for this family of compounds. Some particular compounds such as 2-geranylbenzoquinone (**1**) (isolated from the ascidian *Synoicum castellatum* Kott.) (Carroll et al., 1993), 2-geranylhydroquinone (**2**) isolated from *Cordia alliodora* (ex Ruiz & Pav.) Oken tree (Manners and Jurd, 1977), *Phacelia crenulata* Torr. ex S. Watson (Reynolds and Rodriguez, 1979), *Aplidium antillense* Gravier (Benslimane et al., 1988) and the tunicate *Amaroucium multiplicatum* Sluiter (Sato and Shindo, 1989), have shown antimicrobial, anticancer, protective and antioxidant effects, among others (Figure 1) (Benslimane et al., 1988; De Rosa et al., 1994; Reynolds and Rodriguez, 1979; Rudali and Menetrier, 1967; Rudall, 1966; Sato and Shindo, 1989).

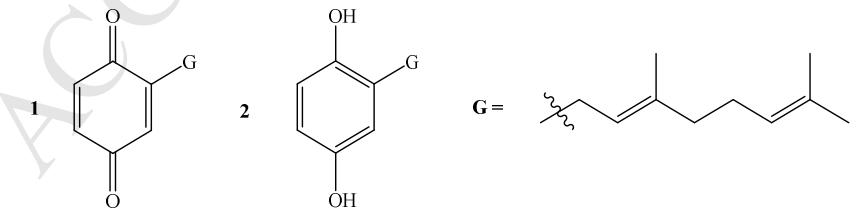


Figure 1. Structure of geranylphenols with biological activity

Based on the data mentioned above, our research group has been devoted in the last years to the synthesis of linear geranylphenols, which were obtained by direct geranylation reactions between geraniol and the corresponding phenol derivatives, using $BF_3{\cdot}OEt_2$ as the first catalyst and $AgNO_3$ as the second one (Espinoza et al., 2014). Eight of them have been previously evaluated for their *in vitro* inhibitory effect of the mycelial growth of *Botrytis cinerea,* a fungus that affects important agricultural crops (Espinoza et al., 2014). Analysis of the results suggested that compounds having hydroxyl groups attached to the aromatic nucleus (compounds **2**, **3** and **4**, Figure 2) exhibited a higher percentage of inhibitory activity of the growth of *B. cinerea* mycelium. In addition the biological activity, decreased or was gone when the compounds were acetylated (Espinoza et al., 2014).

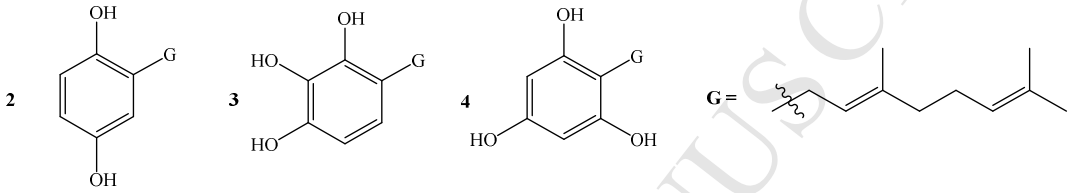


Figure 2. Geranylphenols that showed inhibitory effect over *B. cinerea*.

In order to deepen the knowledge on the antifungal behavior of geranylphenols, we report here the antifungal activity of a series of 24 geranylphenols, two of them new ones (**7** and **9**), against standardized as well as clinical isolates of clinically important yeasts belonging to *Candida* and *Cryptococcus* genera in order to get structure-antifungal activity relationships, and to know the potential usefulness of these compounds as hits and heads of series for their further development.

2. Materials and Methods:

2.1 Chemistry

2.1.1 Synthesis

Unless otherwise stated, all chemical reagents purchased (Merck, Darmstadt, Germany or Aldrich, St. Louis, MO, USA) were of the highest commercially available purity and were used without previous purification. IR spectra were recorded as thin films in a FT-IR Nicolet 6700 spectrophotometer (Thermo Scientific, San Jose, CA, USA) and frequencies are reported in cm^{-1}. Low resolution mass spectra were recorded on an Agilent 5973 spectrometer (Agilent Technologies, Santa Clara, CA, USA) at 70 eV ionizing voltage, in a GC 6890N DB-5 m, 30 m × 0.25 mm × 0.25 µm column, and data are given as *m/z* (% rel. int.). High resolution mass spectra were recorded on an LTQ Orbitrap XL spectrometer (Thermo Scientific, San Jose, CA, USA) by applying a voltage of 1.8 kV in the positive and 1.9 kV in the negative, ionization mode. The spectra were recorded using full scan mode, covering a mass range from *m/z* 100–1,300. The resolution was set to 50,000 and maximum loading time for the ICR cell was set to 250 ms. ^{1}H, ^{13}C, ^{13}C DEPT-135, sel. gs-1D ^{1}H NOESY, gs-2D HSQC and gs-2D HMBC spectra were recorded in $CDCl_3$ solutions and are referenced to the residual peaks of $CHCl_3$ at δ = 7.26 ppm and δ = 77.0 ppm for ^{1}H and ^{13}C,

respectively, on a Bruker Avance 400 Digital NMR spectrometer (Bruker, Rheinstetten, Germany), operating at 400.1 MHz for ^{1}H and 100.6 MHz for ^{13}C. Chemical shifts are reported in δ ppm and coupling constants (*J*) are given in Hz. Silica gel (Merck 200–300 mesh) was used for C.C. and silica gel plates HF_{254} for TLC. TLC spots were detected by heating after spraying with 25% H_2SO_4 in H_2O.

