Activity of Extracts and Isolated Naphthoquinones from *Kigelia pinnata* against *Plasmodium falciparum*

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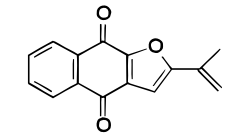
Four naphthoquinoids from *Kigelia pinnata* rootbark were assessed in vitro against chloroquine-sensitive (T9−96) and -resistant (K1) *Plasmodium falciparum* strains and for cytotoxicity using KB cells. 2-(1-Hydroxyethyl)naphtho[2,3-*b*]furan-4,9-dione possessed good activity against both strains [IC_{50} values 627 nM (K1), 718 nM (T9−96)]. Isopinnatal, kigelinol, and isokigelinol exhibited lower activity against both strains.

Isolated plant constituents or their derived structures have been an alternative but valuable source in the search for new antimalarial compounds.[1] The sesquiterpene lactone quinghaosu (artemisinin) isolated from *Artemisia annua* (Asteraceae), and lapachol, a hydroxynaphthoquinone present in South American species of Bignoniaceae,[2] are two examples of plant compounds representing leads for new therapeutics. The further development of artemisinin culminated in artemether with improved pharmacokinetic properties,[3] and lapachol provided the template for the synthetic atovaquone.[1] Both compounds are successfully applied in the therapy of resistant malaria infections.[4,5]

Extracts of *Tabebuia ochracea,* belonging to the Bignoniaceae family, have been assessed for antimalarial activity, and several isolated furanonaphthoquinones showed promising activity in vitro and in vivo against both *Plasmodium falciparum* and *Plasmodium berghei*.[6] However, they were not assessed for cytotoxic effects. In this publication, we report for the first time the antimalarial properties of four extracts of the rootbark of *Kigelia pinnata* DC. (Bignoniaceae) and the subsequent isolation of active compounds. The investigations were of particular interest because the compounds were isolated from the fraction that also showed activity against other protozoal parasites.[7] The 2-(1-hydroxyethyl)naphtho[2,3-*b*]furan-4,9-dione (**1**) was isolated along with three other naphthoquinoids, namely, isopinnatal (**2**), kigelinol (**3**), and isokigelinol (**4**), all unique to *K. pinnata.* None of the compounds has been assessed for antimalarial activity previously. The cytotoxicity of the compounds was also assessed, taking into account that naphthoquinones have been reported to exhibit very strong anti-proliferation properties on mammalian cells,[8] making such assessment of cytotoxic effects of the isolated compounds a necessary part of determining their potential clinical value.



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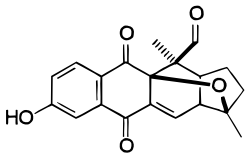

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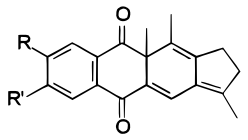

	R	R'
3	OH	H
4	H	OH

Antimalarial activities of the root bark extracts against the chloroquine-resistant strain K1 of *P. falciparum* are summarized in Figure 1. By fragmentation of the dichloromethane (DCM) extract (**B**), which exhibited the greatest antimalarial activity of all tested crude extracts, fraction **B-2** with anti-parasitic constituents was obtained. Further fractionation of **B-2** revealed that **B-2c** showed the clearest antiplasmodial activity. **B-2c** was used for further phytochemical studies, which yielded by bioassay-guided fractionation the furanonaphthoquinone, 2-(1-hydroxyethyl)-naphtho[2,3-*b*]furan-4,9-dione (**1**), and naphthoquinoids isopinnatal (**2**), kigelinol (**3**), and isokigelinol (**4**). Their structures were elucidated and characterized by means of NMR, MS, and co-chromatography with compounds previously isolated.[7,9]

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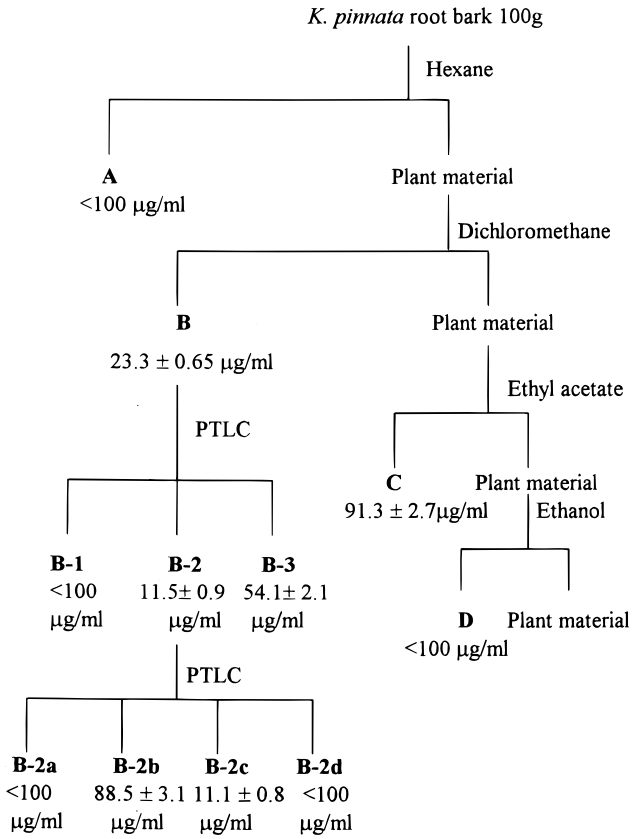

