Lupeol Long-Chain Fatty Acid Esters with Antimalarial Activity from *Holarrhena floribunda*

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An ethnopharmacological investigation was conducted among the Baka pygmies of Dja biosphere reserve (Cameroon) to collect information on the antimalarial plants used in their daily life. *Holarrhena floribunda* is one of those plants. Extracts of the stem barks of *H. floribunda* showed remarkable inhibitory activity against drug-resistant strains of *Plasmodium falciparum* at doses of 1.02−18.53 μg/mL when tested in vitro against two parasite clones designated as Indochina (W-2) and Sierra Leone (D-6). The aqueous extract was the most active against Indochina (W-2), with IC_{50} values of 1.02 μg/mL, while the ethanolic extract appeared to be the most active against Sierra Leone (D-6), with an IC_{50} of 4.33 μg/mL. The bioassay-guided fractionation of the neutral fraction of the crude extract led to the isolation of lupeol (**1**) and its three new long-chain fatty acid ester derivatives, namely, 3-*O*-(3′-hydroxyeicosanoyl)lupeol (**2**), 3-*O*-[(2′-(tetracosyloxy)acetyl]lupeol (**3**), and 3-*O*-[(1″-hydroxyoctadecyloxy)-2′-hydroxypropanoyl]lupeol (**4**). These new compounds displayed some in vitro inhibition activity against the chloroquine-resistant strain FCR-3 isolated from Gambia and the chloroquine-sensitive standard strain 3D7. The hydroxy group of the fatty acid side chain appears to decrease the observed activity.

Due to its high morbidity and mortality, human malaria is an infectious disease of enormous importance in tropical countries. Despite the eradication programs that started more than 80 years ago, malaria is still a threat to over 2 billion people living in areas of high incidence. Although the statistics vary widely, it is estimated that there are 200 million infected humans, along with 150 million new cases every year. It is also estimated to cause more than 2 million deaths annually among which half are children under five years old. Recent investigations have reported alarming deterioration in the effectiveness of the conventional antimalarial drugs.[1−5] Furthermore, resistance to artemisinin, which appears to be the most effective and most promising actual antimalarial drug, has been induced in a rodent malaria model and hence may occur naturally.[6] If resistance to artemisinin emerges, no drug will be available that could offer protection against malaria in all regions of the world. Therefore, the need for novel chemotherapeutic agents is acute.

The treatment of malaria started with quinine, a botanical derivative that has been used for more than 1000 years. After several years of incursion in the synthetic drugs, the final compound to replace quinine appears to be another botanical derivative, artemisinin, isolated from Chinese medicinal herb *Artemisia annua.* Nature may still have much to give to the treatment of this disease as well as several other human devastating ailments such as AIDS and cancer.

As part of phytochemical and pharmacological investigations of antimalarial plants among the Baka pygmies of Dja biosphere reserve (Cameroon), a pharmacological screening of aqueous, ethanolic, and chloroformic extracts of the stem of *Holarrhena floribunda* (Apocynaceae) exhibited significant activity against drug-resistant clones of *Plasmodium falciparum* W-2 and D-6. *H. floribunda* is a species known for its amoebicidic, antidysenteric, febrifugic, antiblennoragic, and diuretic activities.[7,8] Commonly used preparations including palm kernel oil extractions, decoctions, and cold concoctions are given to children with febrile convulsion (probably due to cerebral malaria) in Nigeria[9] and to children and adults by Baka pygmies to fight malaria symptoms. Despite extensive phytochemical and pharmacological investigations of *H. floribunda*,[10−15] no antimalarial bioassay-guided isolation of active constituents from neutral fractions of this species has been reported. Here we present the bioassay-guided fractionation of a neutral fraction of *H. floribunda* stem bark, which led to the isolation of lupeol (**1**) and its three new long-chain fatty acid ester derivatives as well as their evaluation on both chloroquine-sensitive and chloroquine-resistant *P. falciparum* strains.

Results and Discussion

Isolation and Characterization of Compounds. Following the fractionation process, the alkaloid constituents of *H. florbunda* stem were removed from the extract, and their purification and evaluation as antimalarials will be reported elsewhere. The non-alkaloid fraction exhibited a moderate antimalarial activity against chloroquine-sensitive *P. falciparum* strain 3D7. This active extract was fractionated by vacuum liquid chromatography (VLC) on silica gel, into six subfractions (F1 to F6), and the fraction F3 (hexane−CH_2Cl_2 (7:3)) appeared to be the most active. A combination of flash silica gel and Sephadex LH-20 column chromatography (CC) of fraction F3, followed by preparative TLC, yielded compounds **1**−**4**. Compound **1** ($C_{30}H_{50}O$, [M^+] 426) displayed ^1H and ^{13}C NMR spectra exhibiting features characteristic of lup-20(29)-en-3β-ol [δ 4.68 (brs), H-29a; δ 4.57 (brs), H-29b; δ 3.19 (dd, J = 4.8 and 11.6 Hz), H-3; δ 79.3, C-3; δ 151.1, C-20; and δ 109.6, C-29] and signals due to seven tertiary methyl groups, which are also reminiscent of a lupeol-type triterpene.[16,17] The structure was confirmed by comparison of spectroscopic data of our compound to those described for lupeol[16,17] and by its mass (EI) spectrum, which displayed characteristic fragment ions at *m*/*z* 426 [M^+], 218, 207, and 189.[18] The three other compounds displayed, in addition to these characteristic features of lupeol, broadened signals of the long-chain fatty acid unit.[16,19,20] The major differences between the NMR data of compound **1** and the three other compounds are the chemical shifts of H-3 and C-3, which, when contrasted, show significant downfield shifts, with $\Delta\delta_H$ 1.29−1.37 ppm and $\Delta\delta_C$ 2.0−2.4 ppm, respectively. Compounds **2**, **3**, and **4** are therefore lupeol derivatives possessing a long-chain fatty acid unit at C-3. This linkage was confirmed by the observed HMBC correlation from the oxygenated methine proton around δ 4.49−4.57 (1H,

