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In-vitro and in-vivo antimalarial activity of caffeic acid and some of its derivatives

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Abstract

Objectives To explore the in-vitro and in-vivo antimalarial potential of caffeic acid and derivatives.

Methods Two common phenolic acids (caffeic acid and chlorogenic acid) were evaluated for in-vitro and in-vivo antiplasmodial activity in comparison with some semi-synthetic derivatives that were synthesized. An in-vitro assay based on plasmodial lactate dehydrogenase activity, and the classical in-vivo 5-day suppressive test from Peters on an artemisinin-resistant *Plasmodium berghei* strain was used. Parasitic stage sensitivity to ethyl caffeate was determined in this work.

Key findings Phenolic acid esters derivatives showed better antiplasmodial activity than corresponding phenolic acids. The derivative with the highest in-vitro activity being caffeic acid ethyl ester, exhibiting an IC_{50} = 21.9 ± 9.4 μM. Ethyl caffeate and methyl caffeate were then evaluated for antimalarial activity *in vivo* and ethyl caffeate showed a growth inhibition of 55% at 100 mg/kg. Finally, it seems that ethyl caffeate blocks the growth of young parasitic forms.

Conclusions Our study provides evidence for an antimalarial potential of caffeic acid derivatives which are common in several medicinal plants traditionally used against malaria. It also demonstrates the possibility to use such derivatives in the treatment of malaria.

Introduction

Malaria is a major public health problem worldwide, especially in tropical and subtropical countries. The global number of malaria cases and deaths was estimated to have decreased since 2005 due to the expansion of access to rapid diagnostic tests, quality assured artemisinin combination therapy, known as ACT (311 million courses were produced in 2015) and increased funding for malaria control programs (US$ 2.9 billion in 2015).[1] However, malaria is still the most deadly parasitic disease in the world, with an estimated 216 million cases in 2016.[1] For the first time since long ago, the number of registered cases has not decreased between 2015 and 2016 (from 212 to 216 million cases). Children, pregnant women, HIV patients and travellers to sub-Saharan African countries are particularly at risk of severe malaria, if infected with *Plasmodium falciparum*. Malaria remains a major killer of under-fives, claiming the life of one child every 2 min.[1,2]

The emergence and rapid spread of antimalarial drug-resistant strains of *P. falciparum*, of insecticides resistant mosquitoes and the absence of effective vaccine (Mosquirix vaccine currently evaluated shows only a protection of about 30%)[3] made it more difficult to fight against this scourge. This alarming situation highlights the urgent need to discover new antimalarial molecules. Natural phenolic compounds, commonly found in various plants, and some of their simple esters are known for several biological activities, such as anti-inflammatory,[4,5] antioxidant,[6] anti-diabetic,[7] anti-carcinoma,[8] antibacterial[9] and immunomodulatory.[10] Among phenolic metabolites, caffeic acid and derivatives (such as chlorogenic) are the most frequently found and could be

present in high quantities in some plants used as antimalarial traditional medicines, but also in foods (coffee beans, thyme, mint, wine, several fruits and vegetables. . .).[11,12]

Caffeic acid or simple derivatives have never been evaluated for antimalarial activity, even if present in numerous medicinal plants traditionally used as antimalarials. Having found in our previous works on antimalarial traditional plants a moderate antimalarial activity for caffeic acid that could explain in part the activity of some traditional medicines (data not published), we wanted, in this publication, to confirm this finding and to evaluate the in-vitro and particularly in-vivo activity of some semi-synthesized close derivatives to caffeic acid. For in-vivo activity assays, an artemisinin-resistant *Plasmodium berghei* strain was developed, to demonstrate the potential of evaluated compounds against such resistant strains. This study shows for the first time that caffeic acid ethyl ester possess in-vitro antiplasmodial activity against *P. falciparum* and in-vivo antimalarial activity against *P. berghei* in mice.

Materials and Methods

Chemicals and reagents

All chemical solvent and reagent were purchased from LiChrosolv, Merck Germany and HiPerSolv Chromanorm, VWR Chemicals EC, abcr, Acros Organics, Alfa Aesar, Fisher Chemical, Janssen Chimica, Merck, Sigma-Aldrich.

General experimental procedures

Melting points were determined on a Stuart SMP3 capillary apparatus and are uncorrected. The ^{1}H NMR spectra were taken on a Bruker Advance 500 (500 MHz) instrument equipped with cryoprobe in DMSO-d_6 with TMS as an internal standard; chemical shifts are reported in δ values (ppm) relative to internal TMS. The abbreviation s = singlet, d = doublet, t = triplet, q = quadruplet, quint = quintuplet, m = multiplet, and b = broad are used throughout. Elemental analyses (C, H, N, S) were realized on a FlashEA 1112 series (Thermo-Interscience, Louvain-la-Neuve, Belgium) and were within 0.4% of the theoretical values. All reactions were routinely checked by TLC on silica gel Merck 60F254. MS detection was carried out using Micromass ESI-Q-TOF II instrument using ESI ionization in the positive mode (Waters, Zellik, Belgium). The UV spectra were acquired using a U-2910 spectrophotometer.