Figure 1. Fractionation scheme for *K. pinnata* rootbark and activity of extracts against *P. falciparum* K1 strain in vitro (mean IC_{50} values $\pm \sigma$; $n = 6$; chloroquine diphosphate $IC_{50} = 0.119 \pm 0.05$ as positive control).

Table 1. In Vitro Activity Against *P. falciparum* Strain K1 and Clone T9−96[a]

compound	IC_{50} value against K1 strain $\pm \sigma$ in μg [μM]	IC_{50} value against T9−96 clone $\pm \sigma$ in μg [μM]
2-(1-hydroxyethyl)naphtho [2,3-*b*]furan-4,9-quinone (**1**)	0.152 ± 0.129 [0.627]	0.174 ± 0.061 [0.718]
isopinnatal (**2**)	0.258 ± 0.107 [0.763]	0.525 ± 0.494 [1.552]
kigelinol (**3**)	5.139± 0.921 [16.66]	4.682 ± 0.896 [15.20]
isokigelinol (**4**)	4.048± 0.793 [15.20]	3.679 ± 1.335 [11.93]
chloroquine diphosphate	0.119 ± 0.053 [0.228]	0.027± 0.009 [0.051]

[a] The mean IC_{50} values of the test compounds and standard drug ($n = 6$, $\pm \sigma$). (n = number of tests performed in two series).

To obtain preliminary information of a possible mode of action and prediction of cross resistance with chloroquine (CQ), all isolated compounds were assessed for activity on the chloroquine-sensitive (CS) clone T9−96 and chloroquine- resistant (CR) strain K1 of *P. falciparum*. These results, recorded in Table 1, confirm the good antiplasmodial activity of furanonaphthoquinones against *P. falciparum* reported by Perez et al.[6] The isolated 2-(1-hydroxyethyl)naphtho[2,3-*b*]furan-4,9-dione (**1**) was found to be the most active pharmacophore, with no significantly different values against CS or CR *P. falciparum*, indicating the absence of in vitro cross-resistance.[10] The 50% inhibition concentration (IC_{50}) of parasite growth was determined to be 627 nM (152 ng/mL) and 718 nM (174 ng/mL) against the K1 strain and T9−96 clone of *P. falciparum*, respectively. However, the compound was three times less effective than the reference compound CQ against the CR strain K1 (CQ with IC_{50} of 288 nM) and 14 times less active on the CS clone T9−96 (CQ with IC_{50} of 51 nM). The value obtained for CQ was consistent with the value previously reported for K1 and T9-96 with the ^{3}[H]-hypoxanthine incorporation technique.[11]

Table 2. Ratios of the In Vitro Cytotoxicity of the Test Compounds to Activity Against *P. falciparum* Clone T9−96 and Strain K1[a]

compound	ED 50 KB cells in μg/mL [μM]	ratio of activity [ED_{50}KB cells/IC_{50} K1]	ratio of activity [ED_{50}KB cells/IC_{50} T9-96]
2-(1-hydroxyethyl)naphtho [2,3-*b*]furan-4,9-quinone (**1**)	0.94 ± 0.06 [3.9]	6.18	5.40
isopinnatal (**2**)	5.01 ± 0.25 [14.8]	19.42	9.54
kigelinol (**3**)	45.61 ± 8.17 [148]	8.88	9.74
isokigelinol (**4**)	51.51 ± 6.43 [167]	12.72	14.00
chloroquine diphosphate	22.65 ± 2.33 [43.9]	190.37	943.75

[a] $n = 6$, $\pm \sigma$ (n = number of tests performed in two series).