(E)-3-(3,7-dimethylocta-2,6-dienyl)benzene-1,2-diol (**5**), *(E)-4-(3,7-dimethylocta-2,6-dienyl)benzene-1,2-diol* (**7**).

To a solution of catechol (1.0 g, 9.1 mmol) and geraniol (1.4 g, 9.1 mmol) in acetonitrile (25 mL), saturated with $AgNO_3$, was slowly added $BF_3{\cdot}OEt_2$ (0.46 g, 3.2 mmol) dropwise with stirring at room temperature and under a N_2 atmosphere. After the addition was completed, the stirring was continued for 48 h. The end of the reaction was verified by TLC, and then the mixture was poured onto crushed ice (30 g) and extracted with EtOAc (3 × 20 mL). The organic layer was washed with 5% $NaHCO_3$ (15 mL, 5%) and water (2 × 15 mL), dried over Na_2SO_4, and filtered. The solvent was evaporated under reduced pressure. The crude was re-dissolved in CH_2Cl_2 (5 mL) and chromatographed on silica gel with petroleum ether/EtOAc mixtures of increasing polarity (19.8:0.2→0.2:19.8). Two fractions were obtained. Fraction I: 108 mg of viscous reddish oil, 4.8% yield, identified as compound **5**; Fraction II: 239 mg of viscous reddish oil, 10.75% yield, identified as compound **7**.

Compound **5**: IR (cm^{-1}): 3418; 2967; 2922; 1620; 1590; 1475; 1375; 1278. ^{1}H-NMR: 6.78 (d, *J* = 8.4 Hz, 1H, H-6); 6.75 (dd, *J* = 8.4 and 6.8 Hz, 1H, H-5); 6.66 (d, *J* = 6.8 Hz, 1H, H-4); 5.38 (s, 1H, OH-C2); 5.33-5.31 (m, 2H, H-2' and OH-C-1); 5.05-5.04 (m, 1H, H-6'); 3.37 (d, *J* = 7.1 Hz, 2H, H-1'); 2.14-2.12 (m, 2H, H-5'); 2.11-2.10 (m, 2H, H-4'); 1.78 (s, 3H, CH_3-C3'); 1.69 (s, 3H, H-8'); 1.60 (s, 3H, CH_3-C7'). ^{13}C-NMR: 144.3 (C-1); 141.9 (C-2); 138.9 (C-3'); 132.2 (C-7'); 127.4 (C-3); 123.7 (C-6'); 121.7 (C-2'); 121.4 (C-4); 120.7 (C-5); 113.2 (C-6); 39.6 (C-4'); 29.9 (C-1'); 26.3 (C-5'); 25.7 (C-8'); 17.7 (CH_3-C7'); 16,1 (CH_3-C3'). Spectroscopic data of compound **5** were consistent with the literature. (Taborga et al., 2013)

Compound **7**: IR (cm^{-1}): 3402; 2973; 2922; 1605; 1442; 1375; 1278. ^{1}H-NMR: 6.77 (d, *J* = 7.6 Hz, 1H, H-6); 6.70 (s, 1H, H-3); 6.61 (d, *J* = 7.6 Hz, 1H, H-5); 5.29 (t, *J* = 6.7 Hz, 1H, H-2'); 5.10 (t, *J* = 6.4 Hz, 1H, H-6'); 3.24 (d, *J* = 7.2 Hz, 2H, H-1'); 2.11-2.08 (m, 2H, H-5'); 2.05-2.02 (m, 2H, H-4'); 1.68 (s, 6H, CH_3-C3' and H-8'); 1.60 (s, 3H, CH_3-C7'). ^{13}C-NMR: 143.4 (C-2); 141.3 (C-1); 136.1 (C-3'); 135.0 (C-4); 131.5 (C-7'); 124.3 (C-6'); 123.1 (C-2'); 120.7 (C-5); 115.4 (C-3); 115.3 (C-6); 39.7 (C-4'); 33.4 (C-1'); 26.6 (C-5'); 25.7 (C-8'); 17.7 (CH_3-C7'); 16.1 (CH_3-C3'). HRMS: (M + 1) calcd. for $C_{16}H_{22}O_2$: 247.1620, found: 247.1628.

(E)-4-(3,7-dimethylocta-2,6-dienyl)-1,3-phenylene diacetate (**9**).

Ac_2O (1.08 g, 10.6 mmol) was added to a solution of compound **6** (70 mg, 0.284 mmol), DMAP (3.0 mg) and pyridine (1.0 mL) in dichloromethane (30 mL). The end of the reaction was verified by TLC (1 h), and the mixture was extracted with EtOAc (2 × 10 mL). The organic layer was washed with 5% $KHSO_4$ (2 × 10 mL) and water (2 × 10 mL), dried over Na_2SO_4, and filtered. The solvent was evaporated under reduced pressure. Compound **9** was obtained as dark yellow oil (92.2 mg, 98.9% yield). Compound **9**: IR (cm^{-1}): 2964; 2917; 2849; 1770; 1640; 1612; 1495; 1450; 1369; 1198; 1140; 1014; 973; 913. ^{1}H-NMR: 7.22 (d, *J* = 8.4 Hz, 1H, H-5); 6.92 (dd, *J* = 2.1 and 8.4 Hz, 1H, H-6); 6.85 (d, *J* = 2.1 Hz, 1H, H-2); 5.22 (t, *J* = 6.8 Hz, 1H, H-2'); 5.10 (t, *J* = 6.0 Hz, 1H, H-6'); 3.22 (d, *J* = 7.1 Hz, 2H,