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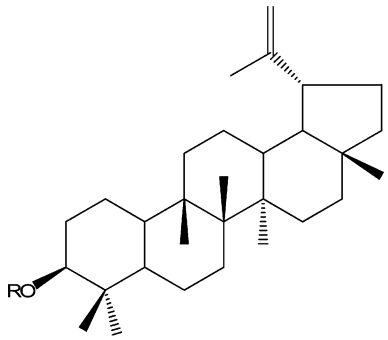
overlapped with H-29b, H-3) to the ester carbonyl carbon at δ_C 170.8−173.0 (C-1′).

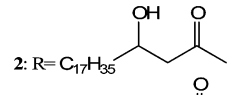
Compound **2** was obtained as a resinous substance showing $[\alpha]^{25}_D$ +7.9 ($CHCl_3$). Its ESIMS spectrum (positive ion mode) displayed a molecular peak at *m/z* 759 ($[M^+ + Na^+]$, $[M^+]$ *m/z* 736), an observation confirmed on its EIMS by the signal at *m/z* 737 ($[M^+ + H]$). Its molecular formula was assigned as $C_{50}H_{88}O_3$ on the basis of HREIMS measurements. In addition to the features characteristic of lupeol mentioned above, a terminal methyl signal at δ 0.82 and a strong methylene proton signal around δ 1.30 indicative of the presence of a fatty acid long chain were observed in its ^{1}H NMR spectrum. These observations were supported by the appearance of ^{13}C signals due to an ester carbonyl group at δ 173.0, a long chain of methylene carbons at δ 24.1−32.3, and a terminal methyl group at δ 14.6. HREIMS spectra indicated a C_{20} fatty acid chain. The ^{13}C NMR spectrum (Experimental Section) indicated the presence of a carbon bearing a hydroxyl group (δ 68.5) in the fatty acid chain, which was attributed to carbon C-3′ according to its ^{1}H−^{1}H COSY, HMQC, and HMBC spectra. In fact, the COSY spectrum displayed a correlation between the two protons at δ 2.98 (H-2′a and H-2′b) and H-3′ at δ 4.02. On the other hand, the HMBC spectrum showed correlations between the protons at δ 2.98 and the carbons at δ 173.0 (C-1′) and 68.5 (C-3′) and correlations between the proton at δ 4.02 and the carbons at δ 173.0 (C-1′), 43.3 (C-2′), and 38.4 (C-4′). These observations confirm the position of the hydroxyl group at C-3′. The absolute configuration of this carbon was not determined. Compound **2** was then deduced as 3-*O*-(3′-hydroxyeicosanoyl)lupeol.

Compound **3** was obtained as a yellowish gum showing $[\alpha]^{25}_D$ +29.0 ($CHCl_3$). It displayed a molecular peak at *m/z* 843 ($[M^+ + Na]$ ($[M^+]$, *m/z* 820) when analyzed by ESIMS, and its formula was assigned as $C_{56}H_{100}O_3$, on the basis of HREIMS measurements. The ^{1}H NMR spectrum showed, along with the typical chemical shifts for lupeol, a pattern of signals attributable to a long-chain fatty acid ester [δ 0.83, overlapped triplet, terminal methyl, and a large broad signal at δ 1.24 ($-CH_2-)_n$]. The main differences between the ^{1}H NMR spectra of **2** and **3** were the appearance of two signals of two protons, a broad singlet at δ 4.24 (H-2′) and a triplet at δ 3.82 (H-1″), in the spectrum of compound **3** as well as the disappearance of the multiplet of one proton at δ 4.02, which in compound **2** is attributed to the presence of H-3′. These observations were supported by the appearance of signals of two oxygenated carbons at δ 69.5 (C-2′) and 72.4 (C-1″) in the ^{13}C NMR spectrum of compound **3** instead of one at δ 68.5 observed in the carbon spectrum of **2**. The HMBC spectrum of **3** displayed correlations between protons at δ 4.24 and the carbonyl carbon at δ 172.2 and the oxygenated carbon at δ 72.4. Correlations between protons at δ 3.82 and one of the carbons of the long-chain fatty acid were also observed, but no correlation between those protons (H-1″) and the carbonyl was present. This suggests that these protons and the carbonyl group are separated by more than three bonds. The fact that the ^{1}H−^{1}HCOSY spectrum of **3** displayed no correlation between H-2′ at δ 4.24 and any other proton proves that they are separated from the rest of the long chain by an oxygen atom. Protons at δ 3.82 are then connected to C-1″ (δ 72.4) and separated from H-2′ by an oxygen atom. This was confirmed by the HMQC spectrum. The rest of the long chain comprised a C_{23} moiety according to HREIMS measurements and confirmed by the presence in the EIMS spectrum of the signals at *m/z* 367 $[CH_3(CH_2)_{23}OCH_2^+]$ and 295 $[CH_3(CH_2)_{20}^+]$. This spectrum even displayed a small but important signal at *m/z* 395 $[CH_3(CH_2)_{23}OCH_2CO^+]$. Compound **3** was then deduced as 3-*O*-[(2′-(tetracosyloxy)acetyl]lupeol.

