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Original article

Synthesis and antifungal activity of terpenyl-1,4-naphthoquinone and 1,4-anthracenedione derivatives

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ABSTRACT

The antifungal evaluation of twenty seven simple and heterocycle-fused prenyl-1,4-naphthoquinones and 1,4-anthracenediones was performed *in vitro* against human pathogenic yeasts (*Candida* spp.) and filamentous fungi (*Aspergillus* spp., *Fusarium* spp., and *Trichophyton* spp.). The synthetic strategy used to obtain the quinone derivatives was initially based on the Diels–Alder cycloaddition between myrcene and several *p*-benzoquinone derivatives, followed by cyclisation of the prenyl side chain in the case of anthracene-1,4-diones. The most promising compounds, displaying MIC values in the low μg/mL range, were those bearing one or two chlorine atoms attached to the quinone ring. Time-kill curves determined for the most potent compounds showed their fungistatic mode of action similar to that of itraconazole.

1. Introduction

The frequency of opportunistic fungal infection has increased drastically, mainly in patients who are immunocompromised due to organ transplant, leukaemia or HIV infection [1]. *Candida albicans* is responsible for most infections caused by fungi; however, the incidence of non-*albicans* species that are resistant, less susceptible or potentially resistant to currently antifungal drugs, such as *Candida parapsilosis*, *Candida dubliniensis*, *Candida krusei*, *Candida glabrata*, *Candida tropicalis*, *Candida guilliermondii* and *Candida lusitaniae*, appears to be increasing [2]. Filamentous fungi infections are less frequent than *Candida* species infection, but are associated with high mortality rates [3]. *Aspergillus* species are the most important in this sense; *A. fumigatus* is the leading etiological agent, but it is joined by *Aspergillus flavus*, *Aspergillus terreus*, *Aspergillus niger* and *Aspergillus nidulans* as the causal agents of pernicious invasive fungal disease. Moreover, *Fusarium* species also cause a broad spectrum of infections in humans, including superficial, invasive and disseminated infections in immunocompromised patients, and the treatment options are limited due to the relative resistance of the fungus to the current antifungal drugs [4]. Dermatophytes are the most common cause of skin disease in tropical countries, being the leader etiological agents *Trichophyton rubrum* and *Trycophyton mentagrophytes*. *T. rubrum* causes between 80 and 90% of all chronic and recurrent infections, and over 90% of cases of onychomycosis [5]. Although dermatophyte onychomycosis is considered as a rather trivial cosmetic problem, in the elderly who suffer diabetes or peripheral vascular problems, the disease can give rise to important complications [5b,6].

Despite the addition of new classes of antifungals, the number of currently available drugs for the treatment of fungal infections remains limited. Many of them are fungistatic rather than fungicidal, while others are associated with a substantial toxicity and display very complex structures [7]. Therefore, there is a continuing need to develop novel and simpler antifungal agents being more effective and less toxic. Interestingly, medicinal plants have been extensively studied with such objectives [8]. Most natural quinones are secondary metabolites of flowering plants and they are associated to many types of biological activity [9]. In the past, a number of studies

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dealt with the antifungal activity of natural and synthetic quinones, mainly substituted 1,4-naphthoquinones, or of compounds with structures containing diverse heterocyclic systems fused to the quinone fragment. Much less interest was focused on tri- and tetracyclic quinone derivatives related to 1,4-anthracenediones or 1,4-naphthacenediones. The results reported up to the present are very broad, both in terms of the antipathogenic activity profile as of the antifungal potency, ranging from μM [10,11] to M levels [12], according published MIC values. Most of the natural naphthoquinones, which had been previously evaluated as antifungals, contained additional functions or substituents attached to the quinone fragment; only a few examples, mainly those related to cordiaquinones [13], contained an alkyl substituent attached to the non-quinonic aromatic ring.

The main aim of this study was thus focused on investigating the influence of a prenyl substitution in the benzene ring of 1,4-naphthoquinones, and that of their further cyclisation to the corresponding anthracene-1,4-dione derivatives, on the antifungal activity of such type of compounds.

2. Chemistry

The structures of the quinone derivatives evaluated in this research are shown in Table 1 and were selected as representative members of different families of simple (**1**–**24**) and heterocycle-fused (**25**–**27**) derivatives of 1,4-naphthoquinone (NQ, **1**–**11**) and 1,4-anthracenedione (AD, **12**–**24**).

Most of the quinone derivatives evaluated in this work were prepared as summarized in Scheme 1. The synthesis of the intermediate prenylquinones was based on an initial catalysed Diels–Alder condensation between myrcene and *p*-benzoquinone (or 2,5-dichlorobenzoquinone) in the presence of $BF_3.OEt_2$, followed by oxidation of the resulting cycloadducts with MnO_2. Controlling the absence or presence of small amounts of catalyst during oxidation, NQ **1** and **2** or AD **12** and **13** could be obtained respectively [14]. Aryloxy- (**4**), arylsulfanyl- (**5**) and bromo derivatives (**3**, **7** and **10**) were easily obtained from **1** as previously reported [15]. The new quinone derivatives were obtained either through nucleophilic addition-elimination or formal substitution from compounds **1**, **2** and **12**, using nitrogen, oxygen and sulphur nucleophiles (Scheme 1), mainly different aliphatic and aromatic amines.

Dichloroquinones **6** and **17** were respectively prepared from **1** and **12** by treatment with $SOCl_2$ [14a]. AD derivatives **14**–**16** were obtained from **12** by Michael addition of the corresponding aliphatic or aromatic amine. Because positions C-2 and C-3 in **12** were not identical, the reactions led to 1:1 mixtures of the two possible regioisomers. When the addition of the amine was performed in the presence of $Cu(OAc)_2$ on quinones **2** (without or with previous side chain hydrogenation) or **13**, compounds **8**, **11** and **23** were obtained in a regioisomeric ratio similar to that of the corresponding starting material. Derivatives **9** and **18**–**24** were obtained from **6** and **17** by formal nucleophilic substitution of one chlorine atom leading to 1:1 mixtures of regioisomers. In the case of acetamide **18**, the substitution was performed with ethanolic NH_4OH, followed by acetylation and chromatographic separation of both regioisomers, while the diethoxy derivative **24** was obtained by direct treatment of **17** with ethanol in the presence of potassium carbonate and 2-aminopyridine. In some cases, each regioisomer was separated by CC to allow independent characterization and biological evaluation, as it is stated in Table 1. The spectroscopic characterization of each regioisomer was based on previously reported two-dimensional HMBC NMR correlations for similar amino-1,4-NQs [14a,15b], and complemented with additional experiments done for compounds **14** and **22**. As an example, representative correlations experimentally observed between the aromatic hydrogens H-9 and H-10 and the carbonyls C-1 and C-4 and carbons C-5 and C-8 are shown in Fig. 1. These correlations served to assign the structures of the regioisomers **14a** and **14b** unequivocally.

Finally, the heterocycle-fused quinones **25**–**27** were prepared by palladium (II)-catalysed oxidative cyclisation as previously reported [15a]. Physical and spectral data found for those previously non-described quinones are included in the experimental section.

3. Antifungal activity

3.1. Antifungal screening

All the synthesized compounds were evaluated *in vitro* against several *Candida* spp., *Aspergillus* spp., dermatophytes, and *Fusarium oxysporum*, but only those NQ and AD derivatives that showed activity at concentrations lower than 32.0 μg/mL (~100 μM), against one or more strains, were considered active and are included in Table 2. Three standard drugs, itraconazole (ITZ), amphotericin B (AMB) and terbinafine (TRB), were used as reference drugs to cover the fungal spectrum widely. Complete evaluation data for all the compounds tested are given as Supplementary material.