Among the tested naphthoquinoids, isopinnatal (**2**) exhibited the most pronounced activity against *P. falciparum*, with IC_{50} values of 763 nM and 1552 nM, respectively, against K1 and T9−96, being only marginally less active than the furanonaphthoquinone (**1**) on the K1 strain. It is noteworthy that isopinnatal was twice as active against the CR K1 strain compared to the CS clone, suggesting a different mode of action from the standard drug. The result further suggests that a full naphthoquinone ring confers the potency to kill parasites effectively, although to a lesser extent than reported for antitrypanosomal activity.[7] Kigelinol (**3**) and isokigelinol (**4**) showed low antimalarial activity, being almost 100 times less potent than CQ. Compared to the structure of plumbagin, a naphthoquinone with good antimalarial activity,[12] the annealed ring decreases electron movement. The possibility to function as an electron carrier[13] or to trigger a radical formation at the quinone structure is therefore reduced and further weakened by the OH group. This precludes a possible induction of a parasitocidal oxidative stress and may explain its weak efficacy. However, no differences were seen between their activities against the two parasite strains tested, suggesting that the position of the hydroxy group on C-8 or C-9 does not influence the effectiveness of the drug.

The assessment of the cytotoxicity of the compound against the human oral epidermoid carcinoma cell line KB summarized in Table 2 confirmed the anti-proliferative properties of naphthoquinones. All compounds exhibited cytotoxicity, the most distinctive being compound **1**, with an ED_{50} value of 3.9 μM (0.90 μg/mL). To predict the selectivity of the established antiplasmodial activity, this was compared with general in vitro cell cytotoxicity.[14] The ratio of cytotoxicity to biological activity for isopinnatal shows clearly that antimalarial efficacy is not due to in vitro cytotoxicity, this being in the range of 10- to 20-fold greater (19.42 for K1 and 9.5 for T9−96) than the IC_{50}. This is encouraging further in vivo investigation. The selectivity of the furanonaphthoquinone was expectantly low with values of 6.18 and 5.4. However, analogues of compound **1** with strong anti-proliferative activity have demonstrated selective toxicity to human cancer cells, whereas DNA damage occurred only at much higher concentrations in normal cells.[15]

The mode of antimalarial action of the furanonaphthoquinone compound **1** is not known. A possible mechanism by inducing oxidative stress, as seen with other naphthoquinones, is questionable, as the fully conjugated ring system of compound **1** does not permit free radicals to be generated. An inhibition of mitochondrial electron transport and respiratory chain by reduced oxygen consumption, as confirmed with atovaquone[16] is likely, as hydroxy-2-(1-hydroxyethyl)naphtho[2,3-*b*]furan-4,9-dione analogues were found to inhibit electron transport in rat liver mitochondria, yet with IC_{50} values around 1000 times higher than the plasmodial inhibitory effect of atovaquone.[17,18] This may suggest that hydroxynaphthoquinones have a much stronger affinity to malarial mitochondria than furanonaphthoquinones by selectively binding to the cavity within the parasitic cytochrome b complex[19] without affecting host mitochondrial functions.[20]

The work reported here further underlines that 2-(1-hydroxyethyl)naphtho[2,3-*b*]furan-4,9-dione (**1**) is a potent antimicrobial agent with antiviral[21] and antitrypanosomal[7] activity. Despite its high toxicity, the structure provides an interesting lead because the synthesis of this natural compound is simple.[22] It is speculated that a minor exchange of the 2-hydroxyethyl group in compound **1** with a methyl group may significantly affect activity as demonstrated for antiviral efficacy.[18] This would increase the lipophilicity of the side chain at the naphthoquinone skeleton and favor the accumulation of the drug in the parasitic mitochondria membrane (D. Warhurst, personal communication).

Experimental Section

Plant Material. Root bark of Zimbabwean *K. pinnata* was authenticated and supplied by Dr. Duguid of Kadoma, Zimbabwe. Voucher specimen KI11R1 for root bark is deposited in the herbarium and museum at King's College, London.

Extraction, Fractionation, and Isolation. The scheme for fractionation is shown in Figure 1. Extraction was carried out as described by Moideen et al.[7] using hexane, DCM, EtOAc, and EtOH as solvents in order to obtain crude extracts **A**−**D**, respectively. Isolation of compounds by means of preparative TLC[7] was undertaken by bioassay-guided fractionation of the active DCM extract B to obtain **B-1**, **B-2**, **B-3**. The final fractionation process involved preparative PTLC separation[7] using **B-2** to yield fractions **B-2a**, **B-2b**, **B-2c**, and **B-2d**. The isolated compounds[7,9] from **B-2c** were identified as 2-(1-hydroxyethyl)naphtho[2,3-*b*]furan-4,9-dione (**1**), isopinnatal (**2**), kigelinol (**3**), and isokigelinol (**4**). All crude extracts, fractions, and isolated compounds were assessed for antiplasmodial activity.