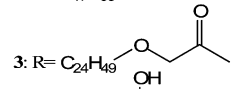
Compound **4** was also isolated as yellowish gum, showing $[\alpha]^{25}_D$ +14.0 ($CHCl_3$). It displayed a molecular peak at *m/z* 805 ($[M^+ + Na]$ ($[M^+]$, *m/z* 782) when analyzed by ESIMS, and its formula was assigned as $C_{51}H_{90}O_5$ according to HRFABMS measurements. Its ^{1}H and ^{13}C NMR spectra were similar to those of compounds **2** and **3**. The ^{1}H NMR spectrum showed, in addition to features of lupeol with a long-chain fatty acid ester, four multiplets at δ 4.86 (1H, m, H-1″), 4.29 (1H, m, H-2′), 4.14 (2H, m, H-3′), and 1.81 (2H, m, H-2″). These observations were confirmed by the ^{13}C NMR spectrum, assisted by HMQC, by the appearance of signals at δ 98.9 (C-1″), 69.1 (C-2′), 68.4 (C-3′), and 36.9 (C-2″). The HMBC spectrum showed correlations between H-3 at δ 4.50 and the C-1′ carbonyl at δ 170.8. Correlations between H-2′ at δ 4.29, H-3′ (δ 4.14), and carbon C-1′ were also observed. The same spectrum displayed correlations between H-2″ at δ 1.81 and C-1″ at δ 98.9, between H-1″ at δ 4.86 and C-2″ at δ 36.9 and C-3′ (δ 68.4). No correlation was observed between H-1″ and C-2′ (δ 69.1) and C-1′ (δ 170.8), indicating that H-1″ and C-2′ are separated by more than three bonds. The chemical shifts of H-1″ and C-1″ are consistent with those of a hemiacetal functionality, indicating that C-1″ is carrying a hydroxyl group and is separated from C-2′ by an oxygen atom. The rest of the fatty acid moiety comprised a C_{16} chain by simple calculation from HRFABMS and confirmed by EIMS, which displayed, in addition to characteristic signal of lupeol, an important but small signal at *m/z* 299 $[CH_3(CH_2)_{16}CH(OH)OCH_2^+]$. This fragment, the only major one obtained from the fatty acid moiety, is indicative of the fragility of the hemiacetal functionality, which was cleaved into small fragments by the high energy of the EIMS, and for the same reason, the HREIMS measurement of this compound was not possible. The absolute configuration of the hydroxylated carbons of the fatty acid moiety was not determined. The structure of **4** was then deduced as 3-*O*-[(1″-hydroxyoctadecyloxy)-2′-hydroxypropanoyl]lupeol.



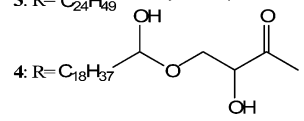
1: R= H



2: R= $C_{17}H_{35}$



3: R= $C_{24}H_{49}$



4: R= $C_{18}H_{37}$

Biological Evaluation. Antimalarial Screening of Crude Extracts. The in vitro assays on crude extracts of the stem of *H. floribunda* were performed by using a modification of the semi-automated microdilution technique described earlier by Desjardins et al.[21] and Milhous et al.[22] Two *P. falciparum* malaria parasite clones, designed as Indochina (W-2) and Sierra Leone (D-6), were utilized in susceptibility testing. The W-2 clone is resistant to chloroquine, pyrimethamine, and sulfadoxine, and the other clone is resistant to mefloquine.[23] The tested extracts were dissolved in H_2O or in a mixture of H_2O−DMSO and serially diluted with media. The uptake of [^{3}H]-hypoxanthine monohydrochloride was used as an index of inhibition of parasite growth. Chloroquine, mefloquine, and quinine were used as reference drugs. The results of the in vitro screening of various extracts of the stem bark of *H.*

Table 1. In Vitro Antimalarial Activity of *H. floribunda* Stem Extracts against W-2 and D-6 Clones of *P. falciparum*

	IC_{50} (μg/mL) ± SD	
type of extract	W-2 clone	D-6 clone
$CHCl_3$	2.29 ± 0.91	18.53 ± 3.54
EtOH	5.22 ± 0.13	4.33 ± 0.25
H_2O	1.02 ± 0.01	5.91 ± 0.98
neutral fraction	23.40 ± 2.95	28.76 ± 3.55
Reference Drugs		
mefloquine	0.0285 ± 0.00	2.55 ± 0.03
chloroquine	1.95 ± 0.15	0.021 ± 0.01
quinine	0.067 ± 0.01	0.025 ± 0.00

floribunda are shown in Table 1. All the extracts inhibited the uptake of hypoxanthine by the plasmodia at low concentrations, with IC_{50}'s ranging from 1.02 to 18.53 μg/mL. The aqueous extract appeared to be the most active against the W-2 strain followed by the chloroformic extract, while the ethanolic extract was the least active, but appeared to be the most active against the D-6 strain, with an IC_{50} of 4.33 μg/mL.

