

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

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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 activities 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-days suppressive test from Peters on an artemisinin-resistant *P. berghei* strain were 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 $IC_{50} = 21.9 \pm 9.4 \mu M$. 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.

Key words

Malaria, *Plasmodium falciparum*, caffeic acid, ethyl caffeate

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 billions 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 travelers to sub-Saharan African countries are particularly at risk of severe malaria, if infected with *P. falciparum*. Malaria remains a major killer of under-fives, claiming the life of 1 child every 2 minutes.^[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 *P. 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 ^1H 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) 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). 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 hours, 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) ; ^1H NMR (DMSO-d_6) δ 3.69 (CH_3 , s, 3H), 6.27 (Ar-CH=CH, d, J = 15.9 Hz, 1H), 6.76 (5-H, d, J = 8.1Hz, 1H), 7.00 (6-H, d, J = 8.2 Hz, 1H), 7.03-7.09 (2-H, m, 1H), 7.48 (Ar-CH=CH, d, J=15.9 Hz, 1H), 9.37 (Ar-OH, bs, 2H). Anal. ($\text{C}_{10}\text{H}_{10}\text{O}_4$) theoretical: C, 61.85; H, 5.19. Found: C, 61.97; H: 5.32. MS m/z : 193 $[\text{M}]^+$, 161, 134. UV_{max} : 218, 295 et 324nm.

Ethyl caffeate (2b). yield = 84%. mp=140.8-143.1 °C (lit. 143-146 °C) ; ^1H NMR (DMSO-d_6) δ 1.24 (CH_2CH_3 , t, J = 7.1 Hz, 3H), 4.15 (CH_2CH_3 , q, J = 7.1 Hz, 2H), 6.25 (Ar-CH=CH, 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-CH=CH, d, J=15.9 Hz, 1H), 9.61 (Ar-OH, bs, 2H). Anal. ($\text{C}_{11}\text{H}_{12}\text{O}_4$) theoretical: C, 63.45; H, 5.81. Found: C, 63.79; H, 5.91. MS m/z : 208 $[\text{M}]^+$, 179, 161 ; UV_{max} : 218, 295 et 324nm.

Isopropyl caffeate (2c). yield = 62%. mp=129.8-135.0 °C (lit. 141-143 °C) ; ¹H NMR (DMSO-*d*₆) δ 1.24 (CH(CH₃)₂, d, J = 6.3 Hz, 6H), 4.99 (CH(CH₃)₂, q, J = 6.2 Hz, 1H), 6.22 (Ar-CH=CH, 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-CH=CH, d, J=15.9 Hz, 1H), 9.35 (Ar-OH, bs, 2H). Anal. (C₁₂H₁₄O₄) theoretical: C, 64.85; H, 6.35. Found: C, 64.78; H, 6.36.

Ethyl cinnamate (2f). yield = 48%. Oil. ¹H NMR (DMSO-*d*₆) δ 1.25 (CH₂CH₃, t, J = 7.1 Hz, 3H), 4.18 (CH₂CH₃, q, J = 7.1 Hz, 2H), 6.50 (Ar-CH=CH, d, J = 16.1 Hz, 1H), 7.43 (3-*H*/4-*H*/5-*H*, m, 3H), 7.67 (Ar-CH=CH, d, J=16.0 Hz, 1H), 7.73 (2-*H*/6-*H*, m, 2H). Anal. (C₁₁H₁₂O₃) 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) ; ¹H NMR (DMSO-*d*₆) δ 1.25 (CH₂CH₃, t, J = 7.1 Hz, 3H), 4.18 (CH₂CH₃, q, J = 7.1 Hz, 2H), 6.50 (Ar-CH=CH, 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-CH=CH, d, J=16.0 Hz, 1H), 9.64 (Ar-OH, s, 1H). Anal. (C₁₁H₁₂O₃) 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) ; ¹H NMR (DMSO-*d*₆) δ 1.24 (CH₂CH₃, t, J = 7.1 Hz, 3H), 4.16 (CH₂CH₃, q, J = 7.1 Hz, 2H), 6.39 (Ar-CH=CH, d, J = 16.0 Hz, 1H), 6.78 (Ar-*H*, m, 2H), 7.55 (Ar-*H*/Ar-CH=CH, m, 3H), 10.01 (Ar-OH, s, 1H). Anal. (C₁₁H₁₂O₃) 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). ¹H NMR (DMSO-*d*₆) δ 1.25 (CH₂CH₃, t, J = 7.1 Hz, 3H), 3.80 (OCH₃, s, 6H), 4.17 (CH₂CH₃, q, J = 7.1 Hz, 2H), 6.54 (Ar-CH=CH, 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-CH=CH, d, J = 15.9 Hz, 1H). Anal. (C₁₃H₁₆O₄) 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 minutes 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 hours. 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 NaHCO₃ aqueous solution (5% w/v), dried over anhydrous