H-1'); 2.29 (s, 3H, $COCH_3$); 2.27 (s, 3H, $COCH_3$); 2.11-2.08 (m, 2H, H-5'); 2.06-2.05 (m, 2H, H-4'); 1.68 (s, 3H, H-8'); 1.68 (s, 3H, CH_3-C3'); 1.60 (s, 3H, CH_3-C7'). ^{13}C-NMR: 169.1 ($COCH_3$); 168.9 ($COCH_3$); 148.9 (C-3); 148.9 (C-1); 137.1 (C-3'); 131.5 (C-7'); 130.9 (C-4); 130.1 (C-5); 124.1 (C-6'); 121.1 (C-2'); 119.0 (C-6); 155.7 (C-2); 39.6 (C-4'); 28.0 (C-1'); 26.5 (C-6'); 25.6 (C-8'); 21.0 ($COCH_3$); 20.8 ($COCH_3$); 17.6 (CH_3-C7'); 16.0 (CH_3 -C3').

2.2 Biology

2.2.1 Microorganisms and media

For the antifungal evaluation, standardized strains from the American Type Culture Collection [(ATCC), Rockville, MD, USA] *C. albicans* ATCC 10231 and *C. neoformans* ATCC 32264 as well as clinical isolates of the Reference Center in Mycology (CEREMIC, CCC, Rosario, Argentina) and Malbrán Institute [(IM), Av. Velez Sarsfield 563, Buenos Aires] were used. The isolates included six strains of *C. albicans,* four non-*albicans Candida* strains (*C. krusei, C. tropicalis, C. glabrata* and *C. parapsilopsis*) and six strains of *C. neoformans* whose voucher specimens are presented in Table 2. Strains were grown on Sabouraud-chloramphenicol agar slants for 48 h at 30 °C, maintained on slopes of Sabouraud-dextrose agar (SDA, Oxoid) and sub-cultured every 15 days to prevent pleomorphic transformations. Inocula were obtained according to reported procedures(CLSI (Clinical and Laboratory Standars Institute), 2008) and adjusted to 1-5 x10^3 cells with colony forming units (CFU)/mL.

2.2.2 Cell line

HDF (human dermal fibroblasts) were obtained from ATCC.

2.2.3 Cell culture

HDF cell line was maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium, containing 10% heat-inactivated fetal bovine serum (FBS), amphotericin (2.5 mg/mL), penicillin (100 U/mL) and streptomycin (100 mg/mL).

2.2.4 Fungal growth inhibition percentage determination.

Microdilution techniques were performed in 96-well microplates according to the guidelines M-27A3 of Clinical and Laboratory Standards Institute (CLSI) for yeasts (CLSI (Clinical and Laboratory Standars Institute), 2008). For the assay, compound test-wells (CTWs) were prepared with stock solutions of each compound in DMSO (maximum concentration ≤ 1%), diluted with RPMI-1640, to final concentrations of 250-3.9 µg/mL. An inoculum suspension (100 µL) was added to each well (final volume in the well = 200 µL). A growth control-well (GCW) (containing medium, inoculum, and the same amount of DMSO used in a CTW, but compound-free) and a sterility control-well (SCW) (sample, medium, and sterile water instead of inoculum) were included for each tested fungus. Microtiter trays were incubated in a moist, dark chamber at 30 °C for 48 h for both yeasts. Microplates were read in a VERSA Max microplate reader (Molecular Devices, Sunnyvale, CA, USA). Tests were performed in triplicate. Reduction of growth for each compound concentration was calculated as follows: % of inhibition = 100 - (OD 405 CTW − OD 405 SCW)/(OD 405 GCW − OD 405 SCW). The means ± SEM were used for constructing the dose-response curves with SigmaPlot 11.0 software that represent % inhibition *vs* concentration of each compound. Two endpoints

were recorded from this assay and the dose-response curves: the Minimum Inhibitory Concentration that results in total fungal growth inhibition (MIC_{100}) and the minimum concentration that inhibits 50 % of the fungal growth (MIC_{50}). Amphotericin B (Sigma-Aldrich, St Louis, Mo, USA) was used as the standard positive drug.

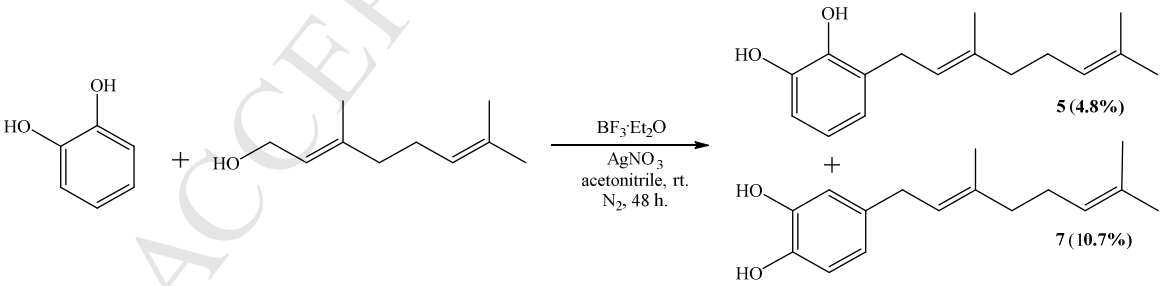
3. Results and Discussion:

3.1. Chemistry

3.1.1. Synthesis

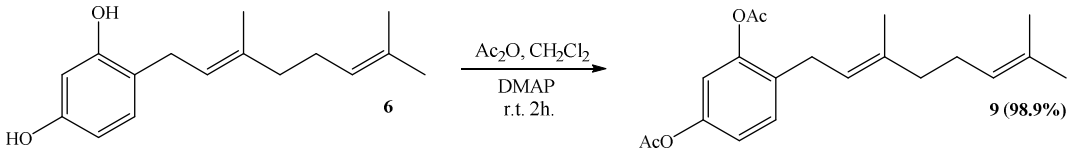
The syntheses of most compounds, as well as their chemical characterization, have been previously reported by our research group. Compounds **5, 6** and **13** were reported in Taborga *et al* (2013); **2, 3, 4, 8, 15, 16, 19, 21**, in Espinoza *et al*. (2014); **10-12, 14, 18, 20, 23** and **24**, in Taborga *et al*., 2015 (Taborga et al., 2015) and **17, 22** and **25**, in Taborga *et al*., 2016 (Taborga et al., 2016). Structures **7** and **9** are new compounds and their syntheses and characterization are reported here for the first time.

