European Journal of Medicinal Chemistry 108 (2016) 134–140

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Research paper

Synthesis and antimalarial activity of quinones and structurally-related oxirane derivatives

Paula F. Carneiro [a], Maria C.R.F. Pinto [a], Roberta K.F. Marra [b], Fernando de C. da Silva [b], Jackson A.L.C. Resende [c], Luiz F. Rocha e Silva [d], Hilkem G. Alves [e], Gleyce S. Barbosa [e], Marne C. de Vasconcellos [e], Emerson S. Lima [e], Adrian M. Pohlit [d], Vitor F. Ferreira [b, *]

[a] Universidade Federal do Rio de Janeiro, Instituto de Pesquisas de Produtos Naturais, 21944-970 Rio de Janeiro, RJ, Brazil
[b] Universidade Federal Fluminense, Instituto de Química, Departamento de Química Orgânica, 24020-150 Niterói, RJ, Brazil
[c] Universidade Federal Fluminense, Instituto de Química, Departamento de Química Inorgânica, 24020-150 Niterói, RJ, Brazil
[d] Instituto Nacional de Pesquisas da Amazônia, Laboratório de Princípios Ativos da Amazônia, Av. André Araújo, 2936 Manaus, Brazil
[e] Universidade Federal do Amazonas, Faculdade de Ciências Farmacêuticas, 69010-300 Manaus, AM, Brazil

ARTICLE INFO

Article history:
Received 3 October 2015
Received in revised form
10 November 2015
Accepted 13 November 2015
Available online xxx

Keywords:
Naphthoquinones
Lapachone
Epoxides
Malaria
Infectious disease

ABSTRACT

A series of eighteen quinones and structurally-related oxiranes were synthesized and evaluated for *in vitro* inhibitory activity against the chloroquine-sensitive 3D7 clone of the human malaria parasite *Plasmodium falciparum*. 2-amino and 2-allyloxynaphthoquinones exhibited important antiplasmodial activity (median inhibitory concentrations (IC_{50}) < 10 μM). Oxiranes **6** and **25**, prepared respectively by reaction of α-lapachone and tetrachloro-*p*-quinone with diazomethane in a mixture of ether and ethanol, exhibited the highest antiplasmodial activity and low cytotoxicity against human fibroblasts (MCR-5 cell line). The active compounds could represent a good prototype for an antimalarial lead molecule.

1. Introduction

Naphthoquinones and derivatives are important due to their demonstrated activity against several pathogenic microorganisms such as *Trypanosoma cruzi* (protozoan that causes American leishmaniasis or Chagas' Disease) [1–3], *Plasmodium falciparum* and *Plasmodium berghei* (protozoans that cause severe human and rodent malaria, respectively) [4,5], and human immunodeficiency virus (HIV) [6] and insects such as the mosquito species *Aedes aegypti* [7]. The naphthoquinones lapachol (**1**) and β-lapachone (**2**) are isolated from South American trees of the genus *Tabebuia* that have been used traditionally by indigenous people to treat many parasitic infections, including malaria [8].

Compound **1** has been used in the past as a co-adjuvant in the treatment of malignant solid tumors but its use was discontinued due to concerns about its toxicity [9,10]. Later, hydroxypropyl-β-cyclodextrin-encapsulated β-lapachone (**2**) was named ARQ501 (ArQule Inc.) and investigated in a phase II clinical [11] in combination with Taxol® or gemcitabine [12,13]. An important variation, the water-soluble prodrug of **2**, ARQ 761 (Code C99146, structure not reported) [14], is currently in clinical trial [15] and requires less solvent in the formulation for intravenous administration and consequently less hemolytic anemia is associated with administration of ARQ 761.

The antitumor mechanism of action of quinones is based on redox cycling that represents a cyclic process of reduction of a compound, followed by (auto)-oxidation of the reaction product and generation of reactive oxygen species (ROS) [16]. Conversely, the reduction reaction of the quinone moiety via acceptance of one or two electrons followed by oxidation by oxygen cause the formation of ROS. Also, it is known that reactive oxygen species formed in excess in the intracellular environment are able to activate the intrinsic pathway of apoptosis by the permeabilization of mitochondria and activation of caspase 9. This may be the mechanism responsible for the cytotoxic action of these substances, both on micro-organisms, as well as on tumor cells [17].

* Corresponding author.
E-mail address: cegvito@vm.uff.br (V.F. Ferreira).

http://dx.doi.org/10.1016/j.ejmech.2015.11.020
0223-5234/

The general structures of compounds **1** and **2** have been widely exploited in medicinal chemistry as prototypes for new candidates against cancer and parasitic infections. Atovaquone (**3**) is synthesized starting from **1** and is used as a therapeutic drug in the treatment or prevention of mild cases of infection by *Plasmodium vivax* (in combination with proguanil) [18–20], although there have been cases of resistance reported [21]. It also can be used for pneumocystosis, toxoplasmosis and babesiosis (usually in combination with azithromycin). Two similar compounds, buparvaquone (**4**) and parvaquone (**5**), are pharmaceuticals for veterinary use (Fig. 1).

Substitution of a carbonyl of naphthoquinone by another group has generated new compounds with important biological activities. Also, epoxynaphthoquinones distributed in nature and the natural 6α-acetoxygedunin (**8**) exhibit interesting biological activities [22]. Substance **6** was synthesized from α-lapachone and was the only oxirane that showed high trypanocidal activity with excellent minimal cytotoxicity in VERO cells. Comparatively, oxirane **7** exhibited similar trypanocidal activity to β-lapachone (**2**). The major mechanism of trypanocidal action of naphthoquinones is by inducing intracellular damage caused by oxidative stress due to the quinonoid moiety. Our group synthesized compounds **6** [23] and **7** [24] and found that they inhibited the serine proteinase of *T. cruzi* leading to interference in the establishment of infections [25–27]. Recently we have shown that oxirane **6** has leishmanicidal effects on *Leishmania (Viannia) braziliensis* and *L. amazonensis* [28]. This compound was able to cause death of promastigote and amastigote forms of *Leishmania* spp. after 3 h of exposure [29].

Recently we reported naphthoquinones with activity against *P. falciparum* [30,31]. These compounds act via ROS production. However, oxiranes act by inhibiting proteinase enzymes (Fig. 2). Protozoans comprise a very diverse group of unicellular eukaryotic organisms [32], which include *Plasmodium*, *Trypanosoma* and *Leishmania* parasites, among others. Based on the good biological activity obtained with oxiranes derived from naphthoquinones against *T. cruzi* and *Leishmania* spp. we decided to synthesize a series of oxiranes and to investigate their activity against the chloroquine-sensitive 3D7 clone of the human malaria parasite *P. falciparum*. It was assumed that these oxiranes would have antimalarial activity. The purpose of this study was to prepare oxirane derivatives of naphthoquinones and test them for antiplasmodial activity and cytotoxicity as a means to search for new antimalarial compounds. Herein we report our findings on the antiplasmodial and cytotoxic activity of these naphthoquinone-derived oxiranes.