Fischer esterification (for 2a-g)

A solution of the suitable carboxylic acid **1**-**2** (for instance **1**, 200 mg, 1.11 mmol) or methyl ester **3** in the appropriate alcohol (8 ml) was heated under reflux with concentrated sulfuric acid (0.2 ml). After 2 h, the mixture was cooled down and ethyl acetate was added. The resulting organic phase was washed by a $NaHCO_3$ aqueous solution (5% w/v), water and then dried over anhydrous magnesium sulfate. After filtration of the drying agent, removal of the solvent under vacuo gave the pure expected compound.

Methyl caffeate (2a)

Yield = 95%. mp = 155.6–158.9 °C (lit.: 158 °C); ^{1}H NMR (DMSO-d_6) δ 3.69 (C*H*$_3$, s, 3H), 6.27 (Ar-CH = C*H*, d, J = 15.9 Hz, 1H), 6.76 (5-*H*, d, J = 8.1 Hz, 1H), 7.00 (6-*H*, d, J = 8.2 Hz, 1H), 7.03–7.09 (2-*H*, m, 1H), 7.48 (Ar-C*H* = CH, d, J = 15.9 Hz, 1H), 9.37 (Ar-O*H*, bs, 2H). Anal. ($C_{10}H_{10}O_4$) theoretical: C, 61.85; H, 5.19. Found: C, 61.97; H: 5.32. MS m/z: 193 $[M]^+$, 161, 134. UV_{max}: 218, 295 et 324 nm.

Ethyl caffeate (2b)

Yield = 84%. mp = 140.8–143.1 °C (lit. 143–146 °C); ^{1}H NMR (DMSO-d_6) δ 1.24 (CH$_2$C*H*$_3$, t, J = 7.1 Hz, 3H), 4.15 (C*H*$_2$CH$_3$, q, J = 7.1 Hz, 2H), 6.25 (Ar-CH = C*H*, d, J = 15.9 Hz, 1H), 6.75 (5-*H*, d, J = 8.2 Hz, 1H), 7.00 (6-*H*, d, J = 8.1 Hz, 1H), 7.04 (2-*H*, d, J = 2.1 Hz, 1H), 7.47 (Ar-C*H* = CH, d, J = 15.9 Hz, 1H), 9.61 (Ar-O*H*, bs, 2H). Anal. ($C_{11}H_{12}O_4$) theoretical: C, 63.45; H, 5.81. Found: C, 63.79; H, 5.91. MS m/z: 208 $[M]^+$, 179, 161; UV_{max}: 218, 295 et 324 nm.

Isopropyl caffeate (2c)

Yield = 62%. mp = 129.8–135.0 °C (lit. 141–143 °C); ^{1}H NMR (DMSO-d_6) δ 1.24 (CH(C*H*$_3$)$_2$, d, J = 6.3 Hz, 6H), 4.99 (C*H*(CH$_3$)$_2$, q, J = 6.2 Hz, 1H), 6.22 (Ar-CH = C*H*, d, J = 15.9 Hz, 1H), 6.75 (5-*H*, d, J = 8.1 Hz, 1H), 6.99 (6-*H*, dd, J = 2.1/8.2 Hz, 1H), 7.03 (2-*H*, d, J = 2.1 Hz, 1H), 7.40 (Ar-C*H* = CH, d, J = 15.9 Hz, 1H), 9.35 (Ar-O*H*, bs, 2H). Anal. ($C_{12}H_{14}O_4$) theoretical: C, 64.85; H, 6.35. Found: C, 64.78; H, 6.36.

Ethyl cinnamate (2f)

Yield = 48%. Oil. ^{1}H NMR (DMSO-d_6) δ 1.25 (CH$_2$CH$_3$, t, J = 7.1 Hz, 3H), 4.18 (CH$_2$C*H*$_3$, q, J = 7.1 Hz, 2H), 6.50 (Ar-CH = C*H*, d, J = 16.1 Hz, 1H), 7.43 (3-H/4-H/5-H, m, 3H), 7.67 (Ar-C*H* = CH, d, J = 16.0 Hz, 1H), 7.73 (2-H/6-H, m, 2H). Anal. ($C_{11}H_{12}O_3$) theoretical: C, 68.74; H, 6.29. Found: C, 68.35; H, 6.33.

Ethyl 3-hydroxycinnamate (2d)

Yield = 68%. mp = 62.9–68.5 °C (lit. 69 °C); ^{1}H NMR (DMSO-d_6) δ 1.25 (CH$_2$C*H*$_3$, t, J = 7.1 Hz, 3H), 4.18

(CH_2CH_3, q, J = 7.1 Hz, 2H), 6.50 (Ar-CH = C*H*, d, J = 16.0 Hz, 1H), 6.83 (6-*H*, ddd, J = 1.0/2.5/8.0 Hz, 1H), 7.03 (2-*H*, t, J = 2.0 Hz, 1H), 7.13 (4-*H*, dt, J = 1.2/7.8 Hz, 1H), 7.21 (5-*H*, t, J = 7.8 Hz, 1H), 7.55 (Ar-C*H* = CH, d, J = 16.0 Hz, 1H), 9.64 (Ar-O*H*, s, 1H). Anal. ($C_{11}H_{12}O_3$) theoretical: C, 68.74; H, 6.29. Found: C, 68.35; H, 6.33.