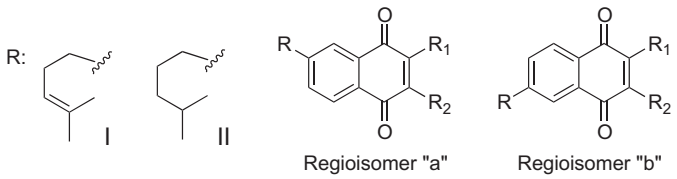
As it can be observed, all the compounds included in Table 2 (one-third of those evaluated) showed good activity against *Trichophyton* species, while some compounds showed important activity against *Candida* species, *A. fumigatus* and *A. flavus*. Only one compound resulted slightly active against *F. oxysporum* and another one against *A. niger*.

Compounds NQ **2a** and **3**, and AD **12**, **13a**, **17** and **18a** showed fairly wide antifungal spectra. Anti-*Candida* activity of AD **17** was relevant because its MIC values were the lowest against the pathogenic yeast *C. albicans* (ATCC 90028) (MIC_{90} = 8.0 μg/mL), and against the less common but not less important yeasts *C. parapsilosis* (MIC_{90} = 2.0 μg/mL), *C. krusei* (MIC_{90} = 1.0 μg/mL) and *C. lusitaniae* (MIC_{90} = 2.0 μg/mL). Also, it is important to highlight the activity of NQ **2a** and AD **13a** against *C. krusei* (MIC_{100} = 2.4 and 2.0 μg/mL, respectively). These three yeasts are important because *C. lusitaniae* is capable of developing resistance to AMB during the course of therapy [3] and *C. parapsilosis* exhibits a consistent trend of decreasing fluconazole susceptibility over treatment time [16]. Notably, *C. krusei* can cause serious infections in immunocompromised patients and it is intrinsically resistant to fluconazole, a currently important drug in the treatment of candidiasis, while displaying decreased susceptibility to AMB and flucytosine [17].

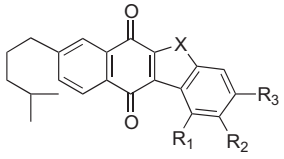
The NQs **2a**, **3** and **6** and the ADs **13a** and **18a**, showed activity against *A. fumigatus* (MIC_{90} range 8.0–22.6 μg/mL), but not against other *Aspergillus* species, while the AD **17** showed the major activity against *A. flavus* and also against *A. fumigatus* (MIC_{90} = 4.8 and 8.0 μg/mL, respectively). In addition, compound **3** was the only compound with a discrete activity against *A. niger* (MIC_{90} = 25.4 μg/mL). None of the tested compounds was active against *A. terreus* (see Supplementary data). The AD **13a**, besides its activity against different species of *Candida* and *A. fumigatus*, showed a moderate activity against *F. oxysporum* (MIC = 16.0 μg/mL) (Table 2). Notably, any activity against this fungus, which leads to high mortality rates, is important because there are limited treatment options due to the resistance of this pathogen to the most important antifungal agents [4].

All compounds shown in Table 2, were active against both dermatophytes tested. NQ **3** and AD **12**, showed the highest activity against *T. mentagrophytes* with MIC_{90} values of 2 and 1.3 μg/mL, respectively. The highest activity against *T. rubrum* was observed for AD **14b**. It is important to emphasize that NQ **6** showed the highest

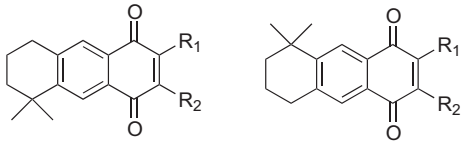
Table 1
Structures of naphthoquinone and anthracenedione derivatives evaluated as antifungals.



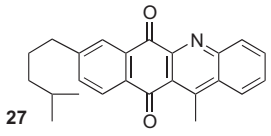
R	**R_1**	**R_2**	Reaction products (*a*/*b* ratio)	Compound tested
I	H	H	**1**	**1**
I	Cl	H	**2** (9:1)	**2a**
II	Br	H	**3** (1:1)	**3a/3b**
II	3,5$(MeO)_2$PhO-	H	**4** (1:1)	**4a**
II	4-MeOPhS-	H	**5** (1:1)	**5a**
II	Cl	Cl	**6**	**6**
II	Br	Br	**7**	**7**
II	Cl	4-MeOPhNH-	**8** (9:1)	**8a**
II	Cl	4-MeOPhO-	**9** (1:1)	**9a/9b**
II	Br	4-MeOPhNH-	**10** (1:1)	**10a**
I	Cl	BnNH-	**11** (9:1)	**11a**



X	R_1	R_2	R_3	
NH	H	CH_3	H	**25**
O	CH_3	H	CH_3	**26**



R_1	**R_2**	Reaction products (*a*/*b* ratio)	Compound tested
H	H	**12**	**12**
Cl	H	**13** (3:1)	**13a**
EtNH-	H	**14**(1:1)	**14b**
4-MeOPhNH-	H	**15** (1:1)	**15a/15b**
4-AcOPhNH-	H	**16** (1:1)	**16a/16b**
Cl	Cl	**17**	**17**
AcNH-	Cl	**18**(1:1)	**18a**
EtNH-	Cl	**19** (1:1)	**19a/19b**
3,4$(Me)_2$PhNH-	Cl	**20** (1:1)	**20a/20b**
4-MeOPhNH-	Cl	**21** (1:1)	**21a/21b**
3,4$(MeO)_2$PhNH-	Cl	**22** (1:1)	**22a/22b**
3,4,5$(MeO)_3$PhNH-	Cl	**23** (1:3)	**23b**
EtO-	EtO-	**24**	**24**



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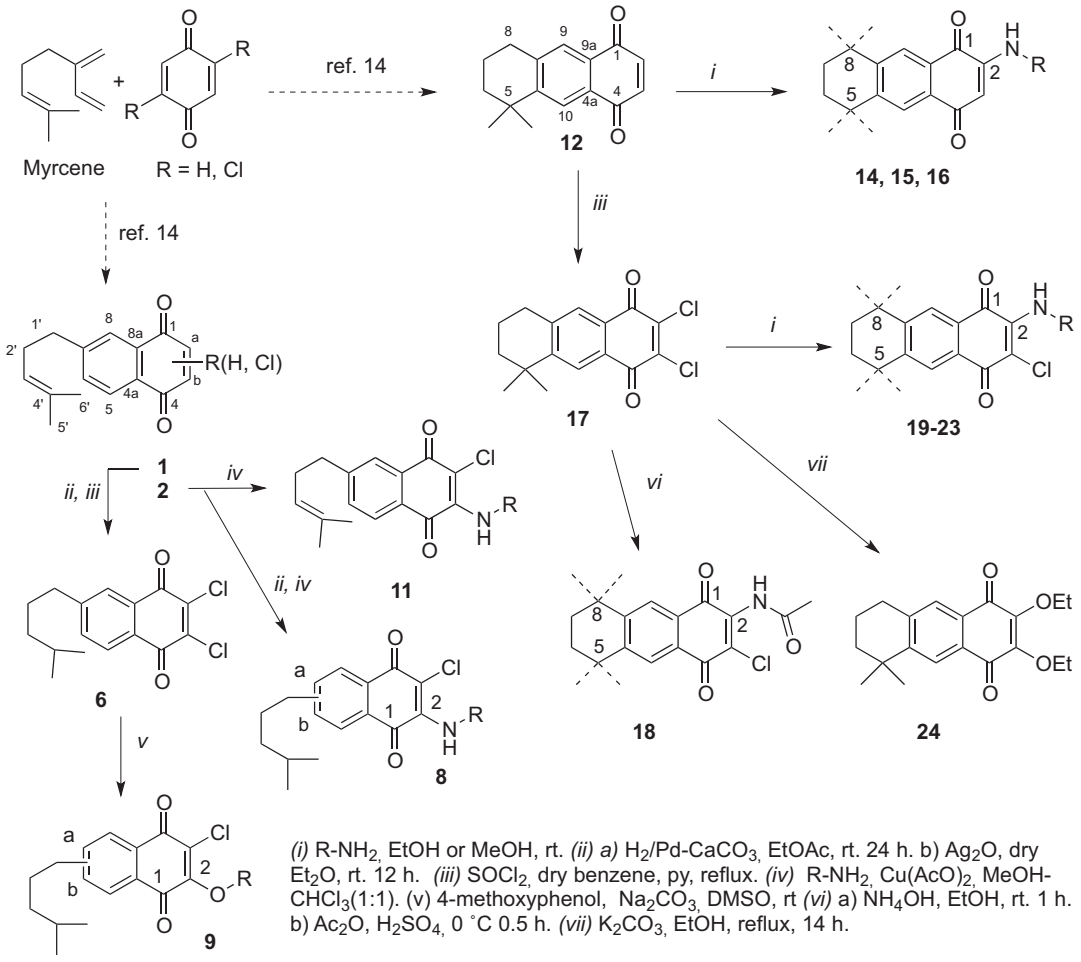
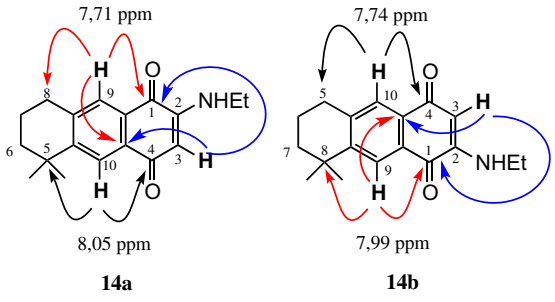
selectivity index (HSI, see definition below), with values >50 for *Trichophyton* spp. (Table 2); then, this compound could be considered as a good candidate for further studies towards the development of a new anti-dermatophytic agent.