Antimalarial Evaluation of Isolated Compounds. The antimalarial evaluation of the isolated compounds (**1**−**4**) was conducted following almost the same method as for the crude extracts but on different strains, as they were tested on chloroquine-sensitive standard *P. falciparum* clone 3D7 and the chloroquine-resistant strain FCR-3 isolated from Gambia. Chloroquine was used here as reference drug, and the results in Figure 1a show the effect of increasing concentrations of CQ on the growth of 3D7 and FCR-3 strains, with IC_{50}'s of 9.09 and 81.81 nM, respectively. FCR-3 displays roughly a 9-fold increase in IC_{50} to CQ. Figures 1b−e show the effect of increasing concentrations of compounds **1**−**4** on the growth of 3D7 and FCR-3 strains, respectively. The IC_{50}'s of all the tested drugs are reported in Table 2. With the exception of compound **4**, which was inactive (IC_{50} > 500 μM), the tested compounds appeared to inhibit growth of both the chloroquine-sensitive and -resistant strains with almost the same IC_{50} for each compound, with a slightly higher activity against the resistant strain [**1**: IC_{50} = 97 μM (FCR-3), 106 μM (3D7); **2**: IC_{50} = 269 μM (FCR-3), 282 μM (3D7)]. The results obtained for lupeol (**1**) are consistent with previously characterized antimalarial effects as found by Khalid et al.[24] The only noticeable difference in the inhibition of the two strains was observed with compound **3** [IC_{50} = 84 μM (FCR-3), 135 μM (3D7)], which appeared to be more active against the chloroquine-resistant than the -sensitive strain.

The fact that all the tested compounds inhibited the chloroquine-sensitive and chloroquine-resistant strains in the same manner suggests that there may be different drug action mechanisms for the lup-20(29)-ene derivatives and chloroquine, but certainly indicates that the chloroquine resistance antiporter does not transport the lup-20(29)-ene type of compounds out of the digestive vacuole, if they accumulate there.

The invasion of erythrocytes by *P. falciparum* merozoites is a key step in the pathogenesis of the malaria disease since it is only after that phase of the parasite's life cycle that the first symptoms appear. In the first step, the merozoite attaches reversibly to the erythrocyte surface followed by apical reorientation and formation of an irreversible junction. This is followed by a parasitophorous vacuole stage and, finally, entry into the vacuole by movement of the junction and resealing of the vacuolar and erythrocytic membranes.[25] According to Ziegler et al.,[26] the in vitro inhibitory activity of lupeol against the *P. falciparum* 3D7 strain is associated with a transformation of the erythrocyte shape toward that of cup-shaped stomatocytes. They observed a good correlation between the IC_{50} value and the membrane curvature caused by lupeol at different concentrations. In their investigations, preincubation of erythrocytes with lupeol, followed by extensive washing, made the cells unsuitable for the parasite growth, suggesting that the compound incorporates into the erythrocyte membrane irreversibly. On the other hand, lupeol-treated parasite culture continued to grow well in untreated erythrocytes. Similar results were obtained with lupeol analogues (betulinic acid, betulinic aldehyde, betulin, etc.).[27] They concluded that the antiplasmodial activity of this type of compounds was indirect, exclusively due to stomatocytic transformation of the host cell membrane and not to toxic effects via action on a drug target within the parasite.[27] Their activity should then be due to the incorporation of the compounds into the lipid bilayer of erythrocytes that may cause the modification of cholesterol-rich membrane rafts, recently shown to be important in parasite vacuolization.[27] Therefore, the three new compounds (**2**, **3**, and **4**), as they are lupeol derivatives, might act in the same mechanistic manner, and the difference observed in the inhibition of different strains with compound **3** may be due to the difference in terms of interaction with the erythrocyte membrane and not to any difference of mode of action. To date, the nature of this difference in interaction with the erythrocyte membrane remains unclear. Thus, an in vivo testing could be a better option since it might provide more information not only on the nature of the interactions with the membranes but also on the possible toxicity of the compounds. In vivo testing is planned in our laboratory in order to check if the combination of those compounds interacting with the erythrocyte membranes and its shape change toward stomatocyte could result in better activity.

It is important to notice that in the new naturally occurring long-chain fatty acids ester derivatives of lupeol the side chain has a positive effect on the antiplasmodial activity, as compound **3** appeared to be more active than the lupeol. However, the activity in this case is markedly affected when the side chain is substituted with hydroxyl groups. Compound **3**, the most active of all the tested compounds, has no hydroxyl group in the side chain, followed by compound **1**, which has no side chain at all. Compound **2**, having one hydroxyl group on the side chain, was less active than compounds **1** and **3**, and compound **4**, with two hydroxyls groups on the side chain, was inactive, although its side chain was similar to compound **3**'s. However, the low number of our samples does not enable any systematization of these observations and further structure−activity studies are necessary.