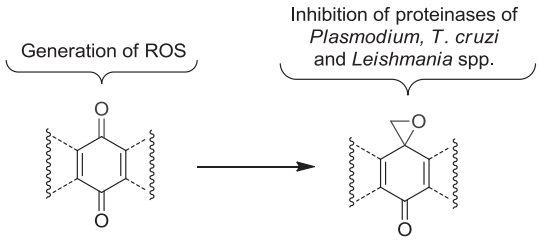


Fig. 2. General rationale used in this study.

2. Results and discussion

The substances used in the screening against the chloroquine-sensitive 3D7 clone of *P. falciparum* were prepared in one step from the appropriate quinone by adding a freshly prepared solution of diazomethane in ether (Scheme 1). The reaction proceeds by nucleophilic attack of diazomethane on the more electrophilic carbonyl of quinones **9**–**17** and fluorenone (**18**) yielding oxiranes **6**, **7**, **19**–**23**, **25** and the unexpected non-oxirane compounds **24**, **26** and **27**. In general, reaction occurred at carbonyl C-1 due to the higher electrophilicity provided by the adjacent heteroatom substituent.

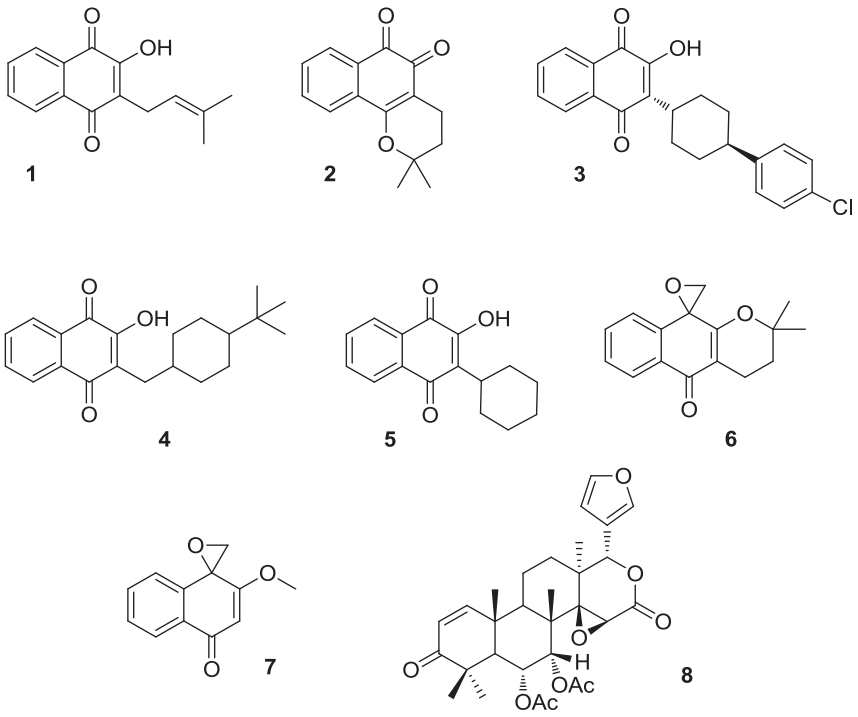
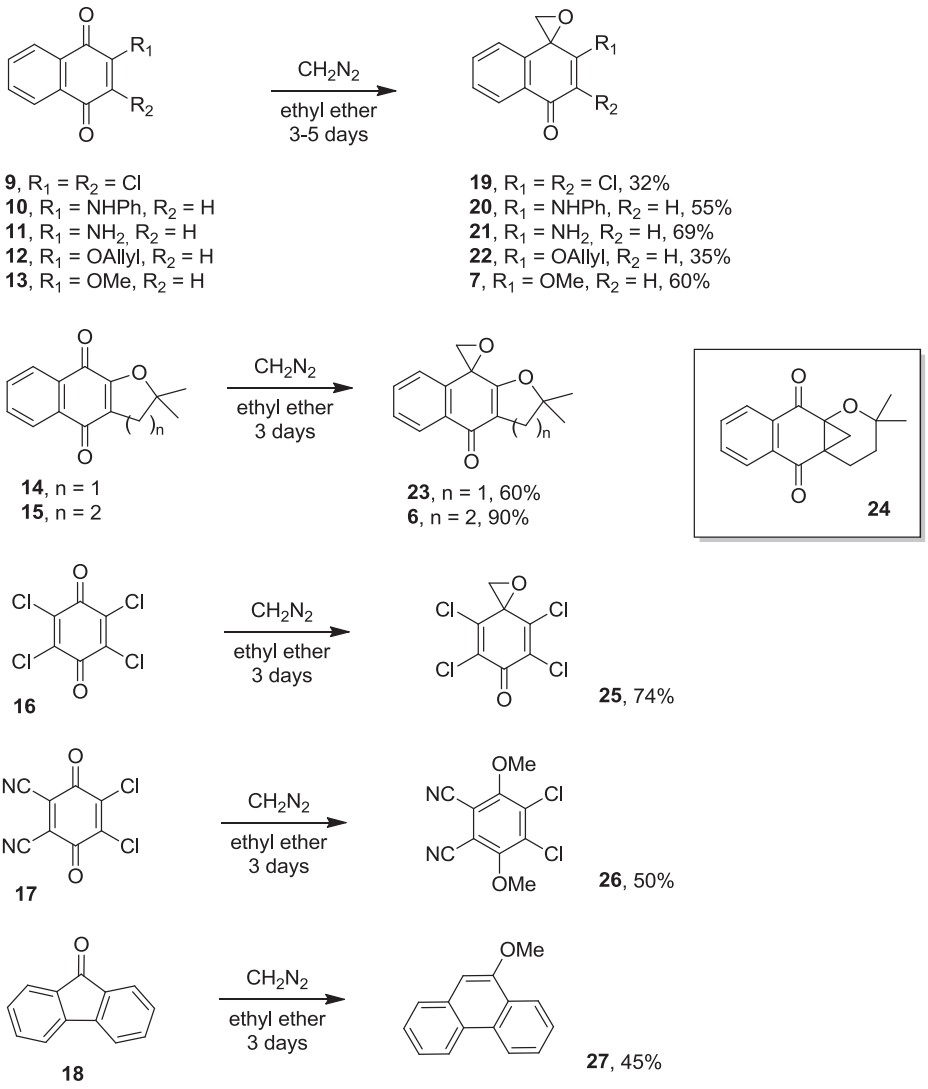


Fig. 1. Natural and synthetic substances with activity against parasitic infections.



Scheme 1. General reactions used for preparing the oxiranes (and other products).

Although substance **23** has been described previously, the NMR data presented in the literature for this compound was not consistent with this structure [33]. The hydrogen chemical shifts less than δ_H 3.00 ppm for CH_2 of the published compound clearly show that reaction (cyclopropanation) occurred at the double bond to form compound **24**. It is noteworthy that the synthesis of **24** and its spectral characterization have been published previously [24]. Therefore, we established that treatment of **14** or **15** with diazomethane in ethyl ether can provide **23** (δ_H 3.70 and 3.44 ppm for the oxirane H atoms) or **24** (δ_H 1.95 and 1.73 ppm for the cyclopropane H atoms) [24], respectively. The correct structures are reported herein. These structures were confirmed by X-ray crystallography and ORTEP projections are presented in Fig. 3.