In order to go further into the potential usefulness of these quinones as antifungal agents, the viability of normal mammalian Vero cells treated with representative elements of both the NQ and AD series and with the antifungals itraconazole, terbinafine, amphotericine B, was evaluated and the corresponding IC_{50} cytotoxicity values determined. All the tested compounds produced a dose-dependent inhibition on the Vero cell line, with R^2 linear regression coefficients higher than 0.7 (Supplementary data). According to the US National Cancer Institute screening program, a pure compound is considered to have a cytotoxic effect if the IC_{50} value is 4.0 μg/mL or lower, after incubation for 48–72 h [18]; on this basis, the compounds reported here should be considered as relatively low cytotoxic on Vero cells (Table 2). In order to quantify their respective selectivity, the highest selectivity index (HSI = IC_{50} for Vero cells/lowest MIC_{90} value of a compound against any pathogenic fungus) was calculated for each compound. It is important to note that while MIC values practically refer to total (90–100%) fungal inhibition, IC values refer to the inhibition of only



Scheme 1. Synthesis of the new 1,4-naphthoquinone and 1,4-anthracenedione derivatives.

50% of Vero cells, and consequently, the actual HSI values included in Table 2 must be higher than those calculated and shown for the tested compounds. This index turns out to be useful to make bioactivity comparisons within each and between both series of compounds, and will aid in the design of more potent and selective compounds. According to literature reports, selectivity index (SI) values higher than 8 are considered indicative of a potential therapeutic agent that would merit further biopharmaceutical and preclinical studies [19]. To this respect, the dichloroquinone NQ **6** came out as the most selective against both dermatophytes (SI > 50), *A. fumigatus* (SI > 25) and *C. lusitaniae* (SI > 9.9), while the dichloroquinone AD **17** showed the highest selectivity against yeasts,



14a **14b**

Fig. 1. Selected long-range H/C connectivities found for regioisomers **14a** and **14b**.

SI > 25 for *C. krusei*, as well as for *C. parapsilosis* and *C. lusitaniae* (SI > 12.5, data not shown).

From the global observation of the data included in Table 2, it could be considered that AD derivatives show wider antifungal spectra and higher potencies than NQ derivatives. Such a comparison seems correct, but can actually be applied only to the case of compounds **1** (inactive) and **12** (MIC_{90}: 4.0 μg/mL/16.6 μM for *C. krusei*), whereas it is not clear for NQ **2a** and AD **13a**, both displaying identical MIC_{90} values for *C. parapsilosis* and very close values, for *C. krusei. C. lusitaniae* and *Trichophyton* spp. However, the AD **13a** proved to be more potent against *C. albicans* and *C. tropicalis* and additionally showed important activity against *F. oxysporum*. Larger differences can be detected when the comparison deals with the presence of chlorine atoms in the molecules. Thus, the dichloro-AD derivative **17** showed a wider spectrum of activity and lower MIC values against *Candida* spp., *Aspergillus* spp., and *Trichophyton* spp. than the dichloro-NQ analogue **6**. However, NQ **6** resulted to be a less cytotoxic compound, and therefore, to have a higher SI value than AD **17** for *Trichophyton* spp. It is interesting to note that the acetamido/chloro-AD **18a** showed a wider spectrum of activity against *Candida* species, *A. fumigatus*, dermatophytes, and also against *F. oxysporum*, in opposition to its dichloro analogue AD **17**.

3.2. Time-kill assays

In order to obtain some insight and information on the mode of action of these compounds, the ADs **13a** and **17**, which showed the

Table 2
Antifungal activity (MIC_{90}) and cytotoxicity (IC_{50}) of selected 1,4-naphthoquinone (NQ) and 1,4-anthracenedione (AD) derivatives.

Comp.	*Cp*	*Ck*	*Cl*	*Ct*	*Ca*1	*Ca*2	HSI (*C.* spp.)	*Afu*	*Afl*	*An*	*Fo*	HSI (*Asp.* spp.)	*Tm*	*Tr*	HSI (*Trich.* spp.)	Vero
2a	**4.0**	**2.4**	**3.2**	11.3	>32	>32	10.9 (*Ck*)	11.3	>32	>32	32	1.4 (*Afu*)	**6.4**	**4.0**	6.5 (*Tr*)	26.1
3	12.7	**5.0**	**4.0**	12.7	25.4	>32	1.0 (*Cl*)	17.4	>32	25.4	32	>1.4 (*Afu*)	**2.0**	**4.0**	2.1 (*Tm*)	4.1
6	>32	>32	20.2	>32	>32	>32	>**9.9** (*Cl*)	**8.0**	>32	>32	>32	>**25** (*Afu*)	**4.0**	**4.0**	**>50** (*Tm, Tr*)	>200
7	>32	>32	>32	>32	>32	>32	na	>32	32	>32	>32	na	>32	32	na	<25
8a	>32	>32	16.0	32	>32	>32	2.2 (*Cl*)	>32	>32	>32	>32	na	**5.0**	12.7	7.1 (*Tm*)	35.6
12	**4.8**	**4.0**	**5.0**	**5.0**	18.0	22.6	2.2 (*Ck*)	>32	> 32	>32	>32	na	**1.3**	**4.0**	6.6 (*Tm*)	8.6
13a	**4.0**	**2.0**	**5.0**	**8.0**	20.2	16.0	>**12.5** (*Ck*)	16	> 32	>32	16	>1.6 (*Afu*)	**3.2**	**5.0**	>7.8 (*Tm*)	109
14b	>32	>32	>32	>32	>32	>32	na	>32	>32	>32	>32	na	**5.0**	**3.2**	**11.1** (*Tr*)	35.6
17	**2.0**	**1.0**	**2.0**	**5.0**	>32	**8.0**	>**25** (*Ck*)	**8.0**	**4.8**	>32	>32	>5.2 (*Afl*)	**6.1**	**8.0**	>4.1(*Tm*)	<25
18a	**8.0**	**2.8**	**2.2**	**4.0**	**4.0**	**8.0**	**10.1** (*Cl*)	22.6	>32	>32	32	0.9 (*Afu*)	**3.2**	**5.0**	7.1 (*Tm*)	22.3
22	>32	>32	>32	>32	>32	>32	na	>32	>32	>32	>32	na	25.4	25.4	2.0 (*Tm, Tr*)	50.5
ITZ	0.25	0.5					>100(***Ck***)									>50
AMB								2.0	4.0			**7.1(*Afl*)**				28.6
TRB													0.03	0.08	386 (***Tr***)	30.9