Of the whole series, compounds **5** and **6** have been previously synthesized by the traditional method used for another geranyl methoxy-phenols (Fedorov et al., 2006), i.e. direct coupling between geraniol and pyrocatechol or resorcinol in dioxane solution catalyzed with $BF_3{\cdot}Et_2O$. The yields of this reaction were 4.8% and 11.9% for compounds **5** and **6**, respectively (Taborga et al., 2013). However, when the geranylation of these hydroquinones was carried out following the modified Electrophilic Aromatic Substitutions (EAS) reaction (Scheme 1), compound **5** was obtained with identical yield, whereas compound **6** was obtained with 15.0 % yield that is slightly higher than the obtained with the traditional method. Additionally, in the reaction of pyrocathecol, the new compound **7** was obtained with 10.7 % yield. The chemical structure of **7** was mainly determined by NMR spectroscopic techniques and the results are detailed in section 1.2. The spectroscopic data of compounds **5** and **6** were consistent with those previously reported (Taborga et al., 2013).



Scheme 1. Synthesis of compounds **5** and **7.**

The new compound **9** was obtained from **6** by standard acetylation (Ac_2O and DMAP in CH_2Cl_2) with 98.9% yield (see Scheme 2). The presence of a diacetylated derivative was confirmed by NMR spectroscopy: in the ^{1}H-NMR spectrum two singlets at δ_H = 2.29 and 2.27 ppm (each 3H, CH_3CO) were observed, whereas in the ^{13}C-NMR spectrum signals at δ_C = 21.0 ($CO\underline{C}H_3$), 20.8 ($CO\underline{C}H_3$), 169.1 and 168.9 ppm (C=O) were found.



Scheme 2. Synthesis of compound **9**.

3.1.2 Structure determination

The chemical structure of the new compound **7** was mainly established by NMR spectroscopy. Thus, in this section the NMR data used to determine the chemical structure of geranylphenol **7** is discussed in detail, whereas those corresponding to the novel acetylated derivative **9** have been given in the previous paragraph.

The ^{1}H NMR spectrum of compound **7** shows aromatic signals at δ_H = 6.77 (d, J = 7.6 Hz, 1H); δ_H = 6.70 (s, 1H) and δ_H = 6.61 (d, J = 7.6 Hz, 1H), which were assigned to hydrogen atoms H-6, H-3 and H-5, respectively. These results confirm the aromatic mono-substitution at 4-position. Additionally, in the HMBC spectrum, the signal at δ_H = 3.24 ppm assigned to H-1' (d, J = 7.2 Hz, 2H, H-1') shows $^{3}J_{H\text{-}C}$ coupling with C-3 (δ_C = 115.4), C-5 (δ_C = 120.7 ppm) and C-3' (δ_C = 136.1 ppm), and $^{2}J_{H\text{-}C}$ coupling with C-4 and C-2' (δ_C = 135.0 and 123.1 ppm, respectively). These HMBC correlations are shown in Figure 3. In order to establish the *E* geometry of the C-2'-C-3' double bond of the geranyl chain, selective 1D NOESY NMR experiments were recorded for compound **7**. These correlations are shown in Figure 3, where the most important ones correspond to those observed between the H-1' and the methyl group in the C-3' position.

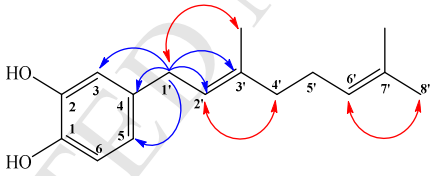


Figure 3. Most important 2D ^{1}H-^{13}C HMBC correlations (blue) and selective 1D NOESY correlations (red) observed for compound **7.**

3.2 Biology

3.2.1 Antifungal activity

The antifungal properties of the twenty four geranylphenols were investigated against clinical important fungal species. Standardized strains of *Cryptococcus neoformans* and *Candida albicans* of the American Type Culture Collection (ATCC) and also clinical isolates of *C. neoformans* and *C. albicans* and non-*albicans Candida* strains were used as targets for the antifungal assays.

Results were expressed as the percentages of inhibition of each fungus in the range 250-3.9 µg/mL by using the standardized microbroth dilution method CLSI M-27A3 (CLSI, 2008) which assures reproducible results.

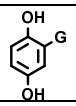
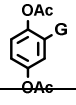
The selection of *C. neoformans* was due to the fact that this opportunistic yeast is the main cause of cryptococcal meningoencephalitis which has a high mortality rate among patients with profoundly impaired infections (Trpković et al., 2012). Even though new antifungal drugs have

been developed in recent years (Roemer and Krysan, 2014), the availability of antifungal agents with anti-cryptococcal activity is still limited and sometimes the strains develop quickly resistance (Perkins et al., 2005). This scenario has motivated the search of new compounds with anti-*C. neoformans* properties (Cordeiro et al., 2012).