This investigation confirms the antimalarial activity of *H. floribunda*, a plant used by the Baka pygmies of Dja biosphere reserve of Cameroon, to fight children's febrile convulsions and malaria in adults. The low activity of the isolated compounds compared to those of the crude extracts indicates that those compounds alone are not solely responsible for the antimalarial activity of the stem bark of *H. floribunda*, and in fact might not even be an important part. Therefore, the study of the alkaloidic fraction of the same plant is in progress in our laboratory. However, there may be synergistic effects with other components of these complex mixtures, effects that can be lost when the components are tested individually. In the present report, we also found that the new long-chain fatty acid ester derivatives of lupeol possess inhibitory activity on both chloroquine-sensitive and chloroquine-resistant strains, almost at the same level. Rather than direct toxic interaction with a drug target in the parasite, the antiplasmodial activity of these types of compounds might be associated with the membrane modification of the host cells. Although the established link between erythrocyte membrane modification and antiplasmodial activity may provide a novel target for potential antimalarial drugs, the case of lup-20(29)-ene type compounds seems to be unsuitable to the development of new drugs, as they interact with the membrane in an irreversible manner. However, the nature and the manner of this interaction are still to be confirmed by in vivo testing. The structure−activity relationships observed during this investigation may be useful in understanding the antiplasmodial activity of these types of compounds and indicative of the influence that a long fatty acid ester side chain may have on this particular antiplasmodial mode of action.

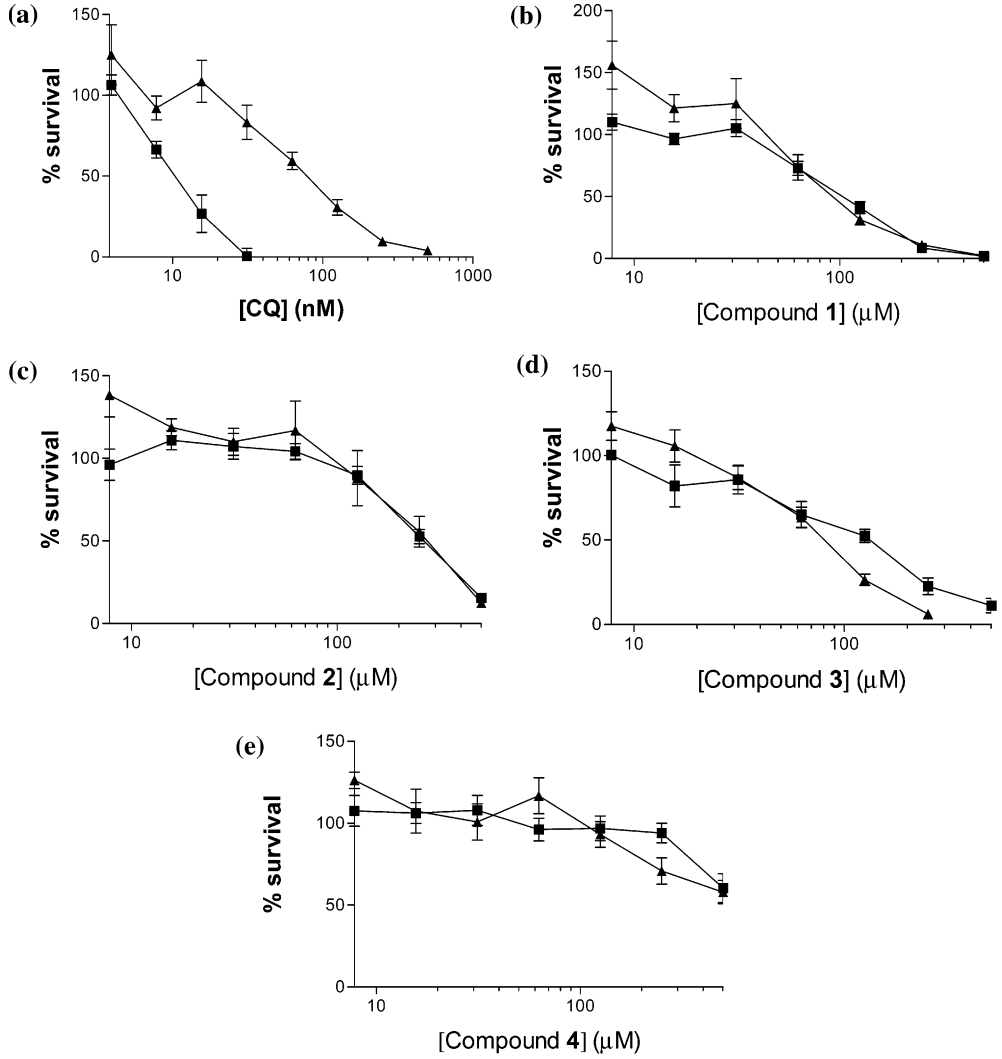


Figure 1. Effects of increasing concentrations of drugs on the in vitro growth of chloroquine-sensitive (3D7) and -resistant (FCR-3) strains of *Plasmodium falciparum.* Parasitized red blood cells were exposed for 48 h to increasing concentrations of chloroquine diphosphate (3.9−500 nM) (a), **1** (7.8−500 μM) (b), **2** (7.8−500 μM) (c), **3** (7.8−500 μM) (d), and **4** (7.8−500 μM) (e). The incorporation of [^{3}H]-hypoxanthine was used to measure the effects of each drug on survival of 3D7 (■) and FCR-3 (▲) strains. Results are expressed as % survival as compared to control, in the absence of added drugs. Each graph represents the means of experiments done 2−3 times with each drug concentration done in quadruplicate.