Ethyl 4-hydroxycinnamate (2e)

Yield = 67%. mp = 70.0–71.0 °C (lit. 73–74 °C); ^{1}H NMR (DMSO-d_6) δ 1.24 (CH_2CH_3, t, J = 7.1 Hz, 3H), 4.16 (CH_2CH_3, q, J = 7.1 Hz, 2H), 6.39 (Ar-CH = C*H*, d, J = 16.0 Hz, 1H), 6.78 (Ar-*H*, m, 2H), 7.55 (Ar-H/Ar-C*H* = CH, m, 3H), 10.01 (Ar-O*H*, s, 1H). Anal. ($C_{11}H_{12}O_3$) theoretical: C, 68.74; H, 6.29. Found: C, 68.48; H, 6.41.

Ethyl 3′,4′-dimethoxycinnamate (2g)

Yield = 47%. m.p.: 56,1 °C (lit.: 56 °C). ^{1}H NMR (DMSO-d_6) δ 1.25 (CH_2CH_3, t, J = 7.1 Hz, 3H), 3.80 (OCH_3, s, 6H), 4.17 (CH_2CH_3, q, J = 7.1 Hz, 2H), 6.54 (Ar-CH = C*H*, d, J = 15.9 Hz, 1H), 6.99 (5-*H*, d, J = 8.3 Hz, 1H), 7.23 (6-*H*, dd, J = 1.9/8.3 Hz, 1H), 7.36 (2-*H*, d, J = 1.9 Hz, 1H), 7.58 (Ar-C*H* = CH, d, J = 15.9 Hz, 1H). Anal. ($C_{13}H_{16}O_4$) theoretical: C, 66.09; H, 6.83. Found: C, 66.47; H, 6.96.

Methyl 3′,4′-dimethoxycinnamate (3)

A mixture of caffeic acid (**1a**, 500 mg, 2.78 mmol) and anhydrous potassium carbonate (1.92 g) in acetone (30 ml) was stirred during 15 min at 40 °C. Dimethylsulfate (2.6 ml, 27 mmol) was added to the medium, and the mixture was heated under reflux at 70 °C during 5 h. The solid was removed by filtration and washed with acetone. The filtrate was evaporated under vacuo. To the residue was added ethyl acetate; the resulting solution was washed with water and a $NaHCO_3$ aqueous solution (5% w/v), dried over anhydrous magnesium sulfate and evaporated under vacuo. The solid obtained by crystallization in a cold ethanol-water mixture was collected by filtration and characterized as methyl 3′,4′-dimethoxycinnamate (**3**, 0.34 g, 55%). mp = 70.1 °C (lit. 68–69 °C). ^{1}H NMR (DMSO-d_6) δ 3.71 ($COOCH_3$, s, 3H), 3.81 ($(OCH_3)_2$, s, 6H), 6.57 (Ar-CH = C*H*, d, J = 16.0 Hz, 1H), 6.98 (5-*H*, d, J = 8.3 Hz, 1H), 7.23 (6-*H*, dd, J = 1.9/8.3 Hz, 1H), 7.36 (2-*H*, d, J = 1.9 Hz, 1H), 7.59 (Ar-C*H* = CH, d, J = 16.0 Hz, 1H). Anal. ($C_{12}H_{14}O_4$) theoretical: C, 64.85; H, 6.35. Found; C, 64.80;H, 6,25.

Fischer esterification (for 5a-b)

A solution of the chlorogenic acid **4** (0.15 g, 0.42 mmol) in the appropriate alcohol (20 ml) was heated under reflux with concentrated sulfuric acid (0.2 ml). After 1 h at 70 °C, the mixture was cooled down, and water (10 ml) and ethyl acetate (10 ml) were added. The solution was extracted three times with AcOEt, and the combined organic phases were dried with $MgSO_4$ and concentrated *in vacuo*.

Methyl chlorogenate (5a)

92 mg, yield = 59%. m.p.: 160 °C (dec). ^{1}H NMR (DMSO-d_6) δ 1.76 (6′-*H*, dd, J = 13.1, 7.8 Hz, 1H), 1.91 (2′-*H*, dd, J = 13.2, 4.0 Hz, 1H), 2.10 (2′-*H*/6′-*H*, m, 2H), 3.58 (4-*H*, m, 1H), 3.56 (CH_3, s, 3H), 3.89 (5′-*H*, m, 1H), 5.07 (3′-H, td, J = 6.9, 4.0 Hz, 1H), 6.11 (Ar-CH = C*H*, d, J = 15.6 Hz, 1H), 6.77 (5-*H*, d, J = 8.0 Hz, 1H), 6.96 (6-*H*, dd, J = 2.1/8.2 Hz, 1H), 7.01 (2-*H*, d, J = 2.1 Hz, 1H), 7.39 (Ar-C*H* = CH, d, J = 15.8 Hz, 1H).