In turn, *C. albicans* is the fourth leading cause of nosocomial bloodstream infection (BSI) in intensive care units, causing fatal invasive candidiasis in a high percentage of patients (Pfaller and Diekema, 2007). As a consequence, new chemical structures with anticandidal activities are highly welcome. In addition although *C. albicans* was in the past the usual species associated with invasive and BSI infections, at present non-*albicans Candida* spp. such as *C. tropicalis, C. glabrata, C. parapsilopsis, C. krusei* and *C. lusitaneae*, constitute more than a half of isolates in human candidiasis (Pfaller and Diekema, 2007).

For a more comprehensive analysis of the antifungal results, the compounds were grouped into two series: (i) compounds with only one geranyl substituent (mono-geranyl) and (ii) compounds with two geranyl moieties (bi-geranyl) on the aromatic ring. Compounds of series (i) were sub-divided in two sub-groups: (i.1) includes compounds with two oxygenated substituents (**2, 5, 6, 7, 8** and **9**); (i.2) includes compounds with three extra substituents (**3, 4, 10, 11, 12, 13, 14, 15, 16** and **17**). All compounds were tested at first against the standardized strains *C. albicans* ATCC 10231 and *C. neoformans* ATCC 32264. Results are showed in Table 1.

Table 1. Antifungal activity, part a

group		Structure	Name	pathogen	250 µg/mL	125 µg/mL	62.5 µg/mL	31.25 µg/mL	15.65 µg/mL	7.8 µg/mL	3.9 µg/mL	MIC_{100} µg/mL	MIC_{50} µg/mL
I	i.1		**2**	Cn	100	100	100	100	0.0	0.0	0.0	31.25	31.25
				Ca	90	45.2±1.1	13.4±0.3	7.6±0.6	5.7±0.5	4.2±1.0	1.1±0.1	>250	125
			**5**	Cn	100	100	100	59.6±3.4	0.0	0.0	0.0	62.5	31.25
				Ca	33.1±2.1	8.5±0.6	6.0±1.3	6.3±0.8	3.4±1.0	1.6±0.1	2.0±0.9	>250	>250
			**6**	Cn	100	100	100	100	100	95.5±2.7	54.0±1.8	7.8	3.9
				Ca	100	97.8±0.8	57.4±3.1	13.9±1.0	3.7±0.6	1.8±0.4	0.0	125	62.5
			**7**	Cn	100	100	100	100	59	27	0	31.2	15.6
				Ca	100	100	96.0±2.3	88.2±1.4	46.5±1.9	13.5±0.8	6.2±0.5	62.5	15.6
			**8**	Cn	68.3±0.9	54.9±0.5	38.3±1.2	34.3±1.3	32.6±0.5	29.7±0.6	24.8±1.0	>250	125
				Ca	29.5±1.3	23.7±1.0	12.2±0.1	10.2±0.7	9.6±0.5	5.8±0.7	0	>250	>250

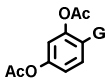
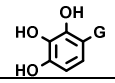
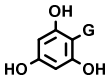
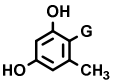
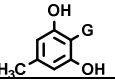
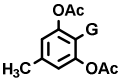
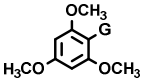
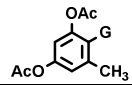
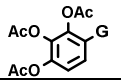
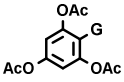
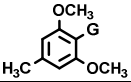
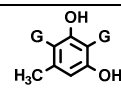
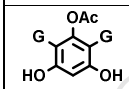
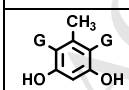
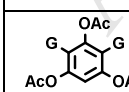
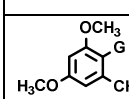
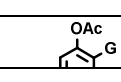
			**9**	Cn	31.2±15.9	15.5±1.1	0.0	0.0	0.0	0.0	2.6	>250	>250
				Ca	25.2±0.2	13.4±0.5	9.8±0.9	4.8±2.6	1.8±0.3	0.6±0.1	0.4±0.1	>250	>250
	i.2		**3**	Cn	100	100	100	100	100	100	100	3.9	1.9
				Ca	100	100	77.9±0.1	66.5±1.8	11.8±0.4	4.5±1.8	2.3±0.7	125	31.2
			**4**	Cn	100	100	100	86.3±5.4	6.6±1.8	1.7±1.1	0.0	62.5	31.2
				Ca	100	66.2±3.1	10.0±0.8	4.0±0.3	2.4±0.2	0.1±0.2	0.0	>250	250
			**10**	Cn	100	100	100	100	100	100	100	3.9	3.9
				Ca	100	100	100	96.8±0.7	72.1±2.5	13.6±0.6	2.1±0.1	31.2	15.6
			**11**	Cn	100	100	100	100	100	100	100	3.9	3.9
				Ca	100	100	100	92.0±2.1	29.4±1.6	6.3±1.8	2.5±0.3	31.2	31.2
			**12**	Cn	65.7±3.8	52.1±3.3	42.6±1.6	32.7±2.0	0.0	0.0	0.0	>250	125
				Ca	58.9±0.1	28.8±1.3	14.2±1.2	6.4±0.7	1.4±0.1	0.0	0.0	>250	250
			**13**	Cn	66.4±2.7	25.5±0.6	15.1±1.3	0.0	0.0	0.0	0.0	>250	250
				Ca	31.0±2.1	19.8±2.3	18.1±1.3	17.6±1.9	13.1±1.8	5.3±0.2	2.6±1.4	>250	>250
			**14**	Cn	15.8±2.3	0.0	0.0	0.0	0.0	0.0	0.0	>250	>250
				Ca	16.0±1.3	8.4±0.8	6.2±0.3	4.8±1.2	4.4±1.4	4.1±1.1	0.0	>250	>250
			**15**	Cn	68.3±3.9	2.3±0.4	0.0	0.0	0.0	0.0	0.0	>250	250
				Ca	20.0±2.7	8.9±0.8	3.7±0.3	3.2±1.0	1.5±0.5	1.4±0.7	0.5 ±0.0	>250	>250
			**16**	Cn	29.6±1.5	7.1±0.1	0.0	0.0	0.0	0.0	0.0	>250	>250
				Ca	20.0±2.7	8.9±0.8	3.7±0.3	3.2±1.0	1.5±0.5	1.4±0.7	0.5±0.0	>250	>250
			**17**	Cn	63.7±1.7	12.2±1.5	0.0	0.0	0.0	0.0	0.0	>250	250
				Ca	9.5±2.1	5.4±0.6	4.1±0.2	3.0±0.6	2.9±0.0	2.1±0.5	2.0±0.4	>250	>250

