Blood shizonticidal activities of phenazines and naphthoquinoidal compounds against *Plasmodium falciparum* in vitro and in mice malaria studies

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Due to the recent advances of atovaquone, a naphthoquinone, through clinical trials as treatment for malarial infection, 19 quinone derivatives with previously reported structures were also evaluated for blood schizonticide activity against the malaria parasite Plasmodium falciparum. *These compounds include 2-hydroxy-3-methylamino naphthoquinones (2-9), lapachol (10), nor-lapachol (11), iso-lapachol (12), phthiocol (13) and phenazines (12-20). Their cytotoxicities were also evaluated against human hepatoma and normal monkey kidney cell lines. Compounds 2 and 5 showed the highest activity against* P. falciparum *chloroquine-resistant blood-stage parasites (clone W2), indicated by their low inhibitory concentration for 50% (IC_{50}) of parasite growth. The therapeutic potential of the active compounds was evaluated according to the selectivity index, which is a ratio of the cytotoxicity minimum lethal dose which eliminates 50% of cells and the in vitro IC_{50}. Naphthoquinones 2 and 5, with activities similar to the reference antimalarial chloroquine, were also active against malaria in mice and suppressed parasitaemia by more than 60% in contrast to compound 11 which was inactive. Based on their in vitro and in vivo activities, compounds 2 and 5 are considered promising molecules for antimalarial treatment and warrant further study.*

Key words: antimalarials - quinones - phenazines - lapachol - *Plasmodium falciparum* - *Plasmodium berghei*

To date, malaria remains a global health problem that affects 225 million people, mostly in tropical and subtropical regions of Africa and Southeast Asia (WHO 2011). A malaria vaccine is currently unavailable and chemotherapy remains the primary intervention for disease control; however, the resistance of *Plasmodium falciparum* to most antimalarial agents is increasing and in some regions of the world, the parasite is becoming resistant to artemisinin derivatives (Dondorp et al. 2009). In addition, the agent *Plasmodium vivax* has become resistant to chloroquine (Price et al. 2009, Graf et al. 2012, Marques et al. 2014), further raising difficulties of controlling malaria. More effective compounds and new treatment strategies are therefore needed, particularly drugs with high therapeutic indexes, novel and/or defined mechanisms of action, easy synthesis and activity against all stages of malarial parasitic infection.

Quinone-based compounds with antimalarial activity, such as hydrolapachol (Hooker 1936) and lapachol, which are both isolated from plants (Fieser & Fieser 1948), have been studied in depth over recent decades. Interest in the antimalarial properties of hydroxy-naphthoquinones has increased since the development of atovaquone (Fry & Pudney 1992, Srivastava et al. 1997), a commercially available drug for malaria chemoprophylaxis (Shapiro et al. 1999) that is used to treat *P. falciparum* malaria (McKeage & Scott 2003) in areas with chloroquine resistance (Aguiar et al. 2012, WHO 2012). These compounds also possess antibacterial and antitumor activities due to their effects on oxidative stress (Lourenço et al. 2011, Cavalcanti et al. 2013).

Phenazines are produced through the heterocyclisation of naphthoquinoidal structures and also possess antimicrobial (van Rensburg et al. 2000), antiviral (Wang et al. 2000) and antimalarial (Makgatho et al. 2000, Andrade-Neto et al. 2004b) activities.

Some quinoidal molecules have demonstrated an improved biological response in relation to their parent compounds due to their increased lipophilicity (de Castro et al. 2013); however, the low bioavailability of atovaquone (Dressman & Reppas 2000) indicates the need for chemical modifications, especially in the alkyl group, to produce new analogues (Schuck et al. 2013). Furthermore, increasing resistance to atovaquone has been reported (Vaidya & Mather 2000, Fivelman et al. 2002).

In this study, we modified the alkyl side chain of atovaquone to create naphthoquinone derivatives containing tertiary amine substitutions (Fig. 1), which were also previously shown to have antimicrobial (Carneiro et al. 2011) and antitumoural (da Silva Júnior et al. 2011) properties. These derivatives were synthesised using the classic Mannich reaction from lawsone and

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evaluated against *P. falciparum* blood parasites in vitro. Compounds were next classified by their selective activity, with the exclusion of toxic compounds. In addition, some phenazines were similarly obtained and evaluated. The compounds with the highest activity and a low toxicity profile in vitro were next evaluated against malaria infection in mice. This study thus reinforces the importance of this class of compounds as treatment for parasitic diseases including *P. falciparum*.