Table 2. In Vitro Antimalarial Activity of Compounds **1**−**4** against the Chloroquine-Sensitive 3D7 and Chloroquine-Resistant FCR-3 Strains of *P. falciparum*

	3D7		FCR-3	
compound	IC_{50} (μM)	IC_{50} (μg/mL)	IC_{50} (μM)	IC_{50} (μg/mL)
1	106	45	97	41
2	282	208	269	198
3	135	111	84	69
4	>500	>391	>500	>391
CQ	0.009	0.004	0.081	0.042

Experimental Section

General Experimental Procedures. Melting points were determined by differential scanning calorimetric techniques on a Thermal Analysis (TA) Instruments DSC 2010 (differential scanning calorimeter). Optical rotations were recorded on a JASCO Model DIP-140 digital polarimeter. UV spectra were recorded in $CHCl_3$ on an HP 8453 UV−visible spectrophotometer, and FTIR (KBr pallet) spectra were measured on an ABB-Bomem, MB series spectrophotometer. ESI spectra were obtained on a Finnigan LCQ^{DUO} and EI-MS, HREIMS, and HRFABMS on Kratos MS25RFA or Kratos analytical spectrometers. ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), COSY, HMQC, and HMBC spectra were recorded in $CDCl_3$ on a Varian Oxford-400 spectrometer. The following Merck chromatographic supports were used: Si gel 230−400 mesh for column and silica gel 60 F_{254} plates for analytical TLC. The lipophilic Sephadex LH-20 was from Sigma and the Si gel GF plates (150 μm, 0.25 and 2 mm thick) for preparative TLC were from Analtech, Uniplate. Chloroquine diphosphate and quinine sulfate were from Sigma-Aldrich, and mefloquine hydrochloride was from Hoffman La Roche.

Materials and Methods. Plant Material. *H. floribunda* stem bark was collected in April 2004 in the Dja biosphere reserve, South Province of Cameroon, and identified at the National Herbarium of Cameroon at Yaoundé, where voucher specimens are deposited (Nos. 29763HNC, 49821HNC, and 7789HNC).

Parasite Culture and Growth Inhibition Essays. The screening of crude extracts was performed by the Kenyan Institute of Medicinal Research of Nairobi. Cultures of the D-6 and W-2 clones were maintained in modified candle jars as described previously by Trager and Jensen.[28] The in vitro efficacies of each extract against *P. falciparum* clone strains D-6 and W-2 were determined by assessing [^{3}H]-hypoxanthine incorporation.[29,30] Briefly, dilutions of each extract at concentrations ranging from 5×10^{-4} to 3×10^{-9} M in DMSO or H_2O were prepared from stock solutions and added in triplicate to wells of sterile flat-bottom 96-well microtiter plates (100 μL, Disposable Products, Adelaide, Australia). Parasite cultures, diluted to a standard 2% parasiteamia and 4% haematocrit, were added to the wells (total volume 200 μL). Cultures were labeled with 0.5 μCi of [^{3}H]-hypo-

xanthine (10 μL/well, Amersham, Australia), incubated for 48 h, harvested, and counted. Data are presented as mean percentage growth inhibition ± SD compared to untreated controls for quadruplicate experiments. The concentrations required to inhibit parasite growth by 50% (IC_{50}) were determined by linear interpolation.[31] Mefloquine, quinine, and chloroquine were used as reference drugs.

For testing the isolated compounds, *P. falciparum* strains 3D7 and FCR-3 (kindly provided by Dr. E. Schurr at the Centre for the Study of Host Resistance, McGill University) were grown in continuous culture as previously described by Trager and Jenson.[32] Washed human erythrocytes (type B^+) from freshly drawn blood were suspended in culture medium (RPMI-1640 from Gibco supplemented with 0.5% Albumax II, 0.32 mM hypoxanthine, 2 mM L-glutamine, 25 mM HEPES, 24 mM $NaHCO_3$, 11 mM glucose) at 5% hematocrit and inoculated with infected erythrocytes. The flasks were incubated in 20 mL of parasite suspension at 37 °C in a T-75 tissue culture flask by candle jar method.[32] FCR-3 was grown in a 5% CO_2 incubator. Both cultures underwent daily changes of medium. The percentage of infected cells (parasitemia) was determined microscopically in thin, Giemsa-stained smears. To assess the growth of chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* (3D7 and FCR-3, respectively), the Desjardins radioisotope method was adopted.[21] Briefly, parasite cultures were washed twice in 10 mL of culture medium (RPMI-1640 from Gibco supplemented with 0.5% Albumax II, 16 μM hypoxanthine, 2 mM L-glutamine, 25 mM HEPES, 24 mM $NaHCO_3$, 11 mM glucose) and diluted to 2% parasitemia in 4% hematocrit (type B^+) in 100 μL of the culture medium and added to each well of 96-well plates. After 30 min incubation at 37 °C, 100 μL of the above culture media containing increasing concentrations of various drugs was added (final dilution; parasitemia 1%, hematocrit 2%) to each well of the 96-well plates and incubated for 24 h at 37 °C. The effect of drugs on the growth of the parasite was assessed through the incorporation of radiolabeled hypoxanthine. Briefly, 20 μL (25 μCi/mL) of [^{3}H]-hypoxanthine monohydrochloride was added to each well of the 96-well plates, and incubation continued for an additional 18−24 h. The parasitized RBCs were harvested on glass fiber filters (Wallac Printed Filtermat A) using the Packard Cell Harvester (FilterMate 96-well), in distilled H_2O as a wash medium. Each filter well was transferred to a separate vial containing 30 mL of scintillation fluid (Betaplate). [^{3}H]-Hypoxanthine monohydrochloride accumulation was determined by fluorometry using the Perkin-Elmer 1450 Microbeta counter. The 50% inhibitory concentration (IC_{50}) values were determined using Prism software (version 3.02). Each concentration of the test substance was tested 2−3 times in quadruplicate. The maximum final concentration of solvents (namely DMSO) was under 2%, and reference wells contained DMSO in the concentration of 0.5%. Chloroquine was used as a reference drug for all experiments.