Ethyl chlorogenate (5b)

57 mg, yield = 35%. m.p. : 140 °C (dec). ^{1}H NMR (DMSO-d_6) δ 1.14 (CH_2CH_3, t, J = 7.1 Hz,3H), 1.76 (6′-*H*, dd, J = 13.1, 7.8 Hz, 1H), 1.91 (2′-*H*, dd, J = 13.2, 4.0 Hz, 1H), 2.10 (2′-*H*/6′-*H*, m, 2H), 3.58 (4-*H*, m, 1H), 3.75 (CH_2CH_3, q, J = 7.1 Hz, 2H), 3.89 (5′-*H*, m, 1H), 5.07 (3′-H, td, J = 6.9, 4.0 Hz, 1H), 6.11 (Ar-CH = C*H*, d, J = 15.6 Hz, 1H), 6.77 (5-*H*, d, J = 8.0 Hz, 1H), 6.96 (6-*H*, dd, J = 2.1/8.2 Hz, 1H), 7.01 (2-*H*, d, J = 2.1 Hz, 1H), 7.39 (Ar-C*H* = CH,d, J = 15.8 Hz, 1H).

Antiplasmodial activity assay

Activity against *P. falciparum* chloroquine-sensitive 3D7 strains was assessed following the procedure already described in Frédérich *et al.*[13] The parasites were obtained from Prof. Grellier (Museum d'Histoire Naturelle, Paris, France). Each compound, fraction and extract was applied in a series of eight twofold dilutions (final concentrations ranging from 0.8 to 100 μg/ml for an extract and from 0.08 to 10 μg/ml for a pure substance) on two rows of a 96-well microplate and were tested in triplicate (n = 3). Parasite growth was estimated by determination of lactate dehydrogenase activity as described previously in Jonville *et al.*[14] Artemisinin (98%; Sigma-Aldrich, Darmstadt, Germany) was used as positive control.

Selection and cloning of artemisinin resistant parasites

Plasmodium berghei sensitive to artemisinin, stored in liquid nitrogen, was used. Two hundred microlitres of parasite suspension was inoculated by caudal intravenous into donor mice. 10^5 infected red blood cells from donor mice

were diluted in 0.2 ml of 9‰ NaCl and inoculated by tail intravenous into two groups of five mice. Immediately, first group received orally an infra therapeutic dose of artemisinin freshly prepared in a solvent system (water : DMSO : Tween-80; 85 : 14.7 : 0.3) consisting of 1.25 mg/kg. These mice received artemisinin treatment once a day for 3 days. After 7 day, parasites which survived this treatment were subinoculated into uninfected mice and treatment was repeated with 2.5 mg/kg. Subsequently, increasing doses have been scaled each week from 2.5 mg/kg to achieve finally 30 mg/kg (Figure S1). To discard the possibility that increases in drug tolerance were due to increased virulence caused by multiples sub-inoculation, an artemisinin sensitive parasite line was maintained in parallel and passaged in untreated mice the same number of times as the drug selected line. The evolution of parasitemia of the two lines within 27 weeks of subinoculation is shown in Figure S2.

Evaluation of artemisinin resistance

Resistance quantification in drug-selected parasites clones was established in the following way. Using a dose–response curve, ED_{50} was assessed in drug-selected parasites and untreated control lines. A resistance index (RI) was determined using the following equation: RI = DE_{50} drug-selected parasites/DE_{50} artemisinin-sensitive parasite. At the end of the experiment, the ED_{50} of the resistant strain and that of the susceptible strain were determined and the results were as following: 36.11 ± 1.39 and 6.36 ± 0.54 mg/kg, respectively, for the isolated resistant strain and the starting sensitive strain, so a RI of 5.67 compared to initial strain.

In-vivo antimalarial assay

Animal experiments were approved by the ethical committee of the «*comité scientifique de recherche et de développement de l'Institut Malgache de Recherches Appliquées*» under the number LEPC-IMRA/2014/07/01, the 18th of July 2014. In-vivo antimalarial activity was evaluated in the previously developed artemisinin resistant strain of *P. berghei* with the method described by Peters.[15] Test compounds were dissolved in 20% Tween and 80% distilled water, different concentration of compound (25, 50 and 100 mg/kg) were prepared. Twenty-five mice were infected intraperitoneally with 10^7 infected red blood cells on day 0. Animals were divided into five groups of five mice each (vehicle control group, artemisinin group and 3 experimental groups receiving, respectively: 25, 50 and 100 mg/kg). One hour postinfection mice were treated with a single dose of test compound by intraperitoneal administration (0.2 ml/mouse per day). Doses of various compounds were administered to mice for 4 days (day 0–3). 24 h after the last treatment, blood smears are prepared from tail incision and per cent reduction in parasitemia was calculated in various groups and ED_{50} was determined from doses–effect curves.