MATERIALS AND METHODS

Chemistry - 2-hydroxy-3-methylamino naphthoquinoidal compounds 2-9 were prepared from lawsone by the classic Mannich reaction, using the appropriate amines and formaldehyde in an alcohol-based solution (Fig. 1) as previously described (Leffler & Hathaway 1948, Lagrota et al. 1988, dos Santos et al. 2000, Lima et al. 2002). The obtained derivatives were then characterised as previously described (Lagrota et al. 1988, dos Santos et al. 2000, Lima et al. 2002) (Fig. 2).

Lapachol (10) was extracted from the heartwood of *Tabebuia* sp. (*Tecoma*) and purified by a series of recrystallisation steps (Pinto et al. 1980). Nor-lapachol (11) was obtained using the Hooker oxidation method (Fieser & Fieser 1948). Iso-lapachol (12) and 2-hydroxy-3-methyl-1,4-naphthoquinone (13) were both synthesised using previously described techniques (Hooker 1936). Phenazine 14 was prepared from lapachol (10) through a two-step process (da Silva Júnior et al. 2009). Compound 15 was obtained from reduced nor-lapachol. Lapachol (10) was catalytically reduced and used to obtain compound 16 using the same reaction (da Silva Júnior et al. 2011) and compound 17 was obtained through a similar process. The phenazine compounds 18-20 were prepared from C-allyl lawsone as previously described (da Silva Júnior et al. 2011) (Fig. 3).

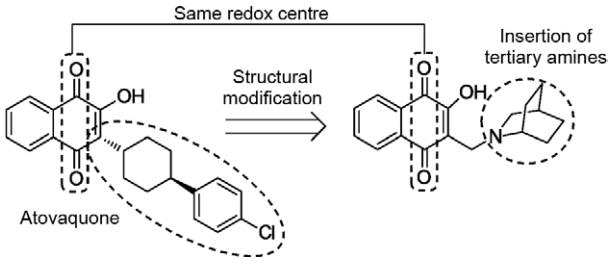

Fig. 1: strategy toward bioactive quinone derivatives.

Cytotoxicity tests with human hepatocellular liver carcinoma cell line (HepG2) - HepG2 (ATT, USA), kindly donated by Universidade Nova de Lisboa, Portugal, and normal monkey kidney cells (BGM) cells, donated by the University of Minas Gerais, were cultured in RPMI-1640 medium (Sigma-Aldrich, USA, ref 6504), containing 40 mg/L gentamicin (Schering-Plough, USA) and 10% heat-inactivated foetal calf serum in 5% CO_2 at 37ºC (Gibco, USA). After reaching confluence, the cell monolayer was then trypsinised, washed with culture medium, aliquoted into a flat-bottomed 96-well plate (Corning, USA, ref 3595) at 5×10^3 cells/well and incubated for 18 h at 37ºC to allow cell adhesion. The test and control compounds were added at various concentrations (188-4,324 µM) and the cell monolayer was incubated for another 24 h. Cytotoxicity was next evaluated as described (Coutinho et al. 2013) with the [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] (MTT) assay by adding 20 µL of 5 mg/mL MTT (Molecular Probes, USA) per well (Denizot & Lang 1986). After 3 h of incubation, the supernatant was discarded, 100 µL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich) were added per well and the optical density was measured (SpectraMax 340PC384, Molecular Devices, USA) at 570 nm for the test conditions and 630 nm for the background wells. Cell viability was expressed as a percentage of the absorbance of the untreated control cells subtracted from the appropriate background measurement. The minimum lethal dose of 50% cells (MLD_{50}) was determined as previously described (de Madureira et al. 2002) and further used to calculate the selectivity index (SI) of the active compounds, defined as the ratio of the MLD_{50} to the IC_{50} (Bézivin et al. 2003).