Extraction and Isolation. The air-dried stem of *H. floribunda* (1 kg) was ground and macerated in a mixture of CH_2Cl_2−MeOH (1:1) for 24 h at room temperature and extracted twice. The resulting extract (103.5 g) was dissolved in MeOH−H_2O (9:1) and extracted with petroleum ether to remove fat. The aqueous residue was treated with 2 N HCl and extracted with $CHCl_3$ to yield the neutral phase. The acid aqueous layer was adjusted to pH 8 with 25% NH_4OH and extracted once to obtain the alkaloidic fraction (35.3 g), as indicated by TLC analysis (hexane−EtOAc−Et_2NH, 24:75:6, Dragendorff spray). The neutral fraction was further washed with H_2O to yield a nonalkaloidic fraction (21.3 g), which displayed a significant antimalarial activity. A 21.3 g portion of this extract was fractionated by VLC on silica gel using a hexane−EtOAc gradient system. The eluate with hexane−EtOAc (95:5 to 90:10) was further purified on Sephadex LH-20 [MeOH−CH_2Cl_2 (1:1)] to afford compound **1** (451 mg). The fraction eluted with hexane−EtOAc (85:15 to 80:20) was chromatographed on Sephadex LH-20, with MeOH−CH_2Cl_2 (1:1), and the different fractions were purified by preparative TLC (hexane−EtOAc 80:20) to yield compounds **2** (108.6 mg) and **3** (319 mg) and (hexane−EtOAc 70:30) compound **4** (379 mg).

Lupeol (1): white microcrystalline powder; mp 213.0 °C (ΔH = 17.2 kJ mol^{-1}) (lit.[18] 212−214 °C); $[\alpha]^{25}_D$ +25.7 (*c* 0.70 in $CHCl_3$) [lit.[18] $[\alpha]^{25}_D$ +26.2 (*c* 0.67 in $CHCl_3$)]; UV($CHCl_3$) $\lambda_{max}(\epsilon)$ 228(60.1), 285(31.8) nm; IR (KBr) ν_{max} 3326, 2931, 1631, 1450, 1377, 1035, 874; EIMS *m*/*z* (%) 425(18) [M^+ − H], 409(23) [M^+ − OH] 218(68), 207-(60), and 189(100); ^{1}H NMR ($CDCl_3$) δ 0.77, 0.80, 0.84, 0.95, 0.97, 1.03 and 1.70 (each 3H, s, H-23, 24, 25, 26 27, 28, and 30), 2.38 (1H, dt, J = 4.0 and 9.6 Hz, H-19), 3.19 (1H, dd, J = 4.8 and 11.6 Hz, H-3), 4.57 (1H, brs, H-29b), 4.68 (1H, brs, H-29a); ^{13}C NMR ($CDCl_3$) δ 39.1, 27.8, 79.3, 39.2, 55.6, 18.7, 34.6, 41.2, 50.7, 37.5, 21.3, 25.5, 38.4, 43.2, 27.8, 35.9, 43.4, 48.3, 48.6, 151.1, 30.2, 40.4, 28.4, 15.8, 16.5, 16.3, 14.9, 18.4, 109.6, 19.7 (C-1−C-30, respectively).

3-*O*-(3′-Hydroxyeicosanoyl)lupeol (2): yellowish gum; $[\alpha]^{25}_D$ +7.9 (*c* 0.76 in $CHCl_3$); UV($CHCl_3$) $\lambda_{max}(\epsilon)$ 231(620.7), 281(199.4) nm; IR (KBr) ν_{max} 3551, 2915, 2847, 1728, 1463, 1382, 1253, 976, 875; HREIMS (*m*/*z*) 736.67430 (calcd for $C_{50}H_{88}O_3$, 736.67335); ESIMS *m*/*z* 759 [M^+ + Na]; EIMS *m*/*z* (%) 737(9) [M^+ + H], 709(10) [M^+ − 28], 425(13) (lupeol), 409(17), 311(7) [$CH_3(CH_2)_{16}CH(OH)CH_2CO^+$], 239(47) [$CH_3(CH_2)_{16}^+$], 218(100), 208(53), and 189(79); ^{1}H NMR ($CDCl_3$) δ 0.83, 0.88, 0.95, 0.98, 0.99, 1.07, and 1.74 (each 3H, s, H-23, 24, 25, 26 27, 28, and 30), 0.86 (3H, t overlapped, H-20′), 1.29−1.31 (brs, H-4′−H-19′), 2.45 (1H, m, H-19), 2.98 (2H, m, H-2′), 4.02 (1H, m, H-3′), 4.57 (1H, overlapped, H-3), 4.58 (1H, overlapped, H-29b), 4.73 (1H, brs, H-29a); ^{13}C NMR ($CDCl_3$) δ 38.7, 28.4, 81.7, 40.3, 55.7, 18.5, 34.5, 41.9, 50.6, 37.4, 23.1, 25.9, 38.1, 43.2, 27.8, 35.9, 43.2, 48.3, 48.6, 151.1, 30.0, 41.2, 29.8, 16.4, 17.0, 16.5, 14.9, 18.4, 109.6, 19.7 (lupeol C-1−C-30, respectively), 173.0, 43.3, 68.5, 38.4 (fatty acid C-1′−C-4′, respectively), 24.1−32.3 (fatty acid C-5′−C-18′), 21.3 and 14.6 (fatty acid C-19′−C-20′).