Table 1 Antifungal activity, part b

ii		**18**	Cn	38.3±2.8	40.4±0.2	16.5±1.8	0.0	0.0	0.0	0.0	>250	>250
			Ca	34.2±1.8	13.0±1.1	5.6±1.4	1.8±0.8	1.8±0.4	0.6 ±0.0	0.0	>250	>250
		**19**	Cn	33.6±1.5	18.0±5.5	0.0	0.0	0.0	0.0	0.0	>250	>250
			Ca	15.0±1.3	7.3±1.8	3.4±0.9	2.3±0.3	1.3±1.2	0.0	0.0	>250	>250
		**20**	Cn	33.2±1.5	30.8±2.2	0.0	0.0	0.0	0.0	0.0	>250	>250
			Ca	26.2±6.1	9.2±2.4	4.5±0.9	2.8±0.2	1.1±0.0	1.2±0.0	1.0±0.4	>250	>250
		**21**	Cn	41.7±12.6	0.0	0.0	0.0	0.0	0.0	0.0	>250	>250
			Ca	14.5±1.1	5.2±1.6	3.6±0.9	0.0	0.0	0.0	0.0	>250	>250
		**22**	Cn	26.0±10.7	24.7±11.9	0.0	0.0	0.0	0.0	0.0	>250	>250
			Ca	9.5±2.1	5.4±0.6	4.1±0.2	3.0±0.6	2.9±0.0	2.1±0.5	2.0±0.4	>250	>250
		**23**	Cn	47.6±1.4	29.1±0.4	0.0	0.0	0.0	0.0	0.0	>250	>250

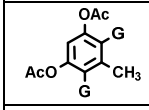
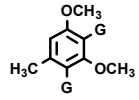
			Ca	31.6±0.7	13.3±2.3	6.9±1.2	4.1±1.4	1.4±0.8	0.4±0.1	0.0	>250	>250
		**24**	Cn	56.0±4.6	10.5±2.8	0.0	0.0	0.0	0.0	0.0	>250	250
			Ca	18.2±0.0	11.1±0.5	6.0±0.2	4.5±0.0	4.4±0.8	2.1±0.1	0.0	>250	>250
		**25**	Cn	62.8±1.8	11.8±1.4	0.0	0.0	0.0	0.0	0.0	>250	250
			Ca	43.5±1.1	24.8±0.1	14.5±1.2	8.4±0.3	6.0±0.5	5.2±1.4	0.0	>250	>250
Amphotericin B			Cn	100	100	100	100	100	100	100	0.25	
			Ca	100	100	100	100	100	100	100	0.50	

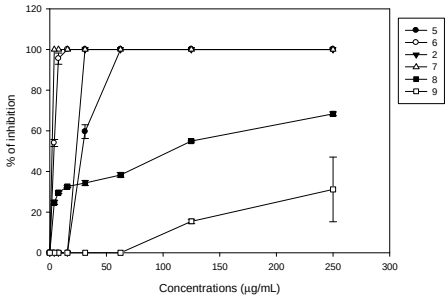
Table 1. Inhibition percentages of the mono- (group i) and bi- geranyl (group ii) phenols and derivatives at different two-fold concentrations (250-3.9 μg/mL) against *Candida albicans* ATCC 10231 (Ca) and *Cryptococcus neoformans* ATCC 32264 (Cn). MIC_{100} and MIC_{50} are included too

From Table 1, it is clear that compounds containing two geranyl substituents (group ii) (no matter the other substituents on the aromatic ring) are very weak inhibitors of *C. neoformans* (all MIC_{100} and almost all MIC_{50} are higher than 250 μg/mL) and of *C. albicans* (MIC_{100} and MIC_{50} > 250 μg/mL).

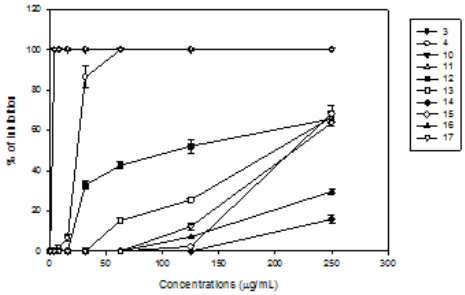
Instead, group (i) with one geranyl substituent, showed better activities against *C. neoformans* than against *C. albicans* which can be easily verified by comparing MIC_{100} or MIC_{50} of each compound against both fungi. As a mere example, from sub-group i.1, **5** possesses MIC_{100} = 62.5 μg/mL against *C. neoformans* and MIC > 250 μg/mL against *C. albicans* and from sub-group i.2, **3** shows MIC_{100} = 3.9 μg/mL against *C. neoformans* and 125 μg/mL against *C. albicans*.