Identification of in-vivo stage sensitivity to ethyl caffeate

Identification of stage sensitivity for ethyl caffeate was performed on *Plasmodium vinckei petteri* purchased from Prof. Grellier (Muséum National d'Histoire Naturelle, Paris, France). The strain was selected for this study because of its high synchronicity.[16] Another advantage when working with this strain is the fact that schizogony occurs 24 h after inoculation and the timing of the various parasitic stages can be set up precisely.[17] For convenience, the intraerythrocytic cycle which occurs within 24 h was subdivided into five different stages: ring (R), young trophozoite (YT), mid-term trophozoite (MT), old trophozoite (OT) and schizont (S). The synchronicity was reinforced by rapid freezing and thawing which eliminates all or most parasite stages except the merozoites. At the time of inoculation, merozoites of *P. vinckei petteri* rapidly penetrate the erythrocytes.[18] When mice were inoculated at T0, R appeared at T0 + 3 h, YTs at T0 + 6 h, MTs at T0 + 12 h and OTs at T0 + 18 h.

Parasitic stages were classified using microscope and the following procedures[19]: R, less than 1/3 of the erythrocyte (RBC) in size, displaying a large vacuole and a tiny ring of cytoplasm; YT, about 1/3 of the RBC in size, having a large vacuole and little or no pigment; MT, between 1/3 and 2/3 of the RBC in size, smaller vacuole than in the previous stage, and containing tine pigment particles; OT, 2/3 of the RBC in size, exhibiting small or no vacuole, dense cytoplasm, coarse and abundant pigment and S more than 2/3 of RBC in size, no vacuole, dense cytoplasm, abundant pigment and exhibiting more merozoites nuclear.

Mice were infected intraperitoneally with 10^7 infected red blood cells. Treatment with ethyl caffeate was started 3 days postinfection when parasitemia was comprised between 1 and 8%. Animals were distributed into six groups: five treated groups and one control group. Each treated group received the same dose of ethyl caffeate at, respectively, H0, H0 + 3, H0 + 6, H0 + 12 and H0 + 18 (when one of five stages defined above was highly predominant). They were treated with a single dose of 100 mg/kg prepared as described for the in-vivo antimalarial test. Following treatment, parasitemia and parasitic pattern (percentage of each stages) were evaluated at H0, H0 + 3, H0 + 6, H0 + 12, H0 + 18, H0 + 24 on thin blood smears and compared with those of control mice. Analysis of these parameters enabled the identification of the most sensitive

stage (or stages) and provided an estimation of the duration of drug action.

Statistical analysis

In-vivo results reflecting inhibition of parasite growth were analysed using the Kruskal–Wallis test. Individual differences between treatments were evaluated using Dunn's test. Analysis was performed using Prism 5.0 (GraphPad Software, La Jolla, CA, USA).

Determination of LogP

Lipophilicity was estimated (clogP) using Molinspiration Cheminformatics http://www.molinspiration.com/.

Results

Synthesis

The compounds of interest were prepared from diversely substituted cinnamic acids **1a-d** using Fischer's method in the appropriate alcohol with concentrated sulfuric acid. This procedure was also followed to carry out a transesterification starting from methyl 3′,4′-dimethoxycinnamate **3** to obtain the corresponding ethyl ester **2g**. Minor modifications of this method were applied to obtain the chlorogenic derivatives **5a-b** (see Figure 1).

In-vitro antiplasmodial activity

Caffeic and chlorogenic acid and the seven synthetic derivatives were evaluated for their in-vitro antiplasmodial activity against *P. falciparum*. Artemisinin was used as positive control and showed an IC_{50} value of 3.9 ng/ml. All results are presented in Table 1.

In-vivo antimalarial activity

Methyl and ethyl esters of caffeic acid (**2a** and **2b**), the most active derivatives, were then evaluated *in vivo* against *P. berghei*. The experiment was conducted on a *P. berghei* strain resistant to artemisinin. This strain was developed in the LEPC laboratory as explained in experimental part. The methyl and ethyl caffeate derivatives showed a dose-dependent antimalarial activity for the following doses (25, 50