Continuous culture of P. falciparum and in vitro tests of drug activity - Blood-stage parasites from the chloroquine-resistant and mefloquine-sensitive *P. falciparum* W2 clone kept as described (Andrade-Neto et al. 2004a) were maintained at 37ºC in human erythrocytes (A^+) in complete medium (RPMI-1640, supplemented with 10% blood group A^+ human serum) that was changed daily as previously described (Trager & Jensen 1976). The effects of the compounds were determined relative to control parasites kept in culture medium only (Rieckmann et al. 1978). Ring-stage sorbitol-synchronised parasites (Lambros & Vanderberg 1979) were used after adjusting the parasitaemia and haematocrit values as specified in each test at a volume of 180 µL/well in 96-well microtitre plates (Corning, ref. 3595) contain-

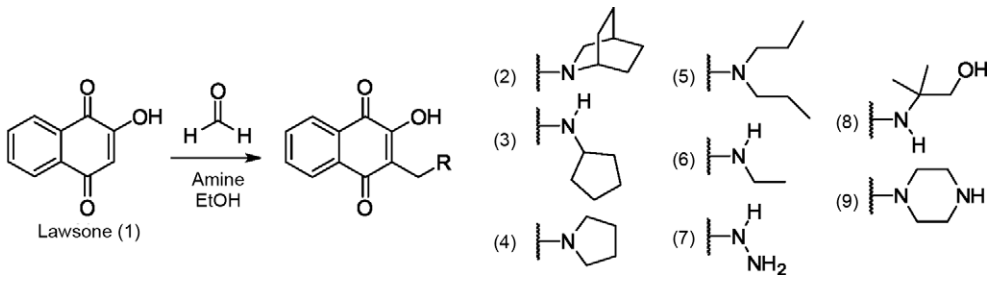

Fig 2: naphthoquinones 2-9 obtained by Mannich reaction.

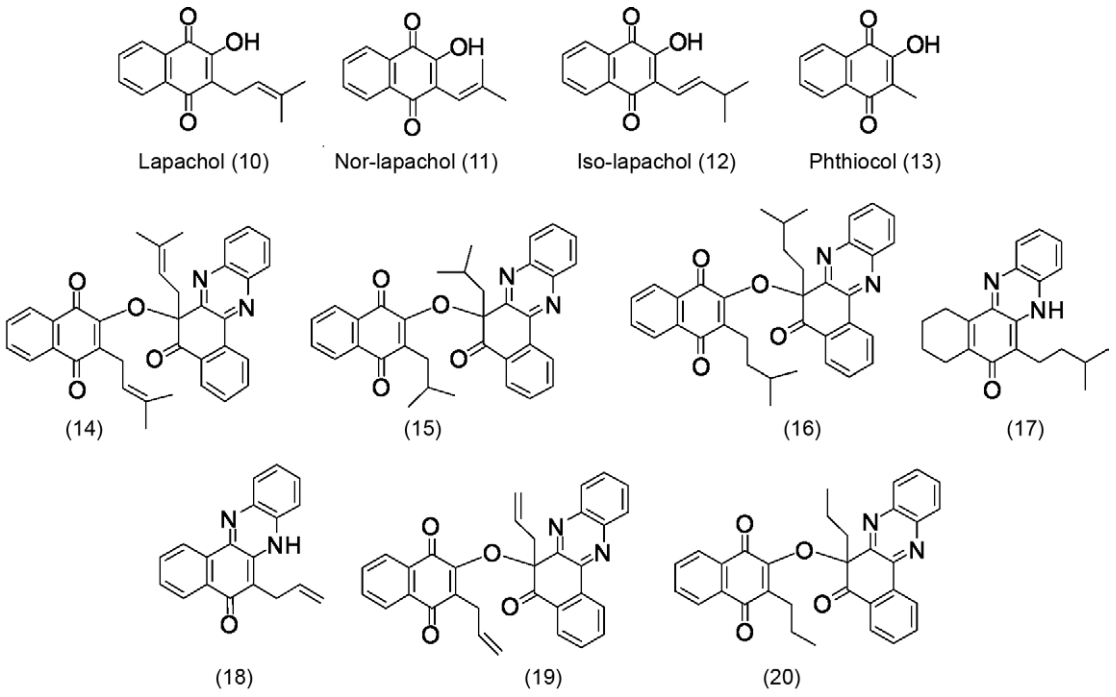

Fig. 3: lapachol (10), nor-lapachol (11), iso-lapachol (12), phthiocol (13) and phenazine compounds 14-20.

ing the diluted compounds in triplicate. The drug activity was measured using the anti-histidine-rich protein II (HRPII) test (Noedl et al. 2002). Chloroquine was used as the antimalarial control in each test.