From the results obtained with *C. neoformans*, the following structure-activity relationships can be drawn: (a) within sub-group (i.1) the phenolic compounds (**2**, **5**, **6** and **7**) clearly show better activities than non-phenolic structures (**8** and **9**); (b) among compounds of sub-group i.2, only di- (**10** and **11**) and tri- hydroxylated (**3** and **4**) compounds showed significant anti-cryptoccocal activity (100% inhibition at concentrations ≤ 250 μg/mL), being **3**, **10** and **11** highly active with MIC_{100} or MIC_{50} = 3.9 μg/mL, similar to the standard drug amphotericin B (Figures 4a-c).

Series i.1



Series i.2



Series ii

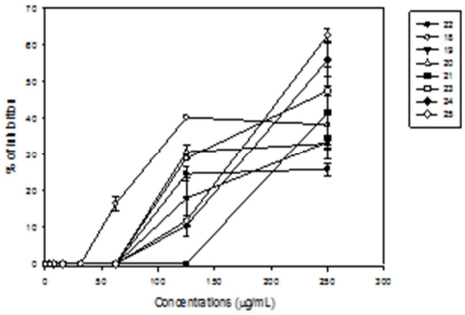


Figure 4. Comparative antifungal activities of compounds of series (i) [(i.1) (i.2)] and (ii) against *C. neoformans*. Amphotericin B inhibits 100 % growth at 0.25 µg/mL against *C. neoformans* (not included).

3.2.2 Second order studies with clinical isolates

In order to gain insight into the potential of the most active compounds against not only standardized strains but on clinical isolates of the tested fungal spp, the most promising compounds were tested against an extended panel of *C. neoformans* strains that were isolated from immunocompromised patients suffering fungal infections. They were also tested against clinical isolates of *C. albicans* and non-*albicans Candida* strains. Results are recorded in Table 2.

Table 2. Minimum Inhibitory Concentrations (MIC_{100} and MIC_{50}) and Minimum Fungicidal activity (MFC) of selected mono-geranyl compounds **6**, **7**, **3**, **10** and **11** against six *C. neoformans,* six *C. albicans* and four *non-albicans Candida* isolated strains. For the sake of comparison, MIC_{100} and MIC_{50} of all compounds against the ATCC standardized strain *C. albicans* ATCC 10231 and *C. neoformans* ATCC 32264 were included.

		3			**6**			**7**			**10**			**11**			Amph. B
Strain	Voucher specimen	MIC_{100}	MIC_{50}	MFC	MIC_{100}	MIC_{50}	MFC	MIC_{100}	MIC_{50}	MFC	MIC_{100}	MIC_{50}	MFC	MIC_{100}	MIC_{50}	MFC	MIC_{100}
C. neoformans	ATCC 32264	3.9	1.9	3.9	7.8	3.9	15.6	31.25	15.6	62.5	3.9	1.9	3.9	3.9	3.9	7.8	0.25
C. neoformans	IM 983040	31.2	15.6	15.6	15.6	7.8	15.6	125	>250	>250	3.9	1.9	7.8	15.6	3.9	15.6	0.12
C. neoformans	IM 972724	31.2	15.6	15.6	15.6	7.8	31.2	250	>250	>250	3.9	1.9	3.9	15.6	7.8	15.6	0.12
C. neoformans	IM 042074	15.6	7.8	31.2	15.6	15.6	31.2	250	>250	>250	7.8	1.9	3.9	15.6	7.8	15.6	0.25
C. neoformans	IM 983036	15.6	7.8	15.6	15.6	15.6	15.6	>250	>250	>250	3.9	3.9	3.9	15.6	15.6	15.6	0.12

C. neoformans	IM 003190	15.6	3.9	15.6	31.2	15.6	31.2	>250	>250	>250	7.8	3.9	7.8	15.6	7.8	15.6	0.25
C. neoformans	IM 003230	15.6	7.8	31.2	15.6	7.8	15.6	250	>250	>250	7.8	3.9	7.8	15.6	7.8	31.2	0.50
C. albicans	ATCC 10231	125	31.2	125	125	62.5	250	125	62.5	>250	31.2	15.6	62.5	31.2	31.2	125	0.25
C. albicans	CCC 125	62.5	31.2	125	125	62.5	250	>250	>250	>250	31.2	15.6	62.5	62.5	31.2	125	0.75
C. albicans	CCC 126	62.5	62.5	62.5	125	62.5	250	>250	>250	>250	31.2	15.6	62.5	62.5	31.2	125	0.50
C. albicans	CCC 127	31.2	31.2	62.5	125	62.5	250	>250	>250	>250	31.2	15.6	62.5	62.5	31.2	125	0.75
C. albicans	CCC 128	62.5	62.5	125	125	62.5	250	>250	>250	>250	31.2	31.2	31.2	31.2	31.2	125	0.56
C. albicans	CCC 129	125	62.5	125	62.5	62.5	62.5	>250	>250	>250	31.2	15.6	125	31.2	31.2	125	0.75
C. albicans	CCC 130	31.2	31.2	31.2	62.5	62.5	62.5	>250	>250	>250	31.2	15.6	62.5	62.5	31.2	125	0.50
C. glabrata	CCC 115	62.5	31.2	62.5	125	125	250	>250	>250	>250	62.5	31.2	125	62.5	31.2	62.5	1.50
C. parapsilopsis	CCC 124	62.5	31.2	125	125	62.5	250	>250	>250	>250	62.5	15.6	125	62.5	31.2	125	0.75
C. krusei	CCC 117	125	62.5	125	125	125	250	>250	>250	>250	62.5	31.2	125	125	62.5	125	0.50
C. tropicalis	CCC 131	62.5	15.6	62.5	125	62.5	250	>250	>250	>250	62.5	15.6	125	62.5	31.2	125	0.50

MIC_{100} and MIC_{50}: concentrations of each compound that produced 100 % or 50 % reduction of the growth control respectively. ATCC= American Type Culture Collection (Illinois, USA); CCC= Center of Mycological Reference (Rosario, Argentina), IM = Malbrán Institute (Buenos Aires, Argentina). *C. albicans = Candida albicans*; *C. glabrata = Candida glabrata; C. parapsilopsis = Candida parapsilopsis; C. krusei = Candida krusei; C. tropicalis = Candida tropicalis; C. neoformans = Cryptococcus neoformans.* Amph B = Amphotericin B.