Values (μg/mL) correspond to the rounded media of Minimum Inhibitory Concentrations (MIC_{90}, fungi) and Cytotoxic Concentrations 50% (IC_{50}, Vero cells) found in triplicate experiments for each compound. Values under 10 μg/mL are bold-faced to facilitate comparisons. *Cp*: *Candida parapsilosis* (ATCC 22019); *Ck*: *C. krusei* (ATCC 6258); *Cl*: *C. lusitaniae* (ATCC 200951); *Ct*: *C. tropicalis* (ATCC 200956); *Ca*1: *C. albicans* (ATTC 10231); *Ca*2: *Candida albicans* (ATCC 90028); *Afu*: *Aspergillus fumigatus* (ATCC 204305); *Afl*: *A. flavus* (ATCC 204304); *An*: *A. niger* (ATCC 16404); *Fo*: *Fusarium oxysporum* (ATCC 48112); *Tm*: *Trycophyton mentagrophytes* (ATCC 24198); *Tr*: *T. rubrum* (ATCC 28188). Vero: mammalian Vero cells (ATCC CCL-81). na: not applicable. HSI: Highest Selectivity Index (IC_{50} Vero/lowest MIC_{90} found for each compound) rounded values.

lowest MIC values against almost all the yeasts tested, were selected to conduct complementary lethality assays. These studies are useful for the evaluation of the pharmacodynamic characteristics of new antimicrobial agents and also allow the determination of whether an agent produces concentration-dependent or time-dependent killing and whether the agent has a fungistatic or fungicidal profile. Time-kill studies are sometimes used to guide therapy in individual patients, and are useful in determining tolerance to the lethal activity of antimicrobial agents and in determining synergy or antagonism between two (or more) antimicrobial agents [20,21].

The analysis of the time-kill curves of compounds **13a** and **17**, as well as those of ITZ and AMB, againts *C. parapsilosis* ATCC 22019 are represented in Fig. 2. As it can be seen in this figure and in other results given as Supplementary material, the behaviour of all those compounds tested were more closely comparable to that of ITZ than that of AMB, thus suggesting a fungistatic rather than a fungicidal profile for these quinone derivatives. At the lowest concentration tested (0.5 × MIC) the yeast growth was not inhibited showing a behaviour similar to the growth control (GC).

As a main conclusion of this research, it can be stated that although a larger number of structural variations and more compounds should be tested, the dichlorinated prenyl-1,4-naphthoquinones and their corresponding cyclised anthracene-1,4-diones display antifungal activity against pathogenic fungi as *Candida* spp., *Aspergillus* spp. and dermatophytes, suggesting that they could be useful as lead compounds for the development of simple and new antifungal drugs readily obtainable from commercial natural products. Additionally, the results of time-kill assays indicate that both types of quinones have a fungistatic behaviour.

4. Experimental section

4.1. Chemistry

NMR spectra were recorded on a Bruker AC 200 at 200 MHz for ^{1}H and 50.3 MHz for ^{13}C in deuterochloroform with TMS as internal standard. Chemical shift (δ) values are expressed in ppm followed by multiplicity and coupling constants (J) in Hz. IR spectra were obtained on a Nicolet Impact 410 spectrophotometer, and wavenumbers are given in cm^{-1}. HRMS were run in a VG-TS-250 spectrometer working at 70 eV. Column chromatography (CC) was performed on silica gel (Merck No 9385) and TLC was carried out on

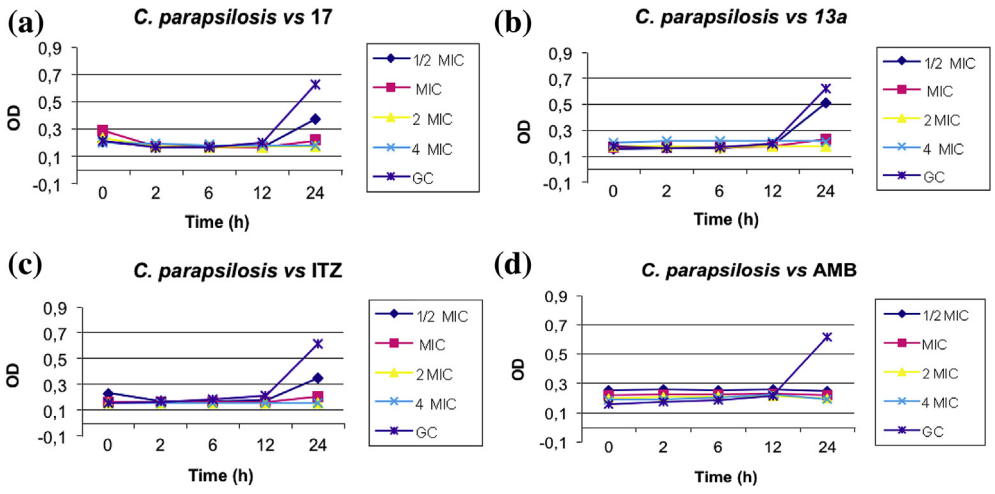


Fig. 2. Time-kill plots of (a) compound **17**, (b) compound **13a**, (c) ITZ and (d) AMB against *C. parapsilosis* ATCC 22019. OD: optical density, arbitrary units.

silica gel 60 F_{245} (Merck, 0.25 mm thick). Solvents and reagents were purified by standard procedures as necessary.

Naphthoquinones **1**, **2**, **3**, **7** and **10** were obtained by means of previously described procedures [14b,15b]. Naphthoquinones **4**, **5**, **25**, **26** and **27** were obtained as described before [15a]. Antracene-1,4-diones **12**, **13** and **17** were also obtained as described before [14a]. Other compounds were prepared as follows.

4.1.1. 2,3-Dichloro-6-(4-methylpentyl)-1,4-naphthoquinone **6**

To a solution of **1** hydrogenated at the side chain [14a] (326 mg, 1.35 mmol) in dry benzene (100 mL), pyridine (3 mL) and recently distilled $SOCl_2$ (8 mL) were added. The mixture was stirred under reflux for 19 h, then water was slowly added and extracted with EtOAc. The organic layer was washed with satd aq $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4 and vacuum-evaporated to yield compound **6** as a viscous oil (85%). ^{1}H NMR ($CDCl_3$) δ: 0.87 (d, 6H, J = 6.6 Hz, H-5′,6′), 1.23 (m, 2H, H-3′), 1.55 (m, 1H, H-4′), 1.65 (m, 2H, H-2′), 2.74 (t, 2H, J = 7.7, H-1′), 7.59 (dd, 1H, J = 8.0, 1.8, H-7), 7.97 (d, 1H, J = 1.8, H-5), 8.08 (d, 1H, J = 8.0, H-8). ^{13}C NMR δ: 22.6 (C5′,6′), 27.9 (C4′), 28.8 (C2′), 36.5 (C1′), 38.5 (C3′), 127.7 (C5), 128.2 (C8), 128.9 (C8a), 131.0 (C4a), 134.8 (C7), 143.4, 143.7 (C2, C3), 151.2 (C6), 175.9 (C1), 176.4 (C4). IR (ν, cm^{-1}): 1677, 1600, 1587, 1291, 1140, 870, 859, 829, 728. HRMS (FAB-POSI, M + 1): calcd: 311.0606, found: 311.0612.