3-*O*-[(2′-(Tetracosyloxy)acetyl]lupeol (3): yellowish gum; $[\alpha]^{25}_D$ +29.0 (*c* 0.62 in $CHCl_3$); UV ($CHCl_3$) $\lambda_{max}(\epsilon)$ 228(253.5), 243(201.7), 283(116.6) nm; IR (KBr) ν_{max} 2923, 2847, 1720, 1467, 1374, 1273, 976, 798; HREIMS (*m*/*z*) 820.73483 (calcd for $C_{56}H_{100}O_3$, 820.73354); ESIMS *m*/*z* (%) 843(100) [M^+ + Na]; EIMS *m*/*z* (%) 821(5) [M^+ + H], 806(13) [M^+ − CH_2], 468(73) [lupeol+$COCH_3$], 426(29) [lupeol], 409(81) [lupeol-OH], 395(4) [$CH_3(CH_2)_{23}OCH_2CO$], 367(13) [$CH_3(CH_2)_{23}OCH_2$], 295(23) [$CH_3(CH_2)_{20}$], 218(64), 207(55), 189(100); ^{1}H NMR ($CDCl_3$) δ 0.77, 0.82, 0.84, 0.86, 0.92, 1.37, and 1.66 (each 3H, s, H-23, 24, 25, 26 27, 28, and 30), 0.83 (3H, t overlapped, H-24″), 1.19−1.38 (brs, H-2″−H-23″), 2.45 (1H, m, H-19), 3.82 (2H, t, H-1″), 4.24 (2H, brs, H-2′), 4.50 (1H, t, J = 7.6 Hz, H-3), 4.52 (1H, d, J = 4.4 Hz, H-29b), 4.66 (1H, d, J = 4.4 Hz, H-29a); ^{13}C NMR ($CDCl_3$) δ 38.7, 28.3, 81.7, 40.3, 55.6, 18.6, 34.5, 42.5, 50.6, 37.3, 23.1, 25.8, 38.3, 43.1, 27.7, 35.9, 43.3, 48.3, 48.6, 150.8, 30.0, 41.1, 29.7, 16.3, 17.0, 16.5, 14.8, 18.4, 109.6, 19.7 (lupeol C-1−C-30, respectively), 172.2, 69.5, 72.4 (fatty acid C-1′, C-2′, and C-1″, respectively), 24.1−38.2 (fatty acid C-2″−C-22″), 21.3 and 14.6 (fatty acid C-23″−C-24″).

3-*O*-[(1″-Hydroxyoctadecyloxy)-2′-hydroxypropanoyl]lupeol (4): yellowish gum; $[\alpha]^{25}_D$ +14.0 (*c* 0.72 in $CHCl_3$); UV ($CHCl_3$) $\lambda_{max}(\epsilon)$ 229(276.6), 283(122.2) nm; IR (KBr) ν_{max} 3491, 2919, 2847, 1736, 1700, 1458, 1378, 967, 875; HRFABMS (*m*/*z*) 782.65486 (calcd for $C_{51}H_{90}O_5$, 782.65398); ESIMS *m*/*z* (%) 805(100) [M^+ + Na] ([M^+], 782); EIMS *m*/*z* (%) 468(7) [lupeol+$COCH_3$], 426(100) [lupeol], 299-(7) [$CH_3(CH_2)_{16}CH(OH)OCH_2$], 218(57), 207(53), 189(59); ^{1}H NMR ($CDCl_3$) δ 0.79, 0.86, 0.89, 0.94, 1.03, 1.36 and 1.69 (each 3H, s, H-23, 24, 25, 26 27, 28, and 30), 0.85 (3H, t overlapped, H-18″), 1.25−1.27 (brs, H-3″−H-17″), 1.81 (2H, m, H-2″), 2.50 (1H, m, H-19), 4.14 (2H, m, H-3′), 4.29 (1H, m, H-2′), 4.50 (1H, t, J = 7.6 Hz, H-3), 4.57 (1H, brs, H-29b), 4.69 (1H, brs, H-29a), 4.86 (1H, m, H-1″); ^{13}C NMR ($CDCl_3$) δ 38.7, 28.3, 81.3, 40.3, 55.7, 18.6, 34.5, 42.0, 50.6, 37.4, 23.1, 25.4, 38.4, 43.1, 27.8, 35.9, 43.3, 48.3, 48.6, 151.1, 30.0, 41.2, 29.7, 16.4, 17.0, 16.6, 14.9, 18.4, 109.6, 19.7 (lupeol C-1−C-30, respectively), 170.8, 69.1, 68.4, 98.9, 36.9 (fatty acid C-1′, C-2′, C-3′, C-1″, and C-2″, respectively), 24.1−32.3 (fatty acid C-3″−C-16″), 21.3 and 14.5 (fatty acid C-17″−C-18″).

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Note Added after ASAP Publication. In the version of the paper published on the Web Dec 16, 2005, an author's name was given incorrectly (David S. Bohle). The version that now appears is correct.

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