Reaction of **17** with diazomethane yielded product **26**. In the ^1H NMR spectrum of **26** the two characteristic doublets of an epoxy group were not observed. Only a singlet was observed at δ_H 4.15 ppm due to the CH_3 groups. In the ^{13}C NMR spectrum (APT type) at δ_C 62.7 ppm and the high resolution mass spectrum exhibited a sodium ion adduct [M+Na^+] at *m/z* 278.9700 consistent with the molecular formula $C_{10}H_6Cl_2N_2O_2$ of compound **26** whose structure was confirmed by X-ray diffraction (Fig. 3).

The reaction of diazomethane with fluorenone (**18**) did not provide the desired oxirane product. Instead, after initial nucleophilic attack by diazomethane at the carbonyl group, ring expansion occurred to form 9-phenanthrol which in turn was methylated by diazomethane to form methyl 9-phenanthryl ether (**27**). In the ^1H and ^{13}C NMR (APT type) spectra signals related to a CH_2 group were not observed. Instead a singlet was observed at δ_H 4.0 that is characteristic of a methoxy group. In the high resolution mass spectrum a hydrogen ion adduct [M+H^+] *m/z* 209.0954 was observed that is consistent with the molecular formula $C_{15}H_{12}O$ of compound **27**. Similar to the result with fluorenone (**18**), quinone **17** did not produce the desired oxirane in the reaction with diazomethane and instead afforded dimethylated derivative **26** whose reaction mechanism has not been elucidated.

A series of quinones **9**–**17** were used for preparing the oxiranes **19**–**25**. The inhibitory concentrations of these compounds against *P. falciparum* 3D7 strain and median toxicities against MRC-5 cells (IC_{50}) were determined and the results are presented in Table 1.

Structurally diverse oxiranes **6**, **19** and **25** were among the most active antiplasmodial compounds and were more active than the naphthoquinono-3,4-dihydro-2*H*-pyran **15**, dichloronaphthoquin

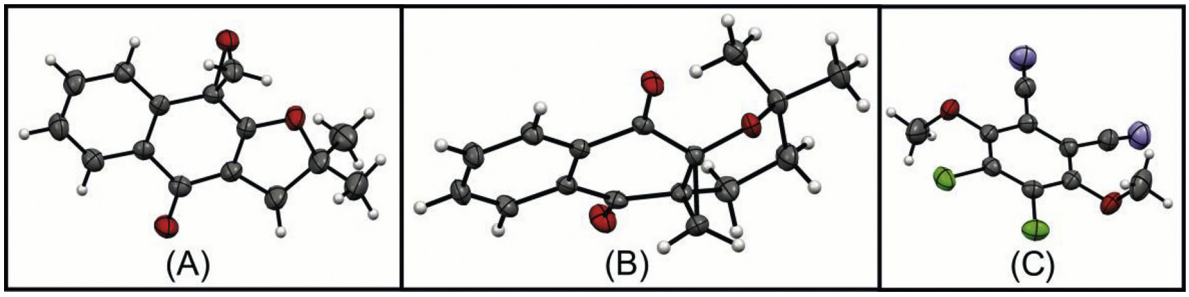


Fig. 3. ORTEP representation (50% probability displacement ellipsoid) of oxirane **23** (A) and cyclopropane **24** (B) derivatives and compound **26** (C). Hydrogen, carbon, nitrogen, chlorine and oxygen atoms are represented in white, gray, blue, green and red, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Median inhibitory concentrations (IC_{50}) against *Plasmodium falciparum* 3D7 strain, toxicities to MRC-5 cells and selectivity indices (SI) of quinones and oxiranes.

Compound	IC_{50} (μM) *P. falciparum* 3D7	IC_{50} (μM) MRC-5 cells	Selectivity index, SI[a]
Naphthoquinones			
9	13.9 (12.6–16.5)	98.2 (90.7–107)	7.2
10	6.30 (4.41–7.42)[b]	>100	>15
11	5.14 (3.18–8.66)[b]	322 (153–681)	63
12	5.18 (4.29–6.77)[b]	64.8 (60.2–64.4)	12
13	>50	5.2 (4.5–5.9)	<0.10
14	>50	56.1 (51.5–60.9)	<1.1
15	>50	>100	ND
Quinones			
16	22.4 (19.5–29.0)	>100	>4.4
17	>50	>100	ND
Oxiranes			
19	6.22 (5.43–8.21)[b]	92.5 (26.1–331)	15
20	>50	135 (112–164)	<2.7
21	>50	>100	ND
22	>50	26.4 (23.7–29.5)	<0.53
7	>50	116 (90.0–148)	<2.3
23	>50	55.7 (45.8–68.1)	<1.1
6	3.71 (3.12–4.60)[b]	>100	>27
25	3.95 (3.58–5.13)[b]	368 (302–449)	93
26	>50	>100	ND
Chloroquine (diphosphate)	0.18 (0.14–0.22)	ND	ND

[a] **SI** = $IC_{50(cytotoxicity)} \div IC_{50(antiplasmodial)}$.
[b] Compounds active against *P. falciparum* ($1 < IC_{50} < 10$ μM) – ND = not determined.

one **9** and tetrachloro-*p*-benzoquinone (**16**) from which these oxiranes were respectively synthesized. Thus, specific improvement of antiplasmodial action may be attributable to the presence of the oxirane moiety in this small group of structurally diverse compounds. However, the oxirane moiety is not in general responsible for enhanced antiplasmodial activity. Thus, the 2-aminonaphthoquinones **10** and **11** and 2-allyloxynaphthoquinone (**12**) were among the most active antimalarial compounds yet their corresponding oxirane derivatives **20**–**22** exhibited no antiplasmodial activity. Thus, among quinones, naphthoquinones having a 2-amino, 2-aminoaryl or 2-oxyalkyl group may be closer to an optimal antiplasmodial structure than other quinones studied herein. Also, it is not clear what the structural requirements are for an oxirane to exhibit enhanced antiplasmodial activity compared to its quinone precursor. None of the compounds tested was more active than chloroquine diphosphate used as drug standard (positive controls). While screening for new antimalarial substances it is normally assumed that active compounds will have IC_{50} values <10 μM [34–36].

The most active compound in this series was oxirane **6** (IC_{50} 3.71 μM), *i.e.* the adduct derived from α-lapachone. Importantly, this compound was also active against *T. cruzi* [23] where it specifically inhibited serine proteinases [25–27]. Also, this compound presented high leishmanicidal activity against *Leishmania (Viannia) braziliensis* and *Leishmania (Leishmania) amazonensis* [28] wherein it inhibited serine proteinases of these parasites [29]. The mechanism of action of oxirane **6** in *P. falciparum* may also be inhibition of the serine proteinase of the parasite. The second most active compound was oxirane **25** that was derived from tetrachlorobenzoquinone **16**. The latter compound has more electrophilic centers that are able to react with an amino group. Both compounds exhibited good selectivity indices, which is an important indication that both are low toxicity antiplasmodial agents.

Aminonaphthoquinones **9**–**11** were quite active with low cytotoxicity leading to high selectivity indices. Oxirane **19** exhibited more antiplasmodial activity and had a higher selectivity index than its precursor **9**.