4.1.2. 3-Chloro-2-(4-methoxyanilino)-6(7)-(4-methylpentyl)-1,4-naphthoquinone **8a (8b)**

A 9:1 mixture of naphthoquinone **2a/2b** hydrogenated at the side chain [14a] (98 mg, 0.36 mmol), *p*-anisidine (45 mg, 0.37 mmol) and $Cu(OAc)_2$ (38 mg, 0.19 mmol) in MeOH–$CHCl_3$ (3 mL–3 mL) was stirred at room temperature for 7 days, and then, a new amount of *p*-anisidine (27 mg, 0.22 mmol) was added and heated to 50 °C for 24 h. Then, the solvent was vacuum-evaporated. The residue was redissolved in diethyl ether and washed with 2 N HCl and brine, dried over anhydrous Na_2SO_4 and vacuum-evaporated to yield a product, which was purified by column chromatography (eluent: Hex/CH_2Cl_2 2:8) affording 41 mg (29%) of a 9:1 mixture of **8a**/**8b** as a viscous oil. ^{1}H NMR ($CDCl_3$) δ: 0.87 (d, 6H, J = 6.6 Hz, H-5′,6′), 1.22 (m, 2H, H-3′), 1.53 (m, 1H, H-4′), 1.66 (m, 2H, H-2′), 2.72 (t, 2H, J = 7.8, H-1′), 7.46/7.55 (dd, 1H, J = 8.0, 1.8, H-7/6), 7.97/7.89 (d, 1H, J = 1.8, H-5/8), 8.00/7.99 (d, 1H, J = 8.0, H-8/5), 3.82 (s, 3H, OCH_3), 6.86/6.91, 7.04 (AB system, J = 8.9/9.3). ^{13}C NMR δ: 22.6 (C5′,6′), 27.9 (C4′), 28.8 (C2′), 36.6 (C1′), 38.5 (C3′), 127.7(126.7) (C5/8), 127.3 (C8/5), 127.7(129.9) (C8a), 131.9 (C4a), 132.9/134.1 (C7/6), 142.0 (C2, C3), 151.6/150.1 (C6/7), 177.8 (C4), 180.3 (C1), 55.5, 113.6, 126.3, 130.5, 157.8 (R–NH). IR (ν, cm^{-1}): 3219, 1674, 1596, 1558, 1500, 1290, 1237, 1042, 828. HRMS (FAB-POSI, M + 1): calcd: 398.1523, found: 398.1547.

4.1.3. 3-Chloro-2-(4-methoxyphenoxy)-6(7)-(4-methylpentyl)-1,4-naphthoquinones **9a** (**9b**)

To a solution of **6** (148 mg. 0.48 mmol) in DMSO (3 mL), Na_2CO_3 (209 mg, 1.97 mmol) and *p*-methoxyphenol (215 mg, 1.73 mmol) were added and kept stirring at room temperature for 8 min. Then, EtOAc was added and the organic phase was washed with 2 N HCl and brine, dried over anhydrous Na_2SO_4 and vacuum-evaporated to yield a product, which was purified by column chromatography (eluent: Hex/CH_2Cl_2 1:9) affording **9** (viscous oil, 32%) as a 1:1 mixture of regioisomers. ^{1}H NMR ($CDCl_3$) δ: 0.86/0.88 (d, 6H, J = 6.6 Hz, H-5′,6′), 1.22 (m, 2H, H-3′), 1.54 (m, 1H, H-4′), 1.67 (m, 2H, H-2′), 2.72 (m, 2H, H-1′), 7.54/7.57 (dd, 1H, J = 7.8, 1.8, H-7/6), 7.98/7.83 (d, 1H, J = 1.8, H-5/8), 8.09/7.93 (d, 1H, J = 7.8, H-8/5), 3.78 (s, 3H, OCH_3), 6.84, 6.96 (AB system, J = 9.1). ^{13}C NMR δ: 22.6 (C5′,6′), 27.9 (C4′), 28.8 (C2′), 36.5 (C1′), 38.5 (C3′), 127.0/127.5 (C5/8), 127.6/127.2 (C8/5), 130.8 (C8a), 131.3 (C4a), 134.7/134.5 (C7/6), 134.5 (C3), 150.5/150.7 (C2), 151.0 (C6/7), 178.1 (C4), 178.6/178.9 (C1), 55.7, 114.8, 117.9, 154.1, 156.2 (R–O). IR (ν, cm^{-1}): 1681, 1592, 1503, 1295, 1246, 1216, 1035, 862, 729. HRMS (FAB-POSI, M + 1): calcd: 399.1363, found: 399.1327.

4.1.4. 2-Benzylamino-3-chloro-6(7)-(4-methyl-3-pentenyl)-1,4-naphthoquinones **11a (11b)**

Following the procedure described above for **8**, the treatment of **2** (327 mg) with benzylamine (0.19 mL, 1.74 mmol) and $Cu(OAc)_2$ (129 mg, 0.65 mmol) for 8.5 days at room temperature gave, after column chromatography (eluent: Hex/CH_2Cl_2 2:8): (a) 42 mg (viscous oil, 9%) of **11a**. (b) 71 mg (viscous oil, 16%) of **11a**/**11b**. ^{1}H NMR ($CDCl_3$) for **11a**, δ: 1.52 (s, 3H, H-6′), 1.66 (s, 3H, H-5′), 2.33 (dd, 2H, J = 15.4,7.3, H-2′), 2.75 (t, 2H, J = 7.3, H-1′), 5.11 (m, 1H, H-3′), 7.41 (dd, 1H, J = 8.0, 1.7, H-7), 7.92 (d, 1H, J = 8.0, H-8), 7.96 (d, 1H, J = 1.7, H-5), 5.05 (d, 2H, J = 6.1, Bn), 7.35 (m, 5H, Bn). ^{13}C NMR δ: 17.6 (C6′), 25.6 (C5′), 29.2 (C2′), 36.3 (C1′), 122.6 (C3′), 126.8 (C5), 127.0 (C8), 127.6 (C8a), 132.6 (C4a, C7), 133.1 (C4′), 144.1 (C2), 150.8 (C6), 177.2 (C4), 180.1 (C1), 48.9, 127.6, 128.0, 129.0, 137.9 (Bn-NH). IR (ν, cm^{-1}): 3355, 1673, 1592, 1568, 1504, 1303, 1066, 740. HRMS (FAB-POSI, M + 1): calcd: 380.1417, found: 380.1466.

4.1.5. 2-Ethylamino-5,5(8,8)-dimethyl-5,6,7,8-tetrahydroanthracene-1,4-diones **14a (14b)**

To a stirred solution of naphthoquinone **12** (1.12 mmol) in ethanol (13 mL), pyridine (3.5 mL) and ethyl amine (63 μL) were added. The mixture was stirred at room temperature for 24 h. After removing the solvent, the crude product was redissolved in ethyl acetate and washed with 2 N HCl and brine, dried over anhydrous Na_2SO_4 and vacuum-evaporated to yield a product, which was purified by column chromatography (eluent: Hex/EtOAc 96:4) affording: (a) 10 mg (3%) of **14a** as a viscous oil. ^{1}H NMR ($CDCl_3$) δ: 1.34 (s, 6H, H-11,12), 1.68 (m, 2H, H-6), 1.82 (m, 2H, H-7), 2.86 (t, 2H, J = 5.8, H-8), 5.67 (s, 1H, H-3), 7.71 (s, 1H, H-9), 8.05 (s, 1H, H-10), 1.33 (t, 3H, J = 7.2, Et-NH), 3.22 (m, 2H, Et-NH). ^{13}C NMR δ: 19.3 (C7), 30.9 (C8), 31.4 (C-11,12), 34.9 (C5), 38.8 (C6), 100.7 (C3), 125.0 (C10), 127.4 (C9), 127.8 (C9a), 131.6 (C4a), 141.0 (C8a), 148.0 (C2), 154.0 (C10a), 182.0 (C1), 183.6 (C4), 13.6, 37.3 (Et-NH). IR (ν, cm^{-1}): 3347, 1673, 1596, 1557, 1511, 1342, 1253, 802. HRMS (ES, M + 1): calcd: 284.1650, found: 284,1668. (b) 46 mg (14%) of **14a**/**14b** (1:1 ratio). (c) 18 mg (6%) of **14b** as a viscous oil. ^{1}H NMR ($CDCl_3$) δ: 1.32 (s, 6H, H-11, 12), 1.69 (m, 2H, H-7), 1.81 (m, 2H, H-6), 2.87 (t, 2H, J = 6.4, H-5), 5.65 (s, 1H, H-3), 7.74 (s, 1H, H-10), 7.99 (s, 1H, H-9), 1.33 (t, 3H, J = 7.2, Et-NH), 3.21 (m, 2H, Et-NH). ^{13}C NMR δ: 19.2 (C6), 31.3 (C5), 31.5 (C-11, 12), 34.5 (C8), 38.7 (C7), 100.6 (C3), 125.2 (C9), 127.2 (C10), 128.5 (C9a), 130.8 (C4a), 144.5 (C10a), 148.2 (C2), 150.6 (C8a), 181.9 (C1), 183.5 (C4), 13.6, 37.3 (Et-NH). IR (ν, cm^{-1}): 3347, 1674, 1596, 1558, 1511, 1341, 1252, 802. HRMS (ES, M + 1): calcd: 284.1650, found: 284.1681.