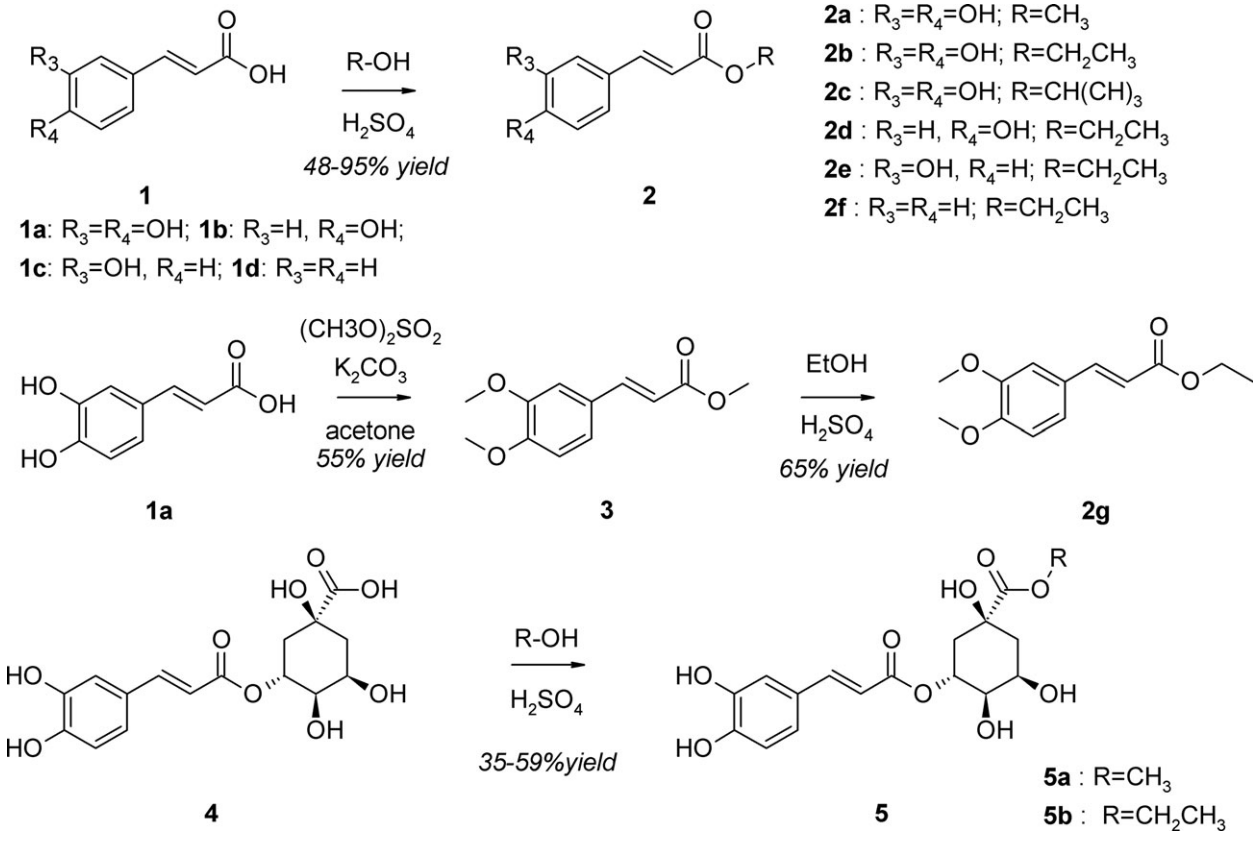

Figure 1 Schemes for obtaining compounds of interest **2a-g** and **5a-b**.

and 100 mg/kg), but only ethyl caffeate at 100 mg/kg showed a statistically significant activity with a growth inhibition of about 55%. The inhibitions of parasites growth are detailed in Figure 2.

Determination of stage specificity for ethyl caffeate

Parasites were able to achieve a full cycle throughout the experiment which allowed us to interpret the results. Indeed, in the control group which received only the solvent, overall parasitemia increased from 7 to 24% in one cycle. At beginning (H0), predominant parasitic form is the YT. Mid-term trophozoites, OTs and S were, respectively, found at H0 + 3 (until H0 + 6), at H0 + 12 and at H0 + 18. This kinetic is taken as reference for result interpretation.

Concerning the group treated at H0 with ethyl caffeate, the parasitemia observed at the end of the cycle (at H0 + 24), is 8% inferior to that observed in control group. This difference is not observed when treatment is given at H0 + 3, H0 + 6 or H0 + 12. We could then conclude that ethyl caffeate is particularly active on YTs as artemether, which makes difference with chloroquine. Detailed kinetics are given in Tables S3–S7.

Discussion

Ethyl and isopropyl, followed by methyl-caffeate presented the best antiplasmodial activity with an IC_{50} around 20 μM against the 3D7 chloroquine sensitive strain of *P. falciparum*. This range of activity is not extremely potent, but caffeic acid is present in numerous plants used as food or medicine and is considered as nontoxic, as probably its simple esters.[11,12] Chlorogenic acid and its esters were significantly less active than caffeic acid and its esters, but these compounds could be transformed in caffeic acid in the digestive track.

Esterification of the carboxylic acid of **1** is correlated with a four times increase of the in-vitro activity. Small alkyl esters present similar IC_{50} values. The importance of the presence of the 2 phenolic functions was confirmed after the evaluation of compounds **2d-g**: for these four compounds, a huge reduction of the in-vitro activity was observed, compared to the corresponding ethyl caffeate (**2b**). The presence of two phenolic functions appeared then mandatory, and this could suggest a link between the antiplasmodial activity and some antioxidant potential, as other phenolic compounds with antioxidant activity such as ellagic acid or gallic acid have demonstrated antiplasmodial activity.[20,21] In this later case, it is also an ethyl ester of gallic acid which has demonstrated better activity.[21] This point has to be investigated in future.