The quinone **17** did not produce the desired oxirane in the reaction with diazomethane and in its place afforded the dimethylated derivative **26** whose reaction mechanism has not been elucidated.

3. Conclusion

In summary, a series of oxiranes was synthesized and analysis

of the antiplasmodial properties of these oxiranes and their precursors was performed. Optimization of naphthoquinone antiplasmodial activity should be possible in future work by varying the substituents at the 2 and 3 positions. Similarly, the oxirane moiety provided greater antiplasmodial activity *vis-à-vis* that of a few structurally diverse quinone precursors. Two oxiranes exhibited good antiplasmodial activity and high selectivity and can be considered for the development of new antimalarial agents with the initial step being the optimization of oxirane structure-activity relationships. The antiplasmodial mechanism of action of these oxiranes is unknown but may be related to the inhibition of *P. falciparum* serine proteinase based on the observation that oxiranes act on this target of *Trypanosoma cruzi* and *Leishmania (Viannia) braziliensis* and *Leishmania (Leishmania) amazonensis*.

4. Experimental section

4.1. Chemistry

Melting points were obtained on a Thomas Hoover apparatus (Philadelphia, USA) and are uncorrected. Analytical grade solvents were used. Column chromatography was performed on silica gel (Acros Organics 0.035–0.070 mm, pore diameter *ca.* 6 nm) and the reactions was monitored by analytical thin-layer chromatography was performed with silica gel plates (Merck, TLC silica gel 60 F254), and the plots were visualized using UV light. Infrared spectra were recorded on a Shimadzu IR Prestige-21 FTIR spectrometer (Kyoto, Japan). 1H and 13C NMR were recorded at room temperature using a VNMRSYS-500 or a Varian MR 400 instrument, in the solvents indicated, with TMS as internal standard. Chemical shifts (δ) are given in ppm and coupling constants (*J*) in Hertz (Hz). Low resolution mass spectra were obtained using Shimadzu GCMSQP2010 Plus and GCMS-QP5000 (70 eV) gas chromatograph-mass spectrometer (Tokyo, Japan) systems with a DB-5MS column. High resolution mass spectra (electrospray ionization) were obtained using a Waters QTOF Micro (Manchester, UK) mass spectrometer. Ions were described in mass-to-charge units (*m/z*) and relative abundance was expressed as a percentage of the base peak intensity. Diazomethane was prepared by reaction of Diazald® (*N*-methyl-*N*-nitroso-*p*-toluenesulfonyl nitrosamide, Steinheim, Germany) with potassium hydroxide.

The quinones **9**, **16**, **17** and compound **18** are commercially available (Sigma–Aldrich, Brazil). The naphtoquinones **10**, **11**, **12**, **13**, **14** and **15** were prepared by standard procedures as described in the literature [37–42]. Oxiranes **6**, **7**, **19**, **22**, **26** and **27** were previously synthesized [23,24,43,44], but proper comparison of NMR spectra was not possible due to lack of spectral data for compounds **20** and **25** in the literature.

The structure of compounds **23**, **24** and **26** were confirmed using single crystal X-ray diffraction experiments. The analysis for **23** and **24** were performed on an Oxford Gemini A-Ultra diffractometer at LabCri/Federal University of Minas Gerais (UFMG), Brazil and for **26** was performed on a Kappa CCD diffractometer at LARE-DRX (Universidade Federal Fluminense), Brazil. The structures were solved by direct methods and refined by full-matrix least-squares on F^2 using the SHELX-2013 package (1). All non-hydrogen atoms were refined with anisotropic displacement parameters. Hydrogen atoms bonded to C atoms were placed at their idealized positions using standard geometric criteria [45].

4.2. General method for the preparation of oxiranes *19*–*27*

The appropriate quinone (1 mmol) was dissolved in 20 mL of a 3:1 mixture of ethyl ether and ethanol. After dissolution of the quinone was complete, diazomethane in ethyl ether (10 mL) was added. The reaction was left at room temperature for 3–5 days. The solvent was evaporated under reduced pressure and the crude product was purified on a column of silica gel by eluting with a gradient of increasing polarity of ethyl acetate/hexane [24].

2,3-Dichloro-4*H*-spiro[naphthalene-1,2′-oxirane]-4-one (**19**) was obtained as a white solid in 52% yield. m.p. 149–150 °C; FT-IR ν_{max} (cm^{-1}, KBr): 3103, 3074, 3045, 2924, 1664, 1595, 1568, 1456, 1325, 1282, 1238, 1165, 1141, 904, 866, 840, 810, 756; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.23 (*d*, *J* = 7.7 Hz, 1H), 7.66 (*t*, *J* = 8.1 Hz, 1H), 7.55 (*t*, *J* = 7.7 Hz, 1H), 7.31 (*d*, *J* = 8.1 Hz, 1H), 3.77 (*d*, *J* = 6.1 Hz, 1H), 3.37 (*d*, *J* = 6.1 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 176.1 (C), 148.4 (C), 137.7 (C), 135.2 (C), 133.8 (CH), 131.0 (C), 129.4 (CH), 127.9 (CH), 123.4 (CH), 58.2 (CH_2), 55.2 (C); MS (70 eV) *m/z* (%): 239 (16, M+), 212 (41), 205 (12), 184 (33), 182 (52), 177 (83), 147 (100), 135 (7), 113 (34), 99 (15), 97 (19), 74 (22), 63 (24), 50 (13); HR-ESI-MS *m/z* calcd. for $C_{11}H_7Cl_2O_2$ $[M+H]^+$: 240.9818. Found: *m/z* 240.9817. Δ = 0.41 ppm.

2-(Phenylamino)-4*H*-spiro[naphthlene-1,2′-oxirane]-4-one (**20**) was obtained as a red solid in 69% yield. m.p. 154–155 °C; FT-IR ν_{max} (cm^{-1}, KBr): 3205, 3178, 3116, 3049, 3035, 2964, 1623, 1593, 1568, 1524, 1497, 1448, 1141, 1131; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.18 (*d*, *J* = 8.8 Hz, 1H), 7.52 (*m*, 2H), 7.43–7.36 (*m*, 2H), 7.29 (*d*, *J* = 7.5 Hz, 1H), 7.22 (*d*, *J* = 8.8 Hz, 2H), 6.58 (*s*, 1H), 6.23 (*s*, 1H), 3.51 (*d*, *J* = 6.1 Hz, 1H), 3.28 (*d*, *J* = 6.1 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 183.2 (C), 155.2 (C), 137.4 (C), 136.2 (C), 133.6 (C), 131.7 (CH), 129.6 (CH), 128.6 (CH), 126.3 (CH), 125.6 (CH), 123.2 (CH), 121.5 (CH), 102.0 (CH), 60.8 (CH_2), 53.8 (C); MS (70 eV) *m/z* (%): 264 (18), 263 (100), 262 (50), 246 (4), 235 (10), 234 (46), 218 (8), 217 (10), 206 (14), 204 (15), 185 (15), 179 (7), 157 (6), 142 (8), 130 (9), 130 (9), 116 (9), 109 (11), 102 (21), 93 (9), 84 (10), 77 (15), 63 (5); HR-ESI-MS *m/z* calcd. for $C_{17}H_{13}NO_2Na^+$: 286.0844. Found: *m/z* 286.0838. Δ = 2.1 ppm.