4.1.6. 2-(4-Methoxyanilino)-5,5(8,8)-dimethyl-5,6,7,8-tetrahydroanthracene-1,4-diones **15a (15b)**

A mixture of naphthoquinone **12** (223 mg, 0.93 mmol) and *p*-anisidine (114 mg, 0.93 mmol) in methanol (15 mL) was stirred at room temperature for 24 h. Treatment of the reaction mixture in the same way as described for **14** afforded a crude product, which was purified by column chromatography over silica gel (eluent: Hex/EtOAc 8:2), yielding: (a) 50 mg (15%) of **15a** as a viscous oil. ^{1}H NMR ($CDCl_3$) δ: 1.34 (s, 6H, H-11,12), 1.69–1.82 (m, 4H, H-6,7), 2.87 (t, 2H, J = 6.4, H-8), 6.18 (s, 1H, H-3), 7.76 (s, 1H, H-9), 8.04 (s, 1H, H-10), 3.82 (s, 3H, OMe), 6.93, 7.19 (AB system, J = 8.8, Ph-NH). ^{13}C NMR δ: 19.3 (C7), 30.9 (C8), 31.4 (C-11,12), 34.9 (C5), 38.7 (C6), 102.5 (C3), 124.8 (C10), 127.6 (C9,9a), 131.3 (C4a), 141.3 (C8a), 145.7 (C2), 154.1 (C10a), 182.1 (C1), 184.3 (C4), 55.6 (OMe), 114.9, 124.8, 130.3, 157.6 (Ph-NH). IR (ν, cm^{-1}): 3319, 1669, 1597, 1558, 1516, 1344, 1248,

828. HRMS (ES, M + 1): calcd: 362.1756, found: 362,1736. (b) 102 mg (30%) of **15a**/**15b** (1:1 ratio). (c) 21 mg (6%) of **15b** as a viscous oil. ^{1}H NMR ($CDCl_3$) δ: 1.35 (s, 6H, H-11,12), 1.70–1.83 (m, 4H, H-6,7), 2.88 (t, 2H, J = 6.4, H-5), 6.16 (s, 1H, H-3), 7.75 (s, 1H, H-10), 8.06 (s, 1H, H-9)), 3.82 (s, 3H, OMe), 6.93, 7.19 (AB system, J = 8.8, Ph-NH). ^{13}C NMR δ: 19.2 (C6), 31.5 (C5), 31.6 (C-11,12), 34.5 (C8), 38.7 (C7), 102.4 (C3), 125.4 (C9), 127.1 (C10), 128.4 (C9a), 130.4 (C4a), 144.7 (C10a), 145.9 (C2), 150.9 (C8a), 182.1 (C1), 184.3 (C4), 55.6 (OMe), 114.9, 124.9, 130.4, 157.6 (Ph-NH). IR (ν, cm^{-1}): 3318, 1672, 1596, 1559, 1515, 1341, 1246, 828. HRMS (ES, M + 1): calcd: 362.1756, found: 362.1736.

4.1.7. 2-(4-Acetoxyanilino)-5,5(8,8)-dimethyl-5,6,7,8-tetrahydroanthracene-1,4-diones ***16a (16b)***

Following the procedure described above, treatment of **12** with *p*-hydroxyaniline in methanol gave a reaction product that was treated with acetic anhydride and pyridine for 20 h. The acetylated product afforded, after column chromatography, quinone **16** (viscous oil, 20%) as a 1:1 mixture of **16a**/**16b** regioisomers. ^{1}H NMR ($CDCl_3$) δ: 1.34 (s, 6H, H-11,12), 1.70-1.83 (m, 4H, H-6,7), 2.88 (t, 2H, J = 6.4, H-5,8), 6.32/6.30 (s, 1H, H-3), 7.77/8.06 (s, 1H, H-9), 8.04/7.75 (s, 1H, H-10)), 2.31 (s, 3H, Ac), 7.13/7.12, 7.26/7.75 (AB system, J = 8.8, Ph-NH). ^{13}C NMR δ: 19.2 (C7/6), 31.4/34.5 (C8), 31.5 (C-11,12), 34.9/31.4 (C5), 38.6 (C6/7), 103.4 (C3), 125.4/127.2 (C10), 127.6/124.9 (C9), 141.5(C9a/4a),145.0 (C4a/9a), 144.8/145.0 (C2), 151.1 (C8a/10a), 154.2 (C10a/8a), 181.8 (C1), 184.3 (C4), 21.1 and 169.4 (Ac), 122.8, 123.6, 131.1/130.3, 135.3 (Ph-NH). IR (ν, cm^{-1}): 3318, 1766, 1678, 1612, 1598, 1192, 921, 755.

4.1.8. General procedure for the preparation of compounds ***18–24***

A mixture of naphthoquinone **17** (0.90 mmol) and the corresponding amine (0.90 mmol) in ethanol (10 mL) was stirred at room temperature until compound **17** disappeared by TLC control. Then, the solvent was vacuum-evaporated and the residue was redissolved in ethyl acetate. The organic layer was washed with 2 N HCl and brine, dried over Na_2SO_4, filtered and evaporated till dryness, affording a crude product, which was purified through column chromatography over silica gel.

4.1.8.1. 2-Acetamido-3-chloro-5,5(8,8)-dimethyl-5,6,7,8-tetrahydroa nthracene-1,4-diones ***18a*** (***18b***).

Following the above procedure, treatment of **17** with 30% NH_4OH for 1 h, gave a reaction product which was acetylated at 0 °C with Ac_2O–H_2SO_4 (6 mL taken from a previously solution prepared with 50 mL of Ac_2O and 0.15 mL of H_2SO_4) for 30 min. The reaction was quenched with ice and extracted with EtOAc. The organic layer was washed with satd aq $NaHCO_3$, and brine, dried over anhydrous Na_2SO_4 and vacuum-evaporated yielding a crude product, which was purified by column chromatography (eluent: Hex/EtOAc 8:2) to afford: (a) 10 mg (8%) of **18a**. M.p. 75–80 °C. ^{1}H NMR ($CDCl_3$) δ: 1.33 (s, 6H, H-11,12), 1.70–1.83 (m, 4H, H-6,7), 2.89 (t, 2H, J = 6.2, H-8), 7.82 (s, 1H, H-10), 8.05 (s, 1H, H-9), 2.29 (s, 3H, Ac). ^{13}C NMR δ: 19.1 (C7), 31.1 (C8), 31.4 (C-11,12), 34.9 (C5), 38.4 (C6), 126.4 (C10), 127.3 (C4a), 128.1 (C9), 129.4 (C9a), 138.8 (C2), 143.8 (C8a) 154.2 (C10a), 177.8 (C1), 180.0 (C4), 24.2 and 166.7 (Ac). IR (ν, cm^{-1}): 3319, 1669, 1597, 1558, 1516, 1344, 1248, 828. IR (ν, cm^{-1}): 3319, 1673, 1597, 1493, 1336, 1291, 750. (b) 34 mg (30%) of **18a**/**18b**. (c) 6 mg (5%) of **18b**. M.p. 140–144 °C. ^{1}H NMR ($CDCl_3$) δ: 1.33 (s, 6H, H-11,12), 1.70–1.83 (m, 4H, H-6,7), 2.88 (t, 2H, J = 6.2, H-5), 7.76 (s, 1H, H-9), 8.11 (s, 1H, H-10), 2.28 (s, 3H, Ac). ^{13}C NMR δ: 19.1 (C6), 31.2 (C5), 31.4 (C-11,12), 34.8 (C8), 38.4 (C7), 124.1 (C4a), 125.9 (C9), 128.6 (C9a,10), 133.4 (C3), 139.0 (C2), 144,7 (C10a) 154.3 (C8a), 177.8 (C1), 180.0 (C4), 24.2 and 166.6 (Ac). IR (ν, cm^{-1}): 3323, 1673, 1597, 1488, 1335, 1295, 1230, 745. HRMS (ES, M + Na): calcd: 354.0867, found: 354.0853.