Many biological activity are attributed to esters derivatives of caffeic acids, such as anti-inflammatory activity, anti-cancer by induction of apoptosis, antimicrobial, immunomodulatory, as described in the introductory part of this manuscript. Furthermore, it has been shown previously that caffeic is absorbed in rats and has an absolute bioavailability in humans around 95%.[22,23] To the best of our knowledge, there are no data available about caffeic acid ethyl ester bioavailability, but a publication showed that caffeic acid phenetyl ester was resorbed in rats.[24] Caffeic acid and derivatives could then explain, at least in part, the oral antimalarial activity of traditional medicinal plants where these compounds are abundant. One hypothesis for better activity of ethyl caffeate compared to caffeic acid could be the ability to reach intracellular targets which

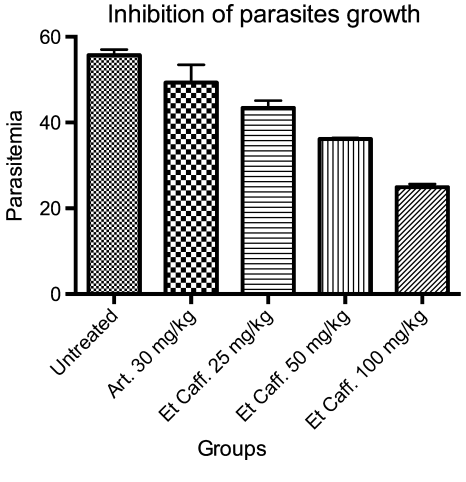

Figure 2 In-vivo antimalarial activity of ethyl caffeate at day 4, following the standard test of Peters, using intra-peritoneal route. Parasitemia (%).

Table 1 In-vitro antiplasmodial activity and Log P estimation of caffeic acid derivatives and analogues against *Plasmodium falciparum* chloroquine-sensitive (3D7) strain

Compound	R	R_3	R_4	IC_{50}[a]	cLog P[b]
1a	H	OH	OH	80.5 ± 22.8	0.9
2a	Methyl	OH	OH	27.3 ± 3.4	1.6
2b	Ethyl	OH	OH	21.9 ± 9.4	1.9
2c	Isopropyl	OH	OH	21.9 ± 19.8	2.3
2d	Ethyl	H	OH	181.6 ± 23.0	2.4
2e	Ethyl	OH	H	95.4 ± 21.7	2.4
2f	Ethyl	H	H	>280	2.9
2g	Ethyl	OCH_3	OCH_3	196.6 (n = 1)	2.6
4	H	OH	OH	>280	−0.4
5a	Methyl	OH	OH	173.8 ± 87.7	0.2
5b	Ethyl	OH	OH	168.9 ± 61.3	0.5

[a]Results expressed in μM ± standard deviation (n = 3–5). [b]Molinspiration Cheminformatics. http://www.molinspiration.com/.

could be related to the better lipophilicity of ester derivatives, as illustrated by Log P value in Table 1. Previous works found in the literature showed that the esterification of caffeic acid or other phenolics increases their lipophilicity thus facilitating their transmembrane passage.[25,26]

In-vivo antimalarial activity evaluation was conducted only on the methyl and ethyl caffeic acid derivatives which were among the three most active compounds and which were the most easily synthesized compounds. In-vivo assay confirmed the best activity and dose-dependent effect for ethyl caffeate.

Only ethyl caffeate was studied for chronotherapy because of its better in-vivo antimalarial activity. Results showed that ethyl caffeate would act preferentially in the 6 first hours of the schizogonic cycle by blocking the growth of young parasites (R and YTs), similarly to what is described for artemisinin derivatives.

Conclusions

Caffeic acid and close derivatives or analogues were shown to exhibit some antiplasmodial activity against *P. falciparum*. Particularly, the ethyl ester of caffeic acid was shown to be the most active derivative of caffeic acid *in vitro* and was also shown for the first time to exhibit interesting activity *in vivo,* with a growth inhibition of *P. berghei* resistant to artemisinin of about 55% at 100 mg/kg. This is not particularly high compared to artemisinin or other standard drugs, but this is interesting considering the non-toxicity of ethyl caffeate, and the abundance of caffeic acid and derivatives in numerous plants, including medicinal plants and food. The presence of phenols seems to be essential for the activity, even if methylated derivative maintain some activity. Caffeic acid derivatives could participate in the antimalarial activity of several plants traditionally used against malaria. It would be interesting, in future, to evaluate the bioavailability and pharmacokinetics parameters of caffeic acid esters, and to evaluate their action in association with standard antimalarial treatments, particularly artemisinins.