2-Amino-4*H*-spiro[naphthlene-1,2′-oxirane]-4-one (**21**) was obtained as a red solid in 55% yield. m.p. 281–283 °C; FT-IR ν_{max} (cm^{-1}, KBr): 3336, 3154, 3080, 3062, 2992, 1662, 1612, 1595, 1538, 1470, 1443, 1273, 1141; ^{1}H NMR (400 MHz, CD_3OD) δ (ppm): 7.94 (*d*, *J* = 7.7 Hz, 1H), 7.45 (*t*, *J* = 7.5 Hz, 1H), 7.37 (*t*, *J* = 7.5 Hz, 1H), 7.16 (*d*, *J* = 7.7 Hz, 1H), 5.67 (*s*, 1H), 3.36 (*d*, *J* = 6.4 Hz, 1H), 3.18 (*d*, *J* = 6.4 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 187.2 (C), 168.6 (C), 140.7 (C), 137.3 (C), 135.1 (CH), 131.8 (CH), 129.1 (CH), 125.5 (CH), 103.2 (CH), 65.0 (CH_2), 56.3 (C); MS (70 eV) *m/z* (%): 187 (56), 172 (4), 171 (9), 160 (9), 159 (60), 143 (11), 131 (19), 130 (63), 115 (14), 103 (25), 102 (100), 89 (12), 76 (21), 63 (12), 51 (15); HR-ESI-MS *m/z* calcd. for $C_{11}H_8NO_2^-$ $[M-H]^-$: 186.0557. Found: *m/z* 186.0560. Δ = 1.6 ppm.

2-(Allyloxy)-4*H*-spiro[naphthalene-1,2′-oxirane]-4-one (**22**) was obtained as a white solid in 35% yield. m.p. 92–94 °C; FT-IR ν_{max} (cm^{-1}, KBr): 3082, 2924, 1656, 1610, 1570, 1460, 1408, 1365, 1325, 1269, 1236, 1203, 1145, 1103, 1060, 1028, 966, 906, 873, 831, 781, 750; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.17 (*d*, *J* = 7.8 Hz, 1H), 7.57 (*t*, *J* = 7.8 Hz, 2H), 7.24 (*d*, *J* = 7.8 Hz, 1H), 6.05–5.94 (*m*, 1H), 6.00 (*s*, 1H), 5.39 (*m*, Hz, 2H), 4.55 (*m*, 2H), 3.72 (*d*, *J* = 6.8 Hz, 1H), 3.31 (*d*, *J* = 6.8 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 184.6 (C), 168.0 (C), 137.1 (C), 132.6 (CH), 132.5 (C), 130.7 (CH), 128.5 (CH), 126.4 (CH), 122.8 (CH), 119.4 (CH_2), 106.9 (CH), 69.7 (CH_2), 57.9 (CH_2); MS (70 eV) *m/z* (%): 228 (100, M+), 213 (61), 199 (12), 185 (15), 181 (9), 152 (16), 128 (24), 115 (29), 102 (6), 89 (12), 77 (14), 63 (12), 51 (10). HR-ESI-MS *m/z* calcd. for $C_{14}H_{13}O_3^+$: 229.0859 ($[M+H]^+$). Found *m/z* 229.0863. Δ = 1.7 ppm.

2,2-Dimethyl-2,3-dihydro-4*H*-spiro[naphtho[2,3-*b*]furan-9,2′-oxiran]-4-one (**23**) was obtained as a white solid in 60% yield. m.p. 123–126 °C; FT-IR ν_{max} (cm^{-1}, KBr): 3066, 2966, 2926, 2858, 1660, 1600, 1568, 1460, 1417, 1375, 1271, 1211, 1165, 1122, 1060, 891, 866, 754; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.18 (*d*, *J* = 7.6 Hz, 1H), 7.52

(*m*, 2H), 7.20 (*d*, *J* = 7.6 Hz, 1H), 3.70 (*d*, *J* = 6.5 Hz, 1H), 3.44 (*d*, *J* = 6.5 Hz, 1H), 2.93 (*s*, 2H), 1.52 (*s*, 3H), 1.51 (*s*, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 181.5 (C), 168.1 (C), 136.2 (C), 134.2 (C), 131.8 (CH), 128.6 (CH), 126.4 (CH), 122.6 (CH), 117.4 (C), 92.2 (C), 57.3 (CH_2), 50.8 (C), 40.2 (CH_2), 28.3 (CH_3), 28.2 (CH_3); MS (70 eV) *m/z* (%): 242 (100, M+), 227 (20), 211 (5), 200 (19), 183 (9), 171 (12), 156 (11), 141 (16), 128 (24),115 (35), 105 (13), 89 (12), 77 (19), 63(12), 43 (45); HR-ESI-MS *m/z* calcd. for $C_{12}H_{11}O_3^+$: 243.1028 $[M+H]^+$. Found: *m/z* 243.1016. Δ = 4.9 ppm.

4,5,7,8-Tetrachloro-1-oxaspiro[2.5]octa-4,7-dien-6-one (**25**) was obtained as a white solid in 74% yield. m.p. 158–160 °C; FT-IR ν_{max} (cm^{-1}, KBr): 3334, 1674, 1613, 1583, 1119, 857; ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 3.70 (*s*, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 146.2 (C), 134.0 (C), 55.1 (CH_2); MS (70 eV) *m/z* (%): 260 (57), 258 (45), 262 (24), 244 (2), 230 (13), 225 (16), 223 (16), 211 (11), 209 (11), 204 (15), 202 (31), 198 (25), 196 (44), 194 (46), 169 (45), 168 (46); HR-ESI-MS *m/z* calcd. for $C_7HCl_4O_2^-$: 258.8707 (100.0%) and 256.8736 (75%) $[M-H]^-$. Found: *m/z* 258.8727 (100%) and *m/z* 256.8745 (75%). Δ = 7.7 and 3.5 ppm, respectively.

4,5-Dichloro-3,6-dimethoxyphthalonitrile (**26**) was obtained as a white solid in 50% yield. m.p. 185–186 °C; FT-IR ν_{max} (cm^{-1}, KBr): 2947, 2925, 2238, 1545, 1463, 1386, 1020, 844; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 4.15 (*s*, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 156.0 (C), 135.2 (C), 111.8 (C), 108.5 (C), 62.7 (CH_3). MS (70 eV) *m/z* (%): 257 (9), 258 (43), 256 (67), 243 (62), 241 (100), 215 (8), 213 (11); HR-ESI-MS *m/z* calcd. for $C_{10}H_6Cl_2N_2O_2Na^+$: 278.9698 $[M+Na]^+$. Found: *m/z* 278.9706. Δ = 2.9 ppm.