4.1.8.2. 3-Chloro-2-ethylamino-5,5(8,8)-dimethyl-5,6,7,8-tetrahydroanthracene-1,4-diones ***19a*** (***19b***).

Following the above procedure, treatment of **17** with ethylamine for 7 days at 80 °C and 2 more weeks at 100 °C, gave out, after column chromatography (eluent: Hex/EtOAc 8:2), the 1:1 mixture of regioisomers **19a**/**19b** (viscous oil, 30%). ^{1}H NMR ($CDCl_3$) δ: 1.31 (s, 6H, H-11,12), 1.68–1.80 (m, 4H, H-6,7), 2.82 (m, 2H, H-8), 7.66/7.96 (s, 1H, H-9), 8.06/7.77 (s, 1H, H-10), 1.30 (m, 3H, Et-NH), 3.88 (m, 2H, Et-NH). ^{13}C NMR δ: 19.1 (C7/6), 30.9 (C8/5), 31.5 (C-11,12), 34.5 (C5/8), 39.9 (C6/7), 125.7 (C10/9), 126.9 (C9a), 127.8 (C9/10), 130.9/126.8 (C4a), 141.5/151.1 (C8a), 144.2 (C3) 154.2/144.8 (C10a), 177.2 (C1), 180.4 (C4), 16.4, 38.6 (Et-NH). IR (ν, cm^{-1}): 1673, 1595, 1561, 1513, 1337, 1294,1255.

4.1.8.3. 3-Chloro-2-(3,4-dimethylanilino)-5,5(8,8)-dimethyl-5,6,7,8-tetrahydroanthracene-1,4-diones ***20a*** (***20b***).

Following the above procedure, treatment of **17** with 3,4-dimethylaniline for 24 h under reflux, gave the 1:1 mixture of regioisomers **20a**/**20b** (viscous oil, 84%). ^{1}H NMR ($CDCl_3$) δ: 1.32 (s, 6H, H-11,12), 1.66–1.82 (m, 4H, H-6,7), 2.82 (m, 2H, H-5, 8), 7.70/8.08 (s, 1H, H-9), 8.09/7.78 (s, 1H, H-10), 2.21 (s, 6H, Me-Ph), 6.78 (m, 1H, Ph-NH), 6.82 (bs, 1H, Ph-NH), 7.03 (d, 1H, J = 7.7, Ph-NH). ^{13}C NMR δ: 19.1 (C7/6), 30.9 (C8/5), 31.4 (C-11,12), 34.6/30.9 (C5), 38.6 (C6/7), 127.0/129.7 (C9a), 128.0 (C9), 129.4 (C10), 130.5/129.7 (C4a), 142.1/151.7 (C8a), 154.3/144.9 (C10a), 177.8 (C4), 180.6 (C1), 19.4, 113.5, 121.7, 125.4, 125.9, 134.1, 135.3, 136.7, 141.6, 141.9 (C2, C3, Ph-NH). IR (ν, cm^{-1}): 3307, 1669, 1593, 1561, 1510, 1337, 1287, 1253, 877. HRMS (ES, M + H): calcd: 394.1568, found: 394.1588.

4.1.8.4. 3-Chloro-2-(4-methoxyanilino)-5,5(8,8)-dimethyl-5,6,7,8-tetrahydroanthracene-1,4-diones ***21a*** (***21b***).

Following the above procedure, treatment of **17** with 4-methoxyaniline for 24 h under reflux, gave the 1:1 mixture of regioisomers **21a**/**21b** (viscous oil, 91%). ^{1}H NMR ($CDCl_3$) δ: 1.33 (s, 6H, H-11,12), 1.75–1.84 (m, 4H, H-6,7), 2.86 (m, 2H, H-5, 8), 7.75/8.04 (s, 1H, H-9), 8.10/7.81 (s, 1H, H-10), 3.82 (s, 3H, MeO-Ph), 6.85, 7.03 (AB system, J = 8.6, Ph-NH). ^{13}C NMR δ: 19.1 (C7/6), 30.9 (C8/5), 31.3 (C-11,12), 34.9/30.9 (C5), 38.5 (C6/7), 125.7 (C10), 126.9/127.7 (C9a), 128.0 (C9), 130.4/129.6 (C4a), 142.0/151.5 (C8a), 154.1/144.8 (C10a), 177.5 (C4), 180.3 (C1), 55.4 (s, 3H, MeO-Ph), 113.5, 126.2, 130.6, 142.0, 157.3 (C2, C3, Ph-NH). IR (ν, cm^{-1}): 3306, 1670, 1593, 1513, 1337, 1289, 1245, 1036, 895. HRMS (ES, M + Na): calcd: 418.1180, found: 418.1176.

4.1.8.5. 3-Chloro-2-(3,4-dimethoxyanilino)-5,5(8,8)-dimethyl-5,6,7,8-tetrahydroanthracene-1,4-diones ***22a*** (***22b***).

Following the above procedure, treatment of **17** with 3,4-dimethoxyaniline for 24 h under reflux, gave the 1:1 mixture of regioisomers **22a**/**22b** (viscous oil, 95%). ^{1}H NMR ($CDCl_3$) δ: 1.33 (s, 6H, H-11,12), 1.68–1.84 (m, 4H, H-6,7), 2.87 (m, 2H, H-5, 8), 7.75/8.04 (s, 1H, H-9), 8.11/7.81 (s, 1H, H-10), 3.85(s, 3H, MeO-Ph), 3.88 (s, 3H, MeO-Ph), 6.63/6.64 (s, 1H, Ph-NH), 6.65 (m, 1H, Ph-NH), 6.80 (d, 1H, J = 8.1, Ph-NH). ^{13}C NMR δ: 19.0 (C7/6), 30.1/34.5 (C8), 31.4 (C-11,12), 34.8/30.8 (C5), 38.5 (C6/7), 125.8 (C10/9), 126.8/127.6 (C9a), 128.0 (C9/10), 130.4/129.6 (C4a), 142.0/151.6 (C8a), 154.3/144.9 (C10a), 177.7 (C4), 180.4 (C1), 55.9 (MeO-Ph), 109.0/109.1, 110.4, 113.7, 116.9/117.0, 130.7, 141.8, 147.1, 148.5 (C2, C3, Ph-NH). IR (ν, cm^{-1}): 3307, 1669, 1593, 1512, 1461, 1337, 1238, 1028, 870. HRMS (ES, M + H): calcd: 426.1466, found: 426.1480.

4.1.8.6. 3-Chloro-2-(3,4,5-trimethoxyanilino)-5,5(8,8)-dimethyl-5,6,7,8-tetrahydroanthracene-1,4-diones ***23a*** (***23b***).