As it can be observed in Table 2, all clinical strains of *C. neoformans* were more susceptible to geranylphenols than all clinical strains of *C. albicans* and non-*albicans Candida* strains similarly to the behavior showed against the standardized strains in Table 1. The only exception was compound **7** that was almost inactive in all the isolated strains with MICs ≥ 250 μg/mL.

It is also clear from Table 2 that compounds **3**, **6**, **10** and **11** are compounds that deserve attention as hits for the development of a new antifungal drug against *C. neoformans*. They inhibit 100% of standardized as well as clinical isolates of *C. neoformans* at very low concentrations (3.9-31.2 μg/mL), and inhibit 50% of their growth at concentrations between 1.9-15.6 μg/mL. In addition, they all are fungicide (kill the fungi rather than inhibit them), thus avoiding recurrence.

3.2.3 In vitro human dermal fibroblasts growth inhibition assay.

The cytotoxic activity of the most active compounds was evaluated following the procedure previously described by our group (Taborga et al., 2016). The IC_{50} obtained from these assays are shown in Table 3.

Table 3. Inhibitory Concentrations (IC_{50}) of selected mono-geranyl compounds **6**, **7**, **3**, **10** and **11** on human dermal fibroblast (HDF)

	The cytotoxicity values on human dermal fibroblast (HDF)				
Compound number	**3**	**6**	**7**	**10**	**11**
IC_{50}	ND	>100μM	ND	>100μM	>100μM

ND = Not Determined

The cytotoxicity assay performed on compounds **6**, **10** and **11** on human dermal fibroblast (HDF), showed values of IC_{50} greater than 100 μM, indicating that the toxicity of these compounds is negligible. This is in agreement with the results previously reported by our group that showed that the toxic activity of this type of compounds depends strongly on the substitution of geranyl chains. Compounds substituted with two geranyl chains on the aromatic ring are more toxic than compounds substituted with only one geranyl chain. The results indicate that the compounds mono-attached by geranyl do not present significant toxic activity in the tests carried out *in vitro* in the non-tumor cell lines (HDF) nor in the tumor cell lines (Taborga et al., 2016).

4. Conclusions:

From the values of inhibition percentages of fungal growth reported in Table 1, we conclude that the compounds having a geranyl substituent (series i) were more active than bi-geranyl compounds (series ii) against *C. neoformans* strains. Additionally, geranylphenols **2**, **3**-**7**, **10**, and **11** were more active than their acetylated or methoxylated derivatives. This effect can be clearly observed by comparing, within sub-series i.2, compounds **4**, **13,** and **16** and **11**, **12** and **17**. Dramatic decrease in biological activity is observed when comparing data between pairs of compounds **2** with **8**; **6** with **9** or **3** with **15**. Therefore the decreasing order of biological activities in this series of compounds could be established as: Ar-OH >> Ar-OCH_3 ≈ Ar-OAc against *C. neoformans* strains. Although the biological activity of these compounds against *C. albicans* decreases, the trend observed is similar. We believe that this effect presumably is caused by the change of polarity in the structures of the compounds from more polar hydroxyl functions to less polar methoxy or acetoxy functions. This effect is also in accordance to the incorporation of two geranyl chains on the aromatic ring (bi-geranyl, series ii). In addition, from the values reported against clinical isolates (Table 2) the compounds **3**, **6**, **10** and **11** (series i) showed interesting MIC_{100} and MIC_{50} values. However, compound **7** showed no activity in the other strains. These results are considered significant because this series of compounds (**3**, **6**, **10** and **11**) can act against to several strains of *Candida* and *C. neoformans.*

Based on the reported data in Table 1 the observed decreasing order in the growth inhibition effect on *C. neoformans* and *C. albicans* assay was **10** ≥ **11** > **3** > **7** ≈ **4**, while a similar effect was observed in clinical isolates for compounds **10** and **11** (Table 2).

Finally, it was observed that the activity of the compounds analyzed is selective towards fungi, considering that the compounds showed growth inhibition of pathogenic fungi, and a negligible cytotoxicity on human non-tumor cell lines. This results making them a very attractive family for the development of future drugs for the treatment of mycotic infections.

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LE, LT and SZ designed research; SZ, LE, LT, MS, EB and HC performed research and analyzed the data; LE, LT, HC and SZ wrote the paper. All authors read and approved the final manuscript.

Conflicts of Interest:

"The authors declare no conflict of interest."

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Highlights

- Geranylphenols have antifungal activity against *Candida albicans* and *Cryptococcus neoformans*.
- The bioactivity of geranylphenols is related to the amount and type of substitution in the aromatic ring.
- Some geranylphenols studied showed similar activity to standard drug Amphotericin B.
- Compound analyzed is selective, showed growth inhibition of fungi, and a negligible cytotoxicity on human cell lines.
- The geranylphenols is a very attractive family for the development of future drugs for the treatment of mycotic infections.