Methyl 9-phenanthryl ether (**27**) was obtained as a white solid in 45% yield. m.p. 64–66 °C; FT-IR ν_{max} (cm^{-1}, KBr): 3445, 3424, 3057, 3009, 2964, 2873, 2836, 1623, 1596, 1459, 1426, 1394, 1309, 1234, 1117, 1095, 829, 766, 743, 722; ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 8.62 (*d*, *J* = 7.9 Hz, 1H), 8.55 (*d*, *J* = 8.0 Hz, 1H), 8.35 (*dd*, *J* = 8.0, 1.0 Hz, 1H), 7.75 (*d*, *J* = 7.9 Hz, 1H), 7.68–7.63 (*m*, 1H), 7.63–7.57 (*m*, 1H), 7.54–7.45 (*m*, 2H), 6.95 (*s*, 1H), 4.05 (*s*, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 153.5 (C), 132.9 (C), 131.2 (C), 127.3 (CH), 127.2 (CH), 127.1 (CH), 126.8 (CH), 126.5 (C), 126.4 (C), 126.3 (CH), 124.2 (CH), 124.1 (CH), 122.5 (CH), 101.9 (CH), 55.4 (CH_3). MS (70 eV) *m/z* (%): 208 (78), 193 (7), 165 (100), 139 (7), 115 (5), 104 (8); HR-ESI-MS *m/z* calcd. for $C_{15}H_{12}OH^+$: 209.0961 $[M+H]^+$. Found: *m/z* 209.0954. Δ = 3.3 ppm.

4.3. Antimalarial assay

The chloroquine-sensitive NF54 isolated 3D7 clone of *P. falciparum* (MRA-102, MR4, ATCC, Manassas, Virginia, USA) was maintained in continuous *in vitro* culture using the Trager and Jensen Method [46]. For the microtest, an initial parasitemia of 1% and hematocrit 2% were used. The substances were dissolved in DMSO at stock concentrations of 10 mg/mL and subsequently diluted in complete culture medium to obtain the seven test concentrations (100–0.14 μg/mL). The test was performed as described by Andrade-Neto and co-workers [47]. Briefly, diluted samples were applied to microplate wells containing parasitized erythrocytes. Each diluted sample was tested in triplicate. The plate was incubated for 48 h at 37 °C. After incubation, the contents of the wells were evaluated by optical microscopy. Inhibition of parasite growth was determined by comparing the sample with untreated controls, according to the formula below:

$$\%\ \text{inhibition} = \frac{(\text{parasitemia of untreated controls} - \text{parasitemia of sample})}{\text{parasitemia of untreated controls}} x\ 100$$

4.4. Cytotoxicity assay

The MRC-5 cell line of human fibroblasts was grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine 100 μg/mL streptomycin and 100 U/mL penicillin, and in-cubated at 37 °C with a 5% atmosphere of CO_2. For assays, the cells were plated in 96-well plates (2.5×10^4 cells/well) and the Alamar Blue™ assay was performed using a previously described procedure [48,49]. After incubation for 24 h, the compounds were individually dissolved in DMSO and the resulting solutions were diluted in culture medium. The resulting dilute solutions of each sample were added to wells at final (well) concentrations of 1.56–100 μg/mL. Control groups had final well concentrations of 0.1% DMSO. The plates were further incubated for 48 h. Three hours before the end of the incubation period, Alamar Blue™ (10 μL) was added to each well. The fluorescent signal was monitored with a multiplate reader using 530–560 nm excitation and 590 nm emission wavelengths.

Acknowledgments

The authors would like to acknowledge the agencies that fund our research: CNPq (National Council of Research of Brazil), CAPES (scholarship), FAPERJ, in particular, through project financing of PRONEX FAPERJ (E-26/110.574/2010) coordinated by Prof. Vitor F. Ferreira (UFF), CNPq (304716/2014-6) coordinated by Fernando de C. da Silva and PRONEX FAPEAM NOSSAPLAM (Edital 023/2009) Project coordinated by Adrian M. Pohlit. This work is part of the activities of ResNet NPND.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.11.020.

References

[1] M.O.F. Goulart, C.L. Zani, J. Tonholo, L.R. Freitas, F.C. de Abreu, A.B. Oliveff, D.S. Rasllam, S. Starling, E. Chiari, Trypanocidal activity and redox potential of heterocyclic-and 2-hydroxy-naphthoquinones, Bioorg. Med. Chem. Lett. 7 (1997) 2043–2048, http://dx.doi.org/10.1016/S0960-894X(97)00354-5.

[2] C.O. Salas, M. Faúndez, A. Morello, J.D. Maya, R.A. Tapia, Natural and synthetic naphthoquinones active against *Trypanosoma cruzi*: an initial step towards new drugs for Chagas disease, Curr. Med. Chem. 18 (2011) 144–161, http://dx.doi.org/10.2174/092986711793979779.

[3] A.V. Pinto, S.L. de Castro, The trypanocidal activity of naphthoquinones: a review, Molecules 14 (2009) 4570–4590, http://dx.doi.org/10.3390/molecules14114570.

[4] T. Müller, L. Johann, B. Jannack, M. Brückner, D.A. Lanfranchi, H. Bauer, C. Sanchez, V. Yardley, C. Deregnaucourt, J. Schrevel, M. Lanzer, R.H. Schirmer, E. Davioud-Charvet, Glutathione reductase-catalyzed cascade of redox reactions to bioactivate potent antimalarial 1,4-naphthoquinones – a new strategy to combat malarial parasites, J. Am. Chem. Soc. 133 (2011) 11557–11571, http://dx.doi.org/10.1021/ja201729z.

[5] E. Perez-Sacau, A. Estévez-Braun, A.G. Ravelo, D.G. Yapu, A.G. Turba, Antiplasmodial activity of naphthquinones related to lapachol an β-lapachone, Chem. Biodivers. 2 (2005) 264–274, http://dx.doi.org/10.1002/cbdv.200590009.

[6] K.W. Stagliano, A. Emadi, Z. Lu, H.C. Malinakova, B. Twenter, M. Yu, L.E. Holland, A.M. Rom, J.S. Harwood, R. Amin, A. Johnson, P. Yves,