Declarations

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References

1. World malaria report 2017. Geneva: World Health Organization; 2017. DOI: ISBN 978-92-4-156552-3.
2. Bellanger A-P *et al.* Malaria outbreak in French troops returning from Côte d'Ivoire. *Scand J Infect Dis* 2011; 43: 230–233.
3. Partnership SCT. Efficacy and safety of RTS, S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial. *Lancet* 2015; 386: 31–45.
4. Chiang Y-M *et al.* Ethyl caffeate suppresses NF-kappaB activation and its downstream inflammatory mediators, iNOS, COX-2, and PGE2 in vitro or in mouse skin. *Br J Pharmacol* 2005; 146: 352–363.
5. Xu S *et al.* Ethyl caffeate ameliorates collagen-induced arthritis by suppressing Th1 immune response. *J Immunol Res* 2017; 2017: 7416792.
6. Chalas J *et al.* Effect of ethyl esterification of phenolic acids on low-density lipoprotein oxidation. *Biomed Pharmacother* 2001; 55: 54–60.
7. Gandhi GR *et al.* Antihyperglycemic activity and antidiabetic effect of methyl caffeate isolated from *Solanum torvum* Swartz. fruit in streptozotocin induced diabetic rats. *Eur J Pharmacol* 2011; 670: 623–631.
8. Lim DY *et al.* (+)-2-(1-Hydroxyl-4-oxocyclohexyl) ethyl caffeate suppresses solar UV-induced skin carcinogenesis by targeting PI3K, ERK1/2, and p38. *Cancer Prev Res (Phila)* 2014; 7: 856–865.
9. Balachandran C *et al.* Antimicrobial and antimycobacterial activities of methyl caffeate isolated from *Solanum torvum* Swartz. Fruit. *Indian J Microbiol* 2012; 52: 676–681.
10. Gangan VD *et al.* Ethyl caffeate ether derivatives as future potential drug. *J Pharm Res* 2014; 88: 818–821.
11. Herrmann K, Nagel CW. Occurrence and content of hydroxycinnamic and hydroxybenzoic acid compounds in foods. *Crit Rev Food Sci Nutr* 1989; 28: 315–347.
12. De P *et al.* Cinnamic acid derivatives as anticancer agents – a review. *Curr Med Chem* 2011; 18: 1672–1703.
13. Frederich M *et al.* Antiplasmodial activity of alkaloids from various *Strychnos* species. *J Nat Prod* 2002; 65: 1381–1386. https://doi.org/10.1021/NP020070E.
14. Jonville MC *et al.* Dimeric bisindole alkaloids from the stem bark of *Strychnos nux-vomica* L. *Phytochemistry* 2013; 87: 157–163.
15. Peters W, Robinson BL. The chemotherapy of rodent malaria. LVI. Studies on the development of resistance to natural and synthetic endoperoxides. *Ann Trop Med Parasitol* 1999; 93: 325–329.
16. Cambie G *et al.* Chronotherapy of malaria: identification of drug-sensitive stage of parasite and timing of drug delivery for improved therapy. *Ann Parasitol Hum Comp* 1991; 66: 14–21.
17. Montalvo-Alvarez AM *et al.* Experimental modifications of the circadian

rythm of *Plasmodium vinckei petteri* following cryopreservation; probable resistance of the merozoïte to thawing. *C R Acad Sci III* 1988; 307: 5–10.
18. Cambie G *et al.* [Timing niches of 3 species of *Plasmodium* coexisting in a rodent in Central Africa]. *C R Acad Sci III* 1990; 310: 183–188.
19. Caillard V *et al. Plasmodium vinckei petteri*: identification of the stages sensitive to arteether. *Exp Parasitol* 1992; 75: 449–456.
20. Soh PN *et al. In vitro* and *in vivo* properties of ellagic acid in malaria treatment. *Antimicrob Agents Chemother* 2009; 53: 1100–1106.
21. Jansen O *et al.* Antiplasmodial activity of *Mezoneuron benthamianum* leaves and identification of its active constituents. *J Ethnopharmacol* 2017; 203: 20–26.
22. Olthof MR *et al.* Chlorogenic acid and caffeic acid are absorbed in humans. *J Nutr* 2001; 131: 66–71.
23. Wang S-J *et al.* Bioavailability of caffeic acid in rats and its absorption properties in the Caco-2 cell model. *Pharm Biol* 2014; 52: 1150–1157.
24. Guo X *et al.* Antitumor activity of caffeic acid 3,4-dihydroxyphenethyl ester and its pharmacokinetic and metabolic properties. *Phytomedicine* 2013; 20: 904–912.
25. Filipe HAL *et al.* Differential targeting of membrane lipid domains by caffeic acid and its ester derivatives. *Free Radic Biol Med* 2018; 115: 232–245.
26. Wu X-W *et al.* Anti-inflammatory phenolic acid esters from the roots and rhizomes of *Notopterygium incisium* and their permeability in the human Caco-2 monolayer cell model. *Molecules* 2017; 22: 935.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article:

Figure S1. Selection and cloning of artemisinin resistant parasites.

Figure S2. *In vivo* evolution of resistance to artemisinin.

Table S1. Ethyl caffeate antimalarial effect against *Plasmodium berghei* strain presenting resistance to artemisinin ($n = 3$).

Table S2. Methyl caffeate antimalarial effect against *Plasmodium berghei* strain presenting resistance to artemisinin ($n = 3$).

Table S3. Growth kinetics, predominant form and global parasitemia during a full cycle of *Plasmodium vinckei petteri* inoculated on mice (control group).

Table S4. Group 2 (Treated at Ho), growth kinetics, predominant parasitic form and global parasitemia during a full cycle of *Plasmodium vinckei petteri* inoculated on mice.

Table S5. Group 3 (Treated at Ho + 3), growth kinetics, predominant parasitic form and global parasitemia during a full cycle of *Plasmodium vinckei petteri* inoculated on mice.

Table S6. Group 4 (Treated at Ho + 6), growth kinetics, predominant parasitic form and global parasitemia during a full cycle of *Plasmodium vinckei petteri* inoculated on mice.

Table S7. Group 5 (Treated at Ho + 12), growth kinetics, predominant parasitic form and global parasitemia during a full cycle of *Plasmodium vinckei petteri* inoculated on mice.