For the HRPII test, the parasites were adjusted to 0.05% parasitaemia and 1.5% haematocrit, placed in plates containing the drugs and incubated for 24 h under standard culture conditions. The contents of six wells (containing no test drugs) were harvested and frozen in microtubes for later use as background measurement. After 48 h of incubation, the plates were frozen at -70ºC and thawed twice. Next, 100 µL of the lysed cells from each well were added to a plate that had been pre-coated overnight at 4ºC with the primary anti-HRPII antibody (MPFM-55A, ICLLAB®, USA) for immunoassay. Phosphate-buffered saline at pH 7.2 supplemented with Tween 20 (Sigma-Aldrich) (0.05%) and bovine serum albumin (Sigma-Aldrich) (4%) was used to block the plates to (avoid non-specific antibody binding) for 3 h at room temperature (RT). After 1 h at RT, the plates were washed and 100 µL/well secondary antibody solution (MPFG-55P ICLLAB®) were added, followed by incubation with 3,3′,5,5′-tetramethylbenzidine chromogen cat. #50-76-00 (KPL, USA) in the dark. The reaction was stopped by adding 1 M sulphuric acid and the absorbance was read (450 nm) using a spectrophotometer (SpectraMax 340PC384, Molecular Devices). Anti-*P. falciparum* drug activity was measured by comparing the drug concentrations that inhibited parasite growth to drug-free control cultures, which were representative of 100% cell growth.

Growth data from the serial drug dilutions to sigmoidal dose-response curves was analysed by curve-fitting software (Microcal Origin Software v.5.0, OriginLab Co, USA) and used to measure inhibitory concentration for 50% (IC_{50}) of parasite growth. Only compounds exhibiting IC_{50} values lower than 5 µM were considered active; those with IC_{50} values between 5-30 µM were considered partially active and those with IC_{50} values higher than 30 µM were considered inactive compounds.

Antimalarial tests against Plasmodium berghei in mice - The antimalarial suppressive test was performed as previously described (Peters et al. 1975) with slight modifications (Andrade-Neto et al. 2004b). Briefly, *P. berghei* (NK65) chloroquine-sensitive blood-stage parasites were stored at -70ºC and passaged weekly in outbred Swiss mice. Adult female mice weighing 20 ± 2 g were inoculated with 1×10^5 infected erythrocytes, randomly distributed into groups of three mice per group after 24 h infection and then drug treated daily by gavage for three consecutive days. All compounds were freshly diluted in 3% DMSO in RPMI medium and used at doses of 100 mg/kg; chloroquine was used at 20 mg/kg and control mice received the drug vehicle. At days 5 and 9 after parasite inoculation, blood was taken from the tails of the mice and used to prepare smears, which were then fixed with methanol, Giemsa-stained and examined microscopically to determine parasitaemia as previously described (Andrade-Neto et al. 2004b). The inhibition of parasite growth by drug treatment was evaluated in relation to the untreated mice, which were representative of 100% parasite growth. Compounds that reduced parasitaemia by more than 40% were considered active, whereas those that reduced parasitaemia by 30-40% or less than 30% were classified as partially active and inactive, respectively.

Ethics - The protocols for animal use in the laboratory were previously approved by the Ethical Committee for Animal Use (L-0046/08), Oswaldo Cruz Foundation, Brazil.

RESULTS

Eight 2-hydroxy-3-methylamino naphthoquinones, seven phenazines and lapachol, nor-lapachol, iso-lapachol and phtiocol were evaluated for their in vitro activity against *P. falciparum* blood-stage parasites. All compounds were assayed using the anti-HRPII test. The standard antimalarial chloroquine was tested as a control agent in parallel and the data are summarised in Table I.

Among the compounds tested, napthoquinones 2 and 5 were active, exhibiting IC_{50} values < 5 µM (1.3 and 1.9 µM, respectively). Compounds 3, 4, 5, 7, nor-lapachol (11) and iso-lapachol (12) were considered partially active, as well as the phenazines 16-18 with IC_{50} within the range of 5 and 30 µM. The other compounds were considered inactive, exhibiting IC_{50} values > 30 µM.

Regarding cytotoxicity against both HepG2 and BGM cells, all molecules exhibited no toxicity based on high MLD_{50} values observed. Only compound 8 was toxic to both cell lines.