Regiocontrolled synthesis and HIV inhibitory activity of unsymmetrical binaphthoquinone and trimeric naphthoquinone derivatives of conocurvone, Bioorg. Med. Chem. 14 (2006) 5651–5665, http://dx.doi.org/10.1016/j.bmc.2006.04.034.
[7] K.A.L. Ribeiro, C.M. Carvalho, M.T. Molina, E.P. Lima, E.L. ópez-Montero, J.R.M. Reys, M.B.F. Oliveira, A.V. Pinto, A.E.G. Santana, M.O.F. Goulart, Activities of naphthoquinones against *Aedes aegypti* (Linnaeus, 1762) (Diptera: Culicidae), vector of dengue and *Biomphalaria glabrata* (Say, 1818), intermediate host of *Schistosoma mansoni*, Acta Trop. 111 (2009) 44–50, http://dx.doi.org/10.1016/j.actatropica.2009.02.008.
[8] L. Willcox, B. Gilbert, Traditional medicinal plants for the treatment and prevention of human parasitic diseases, Ethnopharmacology 1 (2009) 199–208.
[9] H. Hussain, K. Krohn, V.U. Ahmad, G.A. Miana, I.R. Greend, Lapachol: an overview, ARKIVOC (2007) 145–171, http://dx.doi.org/10.3998/ark.5550190.0008.204.
[10] I.T. Balassiano, S.A. Paulo, N.H. Silva, M.C. Cabral, M.G.C. Carvalho, Demonstration of the lapachol as a potential drug for reducing cancer metastasis, Oncol. Rep. 13 (2005) 329–333, http://dx.doi.org/10.3892/or.13.2.329.
[11] R.-Y. Yang, D. Kizer, H. Wu, E. Volckova, X.-S. Miao, S.M. Ali, M. Tandon, S.E. Savage, T.C.K. Chan, M.A. Ashwell, Synthetic methods for the preparation of ARQ 501 (β-lapachone) human blood metabolites, Bioorg. Med. Chem. 16 (2008) 5635–5643, http://dx.doi.org/10.1016/j.bmc.2008.03.073.
[12] H.T. Khong, L. Dreisbach, H.L. Kindler, D.F. Trent, K.G. Jeziorski, I. Bonderenko, T. Popiela, D.M. Yagovane, G. Domba, A phase 2 study of ARQ 501 in combination with gemcitabine in adult patients with treatment naïve, unresectable pancreatic adenocarcinoma, J. Clin. Oncol. ASCO Annu. Meet. Proc. 25 (2007) 15017.
[13] http://www.prnewswire.com/news-releases/arqule-presents-preliminary-interim-phase-1-data-for-arq-501-and-announces-combination-study-with-taxotere-73972182.html (accessed 2.08.15.).
[14] G.M. Cragg, P.G. Grothaus, D.J. Newman, New horizons for old drugs and drug leads, J. Nat. Prod. 77 (2014) 703–723, http://dx.doi.org/10.1021/np5000796.
[15] https://clinicaltrials.gov/ct2/show/NCT01502800 (accessed 2.08.15.).
[16] L.-O. Klotz, X. Hou, C. Jacob, 1,4-Naphthoquinones: from oxidative damage to cellular and inter-cellular signaling, Molecules 19 (2014) 14902–14918, http://dx.doi.org/10.3390/molecules190914902.
[17] M.L. Circu, T.Y. Aw, Reactive oxygen species, cellular redox systems, and apoptosis, Free Radic. Biol. Med. 48 (2010) 749–762, http://dx.doi.org/10.1016/j.freeradbiomed.2009.12.022.
[18] R.J. Weaver, M. Dickins, M.D. Burke, Cytochrome P450 2C9 is responsible for hydroxylation of the naphthoquinone antimalarial drug 58C80 in human liver, Biochem. Pharmacol. 46 (1993) 1183–1197, http://dx.doi.org/10.1016/0006-2952(93)90467-B.
[19] C.J. Canfield, M. Pudney, W.E. Gutteridge, Interactions of atovaquone with other antimalarial drugs against plasmodium falciparum in vitro, Exp. Parasitol. 80 (1993) 373–381, http://dx.doi.org/10.1006/expr.1995.1049.
[20] P. Olliaro, P.D. Wirth, New targets for antimalarial drug discovery, J. Pharm. Pharmacol. 49 (1997) 29–33, http://dx.doi.org/10.1111/j.2042-7158.1997.tb06157.x.
[21] A. Färnert, J. Lindberg, P. Gil, G. Swedberg, Y. Berqvist, M.M. Thapar, N. Lindegårdh, S. Berezcky, A. Björkman, Evidence of *Plasmodium falciparum* malaria resistant to atovaquone and proguanil hydrochloride: case reports, Brit. Med. J. 326 (2003) 628–629, http://dx.doi.org/10.1136/bmj.326.7390.628.
[22] K. Miyashita, T. Imanishi, Syntheses of natural products having an epoxyquinone structure, Chem. Rev. 105 (2005) 4515–4536, http://dx.doi.org/10.1021/cr040613k. T.B. Pereira, L.F.R. e Silva, R.C.N. Amorim, M.R.S. Melo, R.C.Z. de Souza, M.N. Eberlin, E.S. Lima, M.C. Vasconcellos, A.M. Pohlit, *In vitro* and *in vivo* anti-malarial activity of limonoids isolated from the residual seed biomass from *Carapa guianensis* (andiroba) oil production, Malaria J. 13 (2014) 317, http://dx.doi.org/10.1186/1475-2875-13-317.
[23] V.F. Ferreira, A. Jorqueira, A.M.T. Souza, M.N. da Silva, M.C.B.V. de Souza, R.M. Gouvêa, C.R. Rodrigues, A.V. Pinto, H.C. Castro, D.O. Santos, H.P. Araújo, S.C. Bourguig, Trypanocidal agents with low cytotoxicity to mammalian cell line: a comparison of the theoretical and biological features of lapachone derivatives, Bioorg. Med. Chem. 14 (2006) 5459–5466, http://dx.doi.org/10.1016/j.bmc.2006.04.046.
[24] P.F. Carneiro, S.B. do Nascimento, A.V. Pinto, M.C.F.R. Pinto, G.C. Lechuga, D.O. Santos, H.M. dos Santos Júnior, J.A.L.C. Resende, S.C. Bourguignon, V.F. Ferreira, New oxirane derivatives of 1,4-naphthoquinones and their evaluation against *T. cruzi* epimastigote forms, Bioorg. Med. Chem. 20 (2012) 4995–5000, http://dx.doi.org/10.1016/j.bmc.2012.06.027.
[25] A. Jorqueira, R.M. Gouvêa, V.F. Ferreira, M.N. da Silva, M.C.B.V. de Souza, A.A. Zuma, D.F.B. Cavalcanti, H.P. Araújo, D.O. Santos, S.C. Bourguignon, Oxyrane derivative of α-lapachone is potent growth inhibitor of *Trypanosoma cruzi* epimastigote forms, Parasitol. Res. 99 (2006) 429–433, http://dx.doi.org/10.1007/s00436-006-0153-8.
[26] S.C. Bourguignon, H.C. Castro, D.O. Santos, C.R. Alves, V.F. Ferreira, I.L. Gama, F.C. Silva, W.S. Seguis, R.T. Pinho, *Trypanosoma cruzi*: in vitro activity of Epoxy-α-Lap, a derivative of α-lapachone, on trypomastigote and amastigote forms, Exp. Parasitol. 122 (2009) 91–96, http://dx.doi.org/10.1016/j.exppara.2009.03.002.
[27] S.C. Bourguignon, D.F.B. Cavalcanti, A.M.T. de Souza, H.C. Castro, C.R. Rodrigues, M.G. Albuquerque, D.O. Santos, G.G. da Silva, F.C. da Silva, V.F. Ferreira, R.T. de Pinho, C.R. Alves, *Trypanosoma cruzi*: Insights into naphthoquinone effects on growth and proteinase activity, Exp. Parasitol. 127 (2011) 160–166, http://dx.doi.org/10.1016/j.exppara.2010.07.007.