Parasite Culture. Antimalarial activity was assessed against the CR K1 strain and CS T9−96 clone of *P. falciparum*. Both isolates were originally obtained from the WHO Reference Centre in Edinburgh and kept at the London School of Hygiene and Tropical Medicine. The parasites were maintained in a 5% suspension of infected A^+ human red blood cells in RPMI 1640 medium supplemented with 6.9 mg/mL HEPES, 2 mg/mL glucose, 2.33 mg/mL $NaHCO_3$, 50 μg/mL hypoxanthine, 40 μg/mL gentamycin, and 10% A^+ human serum. The medium was changed daily and, prior to sealing the culture flask, was flushed with a gas mixture of 1% O_2, 3% CO_2, and 96% N_2 and kept at 37 °C.[23]

Cell Culture. Toxicity was evaluated on the human oral epidermal carcinoma cell line KB (ECACC, U.K.) The cells were kept in Dulbecco's MEM glutamax-1 + sodium pyruvate + glucose + pyridoxine, complemented with 10% HIFCS. Culture flasks were kept at 37 °C in a 5% CO_2/air mixture.

Test Compounds. The test compounds and extracts were kept at 1 mg/ml in 2% DMSO in RPMI 1640 medium. Prior to use, the compounds were serially diluted in a 96-well microtiter plate. Final concentrations of 100 μg/mL to 411 ng/mL (6 concentrations for crude extracts, 3-fold dilutions) or 50 μg/mL to 1.63 ng/mL (12 concentrations for isolated compounds, 2-fold dilutions) were tested for antimalarial activity. Chloroquine diphosphate was included as a standard drug at concentrations from 2.5 mM to 1.22 nM. For the cytotoxicity assay, the test compounds were dissolved in DMSO and diluted with medium (not exceeding 0.5% DMSO tested) to give final test concentrations from 200 to 0.064 μg/mL in a 5-fold serial dilution.

Antiplasmodial Drug Assay. In vitro drug activity against *P. falciparum* was determined by using a modified lactate dehydrogenase assay;[24,25] 190 μL of blood suspension (1% parasitemia, predominantly ring forms, 2% hematocrit) was dispensed into a 96-well microtiter plate, and 10 μL of the drug solution was added. Additionally, separate wells containing infected and uninfected blood suspensions at 2% hematocrit were used as controls. Solutions of the test substances in unparasitized red blood cell suspension were also included to exclude reduction of NBT (nitro blue tetrazolium) to formazan salt by the compounds themselves. All plates were incubated at 37 °C for 48 h in 1% O_2, 3% CO_2, and 96% N_2 at saturated humidity in a modular incubator. At the end of the incubation period, 20 μL of the parasite suspension was added on a separate microtiter plate to 100 μL of Malstat reagent (Flow Incorporated, USA). The plates were incubated at room temperature for 15 min before adding 20 μL of a 1:1 mixture of NBT (2 mg/mL) and phenazine ethosulfate (0.2 mg/mL) to each well. The plates were protected from light, reincubated for 20 min at room temperature, and read at 650 nm on a microtiter plate reader. All compounds were tested twice in triplicate. The growth of parasites was monitored microscopically with a Giemsa stained thin blood smear. A parasitemia of 3−5% in the control was expected. The IC_{50} calculations were performed using Excel 5 spreadsheet and XL-fit ADD IN (Version 1.02).

Cytotoxicity Assay. Cellular viability in the presence and absence of experimental agents was determined using the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT) microtiter assay.[26] Vigorously grown KB cells in log phase of growth were harvested, washed, and seeded at 25 000 cells/well (100 μL volume) in 96-well microtiter plates. The plates were left for 24 h at 37 °C in 5% CO_2 in air for cell attachment. To each well, 100 μL of the test compound in triplicate or 100 μL of medium was added for the control. All plates were re-incubated at 37 °C for further 48 h before adding 10 μL MTT reagent (5 mg/mL in MEM) to each well. For the blank value, a 10-μL aliquot of the MTT solution was added to an empty outermost well in each plate. After 4 h reincubation, the medium (including blanks) was removed and all wells filled with 200 μL of DMSO. To aid the solubilization of the formazan formed by the reaction of the MTT and the mitochondrial dehydrogenase of the viable cells, the plates were agitated on a rotation platform at room temperature for 15 min. The absorbance of the reaction mixtures with values from the blank subtracted were read at 540 nm on a microplate reader. The mean absorbance value of cells grown in the absence of test compounds was taken as 100% cell survival (control). The percentage of cell growth in each well containing the test compound was calculated by means of the following formula:

$$\frac{A_{(\text{treated cell})}}{A_{(\text{mean of control cell})}} \times 100$$

All results are expressed as mean IC_{50} values ± standard deviation (σ) for each compound and standard drug and are summarized in Table 1.

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