Considering the therapeutic potential of the molecules examined, which was defined as the SI based on the cytotoxicity against HepG2 cells and antiplasmodial activity in vitro, compounds 2 and 5 exhibited the highest SI values of 2,564 and 1,074, respectively, which were similar to or higher than that of chloroquine (SI = 1,535). Seven other molecules exhibited promising SIs ranging from 100-350; these compounds were iso-lapachol (12), three naphthoquinones derived from lawsone (3, 4, 6) and two phenazines (17 and 18). Nor-lapachol (11), the naphthoquinone compound 7, the phenazines prepared from lapachol (14, 16) and phenazine (19) derived from C-allyl lawsone were less active (SI < 100). Other compounds displayed a low SI (10, 13, 15 and 20) and were thus less promising; one compound (8) exhibited an SI value of 10 and was considered toxic. A near two-fold increase in cytotoxicity between HepG2 and BGM cells was observed for compounds 12, 15, 19 and 20.

To determine the in vivo activity of the selected compounds, 2, 5 and 11 were next evaluated against malaria infection caused by *P. berghei*. Both compounds were found to be active by reducing parasitaemia by up to 63%, whereas nor-lapachol (compound 11) was inactive in vivo (Table II). Analysis of the experimental compounds was

TABLE I

Activity of quinones and phenazines against *Plasmodium falciparum* inhibitory concentration for 50% (IC_{50}) measured through the anti-histidine-rich protein II (HRPII) method, cytotoxicity [minimum lethal dose of 50% cells (MLD_{50})] against two different cell lines a human hepatoma (HepG2) and a normal monkey kideny (BGM) and selectivity indexes (SI) a ratio MDL_{50}/IC_{50}

		HepG2		BGM	
Compounds	IC_{50}	MLD_{50}	SI	MLD_{50}	SI
2	1.3 ± 0.4	≥ 3,363.1	2,564	≥ 3,363.1	2,564
3	16.8 ± 3.4	≥ 3,685.8	220	≥ 3,685.8	220
4	14.1 ± 7.0	≥ 3,886.7	276	≥ 3,886.7	276
5	1.9 ± 0.7	2,075.1 ± 316.5	1,074	2,468.4 ± 239.7	1,299
6	30.6 ± 5.7	≥ 4,324.4	141	≥ 4,324.4	141
7	11.2 ± 2.3	331.1 ± 105.3	29	256.6	23
8	59.9 ± 7.7	188.0 ± 34.7	3	321.5 ± 28.3	5.4
9	110.4 ± 12.2	2,497.6 ± 546.9	23	≥ 3,672.4	33
10	93 ± 20,5	1,491.7 ± 321.4	16	1,785.1 ± 888.3	19
11	26.9 ± 0.9	1,698.6 ± 94.8	63	1,608.8 ± 221.8	60
12	12.7 ± 2.0	≥ 4,127.6	324	1,388.9 ± 592.5	109
13	≥ 265.7	5,308.2 ± 8.3	20	≥ 5,314.1	20
14	47.5 ± 1.1	≥ 1,803	38	≥ 1,803	38
15	≥ 94.2	1,041.0 ± 58.5	11	≥ 1,884.6	20
16	20.6 ± 2.6	951.6 ± 115.6	46	632.8 ± 11.4	31
17	9.0 ± 0.1	≥ 3,120.8	348	-	-
18	13.4 ± 2.2	≥ 3,492.5	260	≥ 3,492.5	260
19	60.5 ± 9.2	≥ 2,005.9	33	752 ± 169.9	12
20	≥ 99.5	≥ 1,989.8	20	1,047.6 ± 43.6	10
Chloroquine	0.3 ± 0.02	460.4 ± 97.3	1,535	462.3 ± 2.7	1,541

TABLE II

Antimalarial activity of nor-lapachol (11) and of the naphthoquinones 2 and 5 active in vitro, tested in mice with *Plasmodium berghei*-malaria and orally treated with 100 mg/kg drug dose for three consecutive days

	Inhibition of parasitaemia at days after parasite inoculation (%)		
Compounds[a]	Fifth	Ninth	Activity
2	63	41	Yes
5	63	48	Yes
11	21	21	No
Chloroquine	100	100	Cure

a: the experiment was performed in parallel with chloroquine, which cleared parasitaemia and allowed all mice to survive after treatment with a 20 mg/kg dose.

performed once and in parallel to chloroquine, which served as a control antimalarial agent, clearing parasitaemia even at low doses (20 mg/kg) and maintaining survival of the mice. The other treated animals succumbed to malaria infection with no delay in mortality caused by drug treatment with the new compounds.