[28] F. Souza-Silva, S.B. Nascimento, S.C. Bourguignon, B.A.S. Pereira, P.F. Carneiro, W.S. Silva, C.R. Alves, R.T. Pinho, Evidences for leishmanicidal activity of the naphthoquinone derivative epoxy-α-lapachone, Exp. Parasitol. 147 (2014) 81–84, http://dx.doi.org/10.1016/j.exppara.2014.10.002.
[29] F. Souza-Silva, S.C. Bourguignon, B.A. Pereira, L.M. Côrtes, L.F. de Oliveira, A. Henriques-Pons, L.C. Finkelstein, V.F. Ferreira, P.F. Carneiro, R.T. Pinho, E.R. Caffarena, C.R. Alves, Epoxy-α-lapachone has *in vitro* and *in vivo* anti-leishmania (*Leishmania*) *amazonensis* effects and inhibits serine proteinase activity in this parasite, Antimicrob. Agents Chemother. 59 (2015) 1910–1918, http://dx.doi.org/10.1128/AAC.04742-14.
[30] A. Sharma, I.O. Santos, P. Gaur, V.F. Ferreira, C.R.S. Garcia, D. da Rocha, Addition of thiols to *o*-quinone methide: new 2-hydroxy-3-henylsulfanylmethyl [1,4]naphthoquinones and their activity against the human malaria parasite *Plasmodium falciparum* (3D7), Eur. J. Med. Chem. 59 (2013) 48–53, http://dx.doi.org/10.1016/j.ejmech.2012.10.052.
[31] D.C. Schuck, S.B. Ferreira, L.N. Cruz, D.R. da Rocha, M.S. Moraes, M. Nakabashi, P.J. Rosenthal, V.F. Ferreira, C.R.S. Garcia, Biological evaluation of hydroxynaphthoquinones as antimalarials, Malar. J. 12 (2013) 234, http://dx.doi.org/10.1186/1475-2875-12-234.
[32] L. Monzote, A. Siddiq, Drug development to protozoan diseases, Open Med. Chem. J. 5 (2011) 1–3, http://dx.doi.org/10.2174/1874104501105010001.
[33] E.N. da Silva Junior, M.C.B.V. de Souza, M.C. Fernandes, R.F.S. Menna-Barreto, M.C.F.R. Pinto, F.A. Lopes, C.A. de Simone, C.K.Z. Andrade, A.V. Pinto, V.F. Ferreira, S.L. de Castro, Synthesis and anti-*Trypanosoma cruzi* activity of derivatives from *nor*-lapachones and lapachones, Bioorg. Med. Chem. 16 (2008) 5030–5038, http://dx.doi.org/10.1016/j.bmc.2008.03.032.
[34] E.L. Flannery, A.K. Chatterjee, E.A. Winzeler, Antimalarial drug discovery - approaches and progress towards new medicines, Nat. Rev. Microbiol. 11 (2013) 849–862, http://dx.doi.org/10.1038/nrmicro3138.
[35] A.U. Krettli, J.O. Adebayo, L.G. Krettli, Testing of natural products and synthetic molecules aiming at new antimalarials, Curr. Drug Targets 10 (2009) 261–270, http://dx.doi.org/10.2174/138945009787581203.
[36] M.P. Carrasco, A.S. Newton, L. Gonçalves, A. Góis, M. Machado, J. Gut, F. Nogueira, T. Hänscheid, R.C. Guedes, D.J.V.A. dos Santos, P.J. Rosenthal, R. Moreira, Probing the aurone scaffold against *Plasmodium falciparum*: design, synthesis and antimalarial activity, Eur. J. Med. Chem. 80 (2014) 523–534, http://dx.doi.org/10.1016/j.ejmech.2014.04.076.
[37] C.S. Lisboa, V.G. Santos, B.G. Vaz, N.C. de Lucas, M.N. Eberlin, S.J. Garden, C-H functionalization of 1,4-naphthoquinone by oxidative coupling with anilines in the presence of a catalytic quantity of copper(II) acetate, J. Org. Chem. 76 (2011) 5264–5273, http://dx.doi.org/10.1021/jo200354u.
[38] L.F. Fieser, J.L. Hartwell, The reaction of hydrazoic acid with the naphthoquinones, J. Am. Chem. Soc. 57 (1935) 1482–1484, http://dx.doi.org/10.1021/ja01311a030.
[39] S.C. Hooker, LVII.-The constitution of lapachic acid(lapachol) and its derivatives, J. Chem. Soc. Trans. 61 (1892) 611–650, http://dx.doi.org/10.1039/CT8926100611.
[40] V.F. Ferreira, A.V. Pinto, M.C.F.R. Pinto, M.M. Silva, Catalytic reduction in pyranonaphthoquinone systems, An. Acad. Bras. Ciênc 59 (1987) 329–333.
[41] S.C. Hooker, The constitution of lapachol and its derivatives. Part IV. Oxidation with potassium permanganate, J. Am. Chem. Soc. 58 (1936) 1168–1173, http://dx.doi.org/10.1021/ja01298a029.
[42] L.F. Fieser, The alkylation of hydroxynaphthoquinone II. Carbon alkylation, J. Am. Chem. Soc. 48 (1926) 3201–3214, http://dx.doi.org/10.1021/ja01691a030.
[43] B. Eistert, H. Fink, A. Muller, Reaktionen von α-dicarbonylverbindungen und chinonen mit diazoalkanen, v. umsetzungen substituierter p-benzo- und -naphthochinone mit diazomethan, Chem. Ber. 95 (1962) 2403–2415, http://dx.doi.org/10.1002/cber.19620951012.
[44] B. Eistert, L.S.B. Goubran, C. Vamvakaris, T.J. Arackal, Reaktionen von diazoalkanen mit α-diketonen und chinonen, xxiii. umsetzungen von 2-amino-p-benzo- und -1,4-naphthochinonen mit diazoalkanen, Chem. Ber. 108 (1975) 2941–2946, http://dx.doi.org/10.1002/cber.19751080914.
[45] G.M. Sheldrick, A short history of SHELX, Acta Cryst. A 64 (2008) 112–122, http://dx.doi.org/10.1107/S0108767307043930.
[46] W. Trager, J.B. Jensen, Human malaria parasites in continuous culture, Science 193 (1976) 673–675, http://dx.doi.org/10.1126/science.781840.
[47] V.F. Andrade-Neto, A.M. Pohlit, A.C. Pinto, E.C. Silva, K.L. Nogueira, M.R. Melo, M.C. Henrique, R.C. Amorim, L.F. Silva, M.R. Costa, R.C. Nunomura, S.M. Nunomura, W.D. Alecrim, M.G. Alecrim, F.C. Chaves, P.P. Vieira, *In vitro* inhibition of *Plasmodium falciparum* by substances isolated from Amazonian antimalarial plants, Mem. Inst. Oswaldo Cruz 102 (2007) 359–365, http://dx.doi.org/10.1590/S0074-02762007000300016.
[48] S.A. Ahmed, R.M. Gogal Jr., J.E. Walsh, A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [3H] thymidine incorporation assay, J. Immunol. Methods 15 (1994) 211–224, http://dx.doi.org/10.1016/0022-1759(94)90396-4.
[49] E.S. Lima, A.C.S. Pinto, K.L. Nogueira, L.F.R. Silva, P.D.O. Almeida, M.C. Vasconcellos, S.C.M. Chaves, W.P. Tadei, A.M. Pohlit, Stability and antioxidant activity of semi-synthetic derivatives of 4-nerolidylcatechol, Molecules 18 (2013) 178–189, http://dx.doi.org/10.3390/molecules18010178.