A 3:1 mixture of **13a**/**13b** (0.29 mmol) was treated with 3,4,5-trimethoxyaniline (0.31 mmol) in dioxane (10 mL) for 4 days at 120 °C. Treatment of the reaction mixture as described in the general procedure afforded a reaction product that gave, after column chromatography (eluent: Cl_2CH_2/EtOAc 97:3), (a) 42% of unreacted **13a**/**13b mixture**, (b) 18%

of the 1:9 mixture of **23a**/**23b** as a viscous oil. ^{1}H NMR ($CDCl_3$) δ: 1.34 (s, 6H, H-11,12), 1.71-1.83 (m, 4H, H-6,7), 2.90 (t, 2H, $J = 6.2$, H-5), 8.06 (s, 1H, H-9), 7.84 (s, 1H, H-10), 3.84, 3.86 (s, 9H, MeO-Ph), 6.31 (s, 2H, Ph-NH). ^{13}C NMR δ: 19.0 (C7/6), 31.3 (C5), 31.5 (C-11,12), 34.6 (C8), 38.5 (C6/7), 126.0 (C10/9), 128.2 (C9/10), 129.7 (C9a, 4a), 133.4 (C3), 141.6 (C10a), 145.1 (C2), 151.9 (C8a), 177.8 (C1), 180.5 (C4), 56.3, 61.1 (MeO-Ph), 102.3, 133.4, 136.0, 152.0 (Ph-NH). IR (ν, cm^{-1}): 3316, 1668, 1591, 1560, 1505, 1234, 1128, 735. HRMS (ES, M + H): calcd: 456.1577, found: 456.1543.

*4.1.8.7. 2,3-Diethoxy-5,5-dimethyl-5,6,7,8-tetrahydroanthracene-1,4-dione **24**.* Following the above general procedure, treatment of **17** with 2-aminopyridine and K_2CO_3 in ethanol for 14 h under reflux gave, after column chromatography (eluent: Hex/EtOAc 8:2), the quinone **24** (20%) as a viscous oil. ^{1}H NMR ($CDCl_3$) δ: 1.31 (s, 6H, H-11,12), 1.68–1.78 (m, 4H, H-6,7), 2.85 (t, 2H, $J = 6.2$, H-8), 7.69 (s, 1H, H-9), 7.98 (s, 1H, H-10), 1.37 (t, 3H, $J = 6.8$, EtO), 4.34 (m, 2H, EtO). ^{13}C NMR δ: 19.0 (C7), 31.0 (C8), 31.4 (C-11,12), 34.7 (C5), 38.6 (C6), 125.0 (C10), 127.2 (C9), 128.1 (C9a), 128.9 (C4a), 143.1 (C2,3), 147.5 (C8a), 152.6 (C10a), 182.4 (C1), 187.6 (C4), 15.7, 69.6 (EtO).

4.2. Biological assays

Stock solutions of compounds were prepared in dimethyl sulfoxide (DMSO, Sigma) and frozen at −70 °C until required. The final concentration of DMSO in biological assays was 0.05%. Cell controls with DMSO at 0.05% were used.

4.2.1. Antifungal assay

Minimum inhibitory concentrations (MICs) of 1,4-naphthoquinone and 1,4-anthracenedione derivatives were determined following the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing (AFST-EUCAST) reference procedure for *Candida* species [22] and the Clinical and Laboratory Standards Institute M38-A protocol for filamentous fungi [23]. MICs for dermatophytes were determined using a CLSI M38-A modified method [24]. A preliminary screening of all the compounds included in Scheme 1 was dispensed into 96-well flat-bottom microdilution plates, in triplicate, at concentrations of 32 μg/mL (around 100 μM). The active compounds were then serially diluted (32, 16, 8, 4, 2 μg/mL) and re-assayed to determine the corresponding MIC.

The yeasts *C. parapsilosis* (ATCC 22019), *C. krusei* (ATCC 6258), *C. tropicalis* (CECT 11901), *C. albicans* (ATCC 10231), *C. albicans* (ATCC 90028), *C. lusitaniae* (ATCC 200951), *C. tropicalis* (ATCC 200956), along with the filamentous fungi *A. fumigatus* (ATCC 204305), *A. flavus* (ATCC 204304), *A. terreus* (CDC 317), *A. niger* (ATCC 16404), *F. oxysporum* (ATCC 48112), and the dermatophytes *Trichophyton rubrum* (ATCC 28188) and *T. mentagrophytes* (ATCC 24198) were used to evaluate antifungal activity.

Amphotericine B (AMB; Sigma Chemical Co, MO, USA), itraconazole (ITZ; Sigma Chemical Co, MO, USA) and terbinafine (TRB; Recalcine Laboratories, Santiago de Chile), at a range of 0.031–16 μg/mL, were used as positive controls for non-dermatophyte filamentous fungi, *Candida* spp. and dermatophytes, respectively. The inoculum sizes were $0.5–2.5 \times 10^5$ and $0.4–5 \times 10^4$ CFU/mL for yeasts and filamentous fungi, respectively. For the AFST-EUCAST method, the Minimum Inhibitory Concentrations (MICs) were determined after 24 h of incubation at 35 °C by means of spectrophotometric reading at 405 nm and were defined as the lowest concentrations that resulted in a 90% or higher inhibition of growth. For the CLSI M38-A method, MICs were determined after 48 h of incubation at 35 °C for *Aspergillus* spp. and at 28 °C for *F. oxysporum*. Dermatophyte activity was determined after 6 days of incubation at 28 °C. MICs were defined as the lowest concentrations that resulted in a 90% of inhibition of visible growth. MICs results were expressed as geometric mean (GM) of duplicates of each compound tested three different times against each fungi in different assays.

4.2.2. Time-kill assay

The *in vitro* pharmacodynamic profile of **13a** and **17a** with *C. parapsilosis* (ATCC 22019) was determined by the method of time-kill curves described by Wittebolle et al. [25]. 100 μL of initial inoculum ranging from $1–5 \times 10^5$ CFU/mL was seeded in 96-well microplates with 100 μL of each compound solution at concentrations of 1/2, 1, 2 and 4 × MIC. The samples were incubated at 35 °C with agitation. At 0, 2, 6, 12 and 24 h, and spectrophotometric measures were taken at 405 nm. Time-kill curves with ITZ and AMB were used as fungistatic and fungicidal controls. Experiments were carried out in duplicate in two separate experiments. Time-kill curves were constructed by plotting the measured optical density as a function of time (hours).

4.2.3. Cytotoxicity assay

Cercopithecus aethiops African green monkey kidney cells (Vero cell line ATCC CCL-81) were used. The cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% FBS, 100 units/mL of penicillin, 100 μg/mL of streptomycin, 20 mg/mL of glutamine, 0.14% $NaHCO_3$ and 1% each of non-essential amino acids and vitamin solution. The cytotoxicity of quinones that were active against any fungi, and of the drugs itraconazole, terbinafine and amphotericine B, was examined *in vitro* using an MTT (dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (Sigma, New Jersey, USA) assay as described by Betancur-Galvis et al. [26]. Vero cell monolayers were trypsinised and washed with culture medium, and then plated at 1.25×10^4 cells per well in a 96-well flat-bottomed plate. After 24 h of incubation, each diluted compound was added to the appropriate wells and the plates were further incubated for 48 h at 37 °C in a humidified incubator with 5% CO_2. The minimal dilution of compound that induced 50% growth inhibition of the cells was expressed as Inhibitory Concentration 50 (IC_{50}). The IC_{50} values for each compound were obtained through a linear regression analysis of the dose–response curves generated from the absorbance data with the statistical package R (Development Core Team, Vienna, Austria, 2008). IC_{50} values were expressed as the Mean ± Standard Deviation (M ± SD) of two independent experiments done in quadruplicate. Selectivity indexes (SI) were calculated as the ratio of Vero IC_{50} and MIC values.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.ejmech.2013.06.018. These data include MOL files and InChiKeys of the most important compounds described in this article.

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