DISCUSSION

The synthetic strategy for the preparation of the 2-hydroxy-3-methylamino naphthoquinoidal derivatives (2-9) was used to obtain substances with aliphatic (5-8) and cyclic (2-4 and 9) substituents (Lima et al. 2002, da Silva Júnior et al. 2012). This approach mimics the nonpolar portion of atovaquone. The quinoidal compounds were prepared in only one step using accessible reactants. The structures of these naphthoquinonoid compounds have been previously published, although our study is the first to report their potent activity against *P. falciparum* parasites. The synthetic methods described in this study were completed with commercial reactants and at high yields for quinoidal structures based on atovaquone. These compounds were prepared through a single step synthesis in contrast to the production of atovaquone, which is generally obtained in four steps. The phenazine compounds were easily prepared from lapachol derivatives and their activities have been described as antitumoural (da Silva Júnior et al. 2011) and antimicrobial (Carneiro et al. 2011).

Our results showed that compounds 2 and 5 were active against *P. falciparum* in vitro, exhibiting high SIs and promising therapeutic activity, similar to that of chloroquine. We considered the SI an ideal parameter to evaluate new antimalarials, as this index reflects both drug toxicity and activity, thereby excluding toxic compounds from further study. Using the SI as a screening parameter has also been reported in recent literature (No et al. 2012).

Establishing a structure-activity relationship for the compounds herein described was beyond the scope of this initial study. Investigations into the antimalarial activity of these compounds after structural modification are currently underway in our laboratories and will be reported in future work after establishing a structure-activity relationship.

Compounds 2 and 5, which were highly active in vitro against *P. falciparum*, were also active against malaria in mice by inhibiting parasitaemia; however, they could not prevent mortality from malaria. All compounds were tested at high doses because previous studies have indicated that lapachol has low activity in vitro (Carvalho et al. 1988) compared with the new compounds described. All displayed a low in vivo activity compared do chloroquine. It is possible that these compounds possess low oral bioavailability, similar to most naphthoquinones (Ioset 2008). In addition, slow uptake and/or rapid elimination of their active metabolites have also been previously described (Zani et al. 1997).

In previous work, other phenazines derived from β-lapachone partially active against *P. falciparum* in vitro were only active against malaria in mice when administered subcutaneously, but not orally (Andrade-Neto et al. 2004b). Compounds 2 and 5 had a high therapeutic activity in vitro were tested only through oral administration in malaria-infected mice. These compounds were active despite limiting our study to oral administration.

The mechanism of quinone activity involves the generation of reactive oxygen species due to molecular structures that confer redox properties through reduction by one or two electrons. Oxidative stress and/or the alkylation of lipids, proteins and nucleic acids by naphthoquinones may lead to cell damage (Vilamil-Fernandez et al. 2004), which accounts for their antimalarial (dos Santos et al. 2004) and antileukemic (Cavalcanti et al. 2013) activities. The ability to collapse mitochondrial membrane potential has also been described for atovaquone (Srivastava et al. 1997) and other naphthoquinones active against *P. falciparum* (Schuck et al. 2013), a process that eventually leads to apoptosis or necrosis (Brand et al. 2004). Whether the mechanism of action of these molecules is related to such processes remains to be determined.

The cytotoxicity profiles were similar for all molecules using BGM or HepG2 cell lines. The two-fold differences observed for compounds 12, 15, 19 and 20 may be related to the distinct cellular processes exhibited by these two cells lines, which may have altered their sensitivity to the compounds.

In conclusion, among the 19 compounds evaluated for anti-*P. falciparum* activity, the naphthoquinones derived from lawsone (compounds 2 and 5) showed the highest activities with IC_{50} values < 5 μM. They also showed the highest therapeutic effects which were similar to or higher than that of chloroquine. In addition, the cytotoxicities of the majority of the tested compounds were low. Importantly, compounds 2 and 5 were shown to be active in vivo against malaria, causing a greater than 50% reduction of parasitaemia in mice infected with *P. berghei*. These compounds therefore show promise in the search for new antimalarial agents, with activities similar to that of atovaquone.

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