magnesium sulfate and evaporated under vacuo. The solid obtained by crystallisation 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). ¹H NMR (DMSO-*d*₆) δ 3.71 (COOCH₃, s, 3H), 3.81 ((OCH₃)₂, s, 6H), 6.57 (Ar-CH=CH, 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-CH=CH, d, J = 16.0 Hz, 1H). Anal. (C₁₂H₁₄O₄) 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 hour at 70°C, the mixture was cooled down, 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 MgSO₄ and concentrated *in vacuo*.

Methyl chlorogenate (5a). 92 mg, yield = 59 %. m.p. : 160°C (dec). ¹H 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 (CH₃, s, 3H), 3.89 (5'-*H*, m, 1H), 5.07 (3'-*H*, td, J = 6.9, 4.0 Hz, 1H), 6.11 (Ar-CH=CH, 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-CH=CH, d, J = 15.8 Hz, 1H)

Ethyl chlorogenate (5b). 57 mg, yield = 35%. m.p. : 140°C (dec). ¹H NMR (DMSO-*d*₆) δ 1.14 (CH₂CH₃, 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 (CH₂CH₃, 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=CH, 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-CH=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 2-fold 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) was used as

positive control.

Selection and cloning of artemisinin resistant parasites

Plasmodium berghei sensitive to artemisinin, stored in liquid nitrogen, was used. 200 µl of parasite suspension were inoculated by caudal intravenous into donor mice. 10^5 infected red blood cells from donor mice were diluted in 0.2ml of 9‰ NaCl and inoculated by tail intravenous into 2 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 seven day, parasites which survived this treatment were sub-inoculated 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: $RI = DE_{50} \text{ drug-selected parasites} / DE_{50} \text{ artemisinin-sensitive parasite}$. 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 mg/kg and 6.36 ± 0.54 mg/kg respectively for the isolated resistant strain and the starting sensitive strain, so a resistance index 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 *Plasmodium 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 100mg/kg) were prepared. Twenty-five mice were infected intraperitoneally with 10^7 infected red blood cells on day 0. Animals were divided into 5 groups of 5 mice each (vehicle control group, artemisinin group and 3 experimental groups receiving respectively: 25, 50 and 100mg/kg). One hour post-infection mice were treated with a

single dose of test compound by intraperitoneal administration (0,2 ml/mouse/day). Doses of various compounds were administered to mice for four days (day 0 to 3). 24 hours after the last treatment, blood smears are prepared from tail incision and percent reduction of parasitemia was calculated in various groups and ED₅₀ 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 hours 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, ring appeared at T0+3h, young trophozoites at T0+6h, midterm trophozoites at T0+12h and old trophozoites at T0+18h.

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 fine 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 10⁷ infected red blood cells. Treatment with ethyl caffeate was started 3 days post infection when parasitemia was comprised between 1 % and 8 %. Animals were distributed into 6 groups : 5 treated groups and 1 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 5 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 analyzed using the Kruskal-Wallis test. Individual differences between treatments were evaluated using Dunn's test. Analysis was performed using Prism 5.0.

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 in order 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 *Plasmodium falciparum*. Artemisinin was used as positive control and showed an IC₅₀ value of 3.9 ng/ml. All results are presented in Table I.

In vivo antimalarial activity.

Methyl and ethyl esters of caffeic acid (**2a** and **2b**), the most active derivatives, were then evaluated *in vivo* against *Plasmodium 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 (25mg/kg, 50mg/kg and 100mg/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 young trophozoite. Mid-term trophozoites, old trophozoites and schizonts 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 young trophozoites 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 μ M against the 3D7 chloroquine sensitive strain of *Plasmodium 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 the future.

Many biological activities 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 is 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 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 (ring and young trophozoites), 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 *Plasmodium 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 nontoxicity 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 the future, to evaluate the bioavailability and pharmacokinetics parameters of caffeic acid esters, and to evaluate their action in association to standard antimalarial treatments, particularly artemisinins.

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Tables and Figures:

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

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

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 $\mu\text{M} \pm$ standard deviation (n=3-5).

^b Molinspiration Cheminformatics. <http://www.molinspiration.com/>

Figure 1

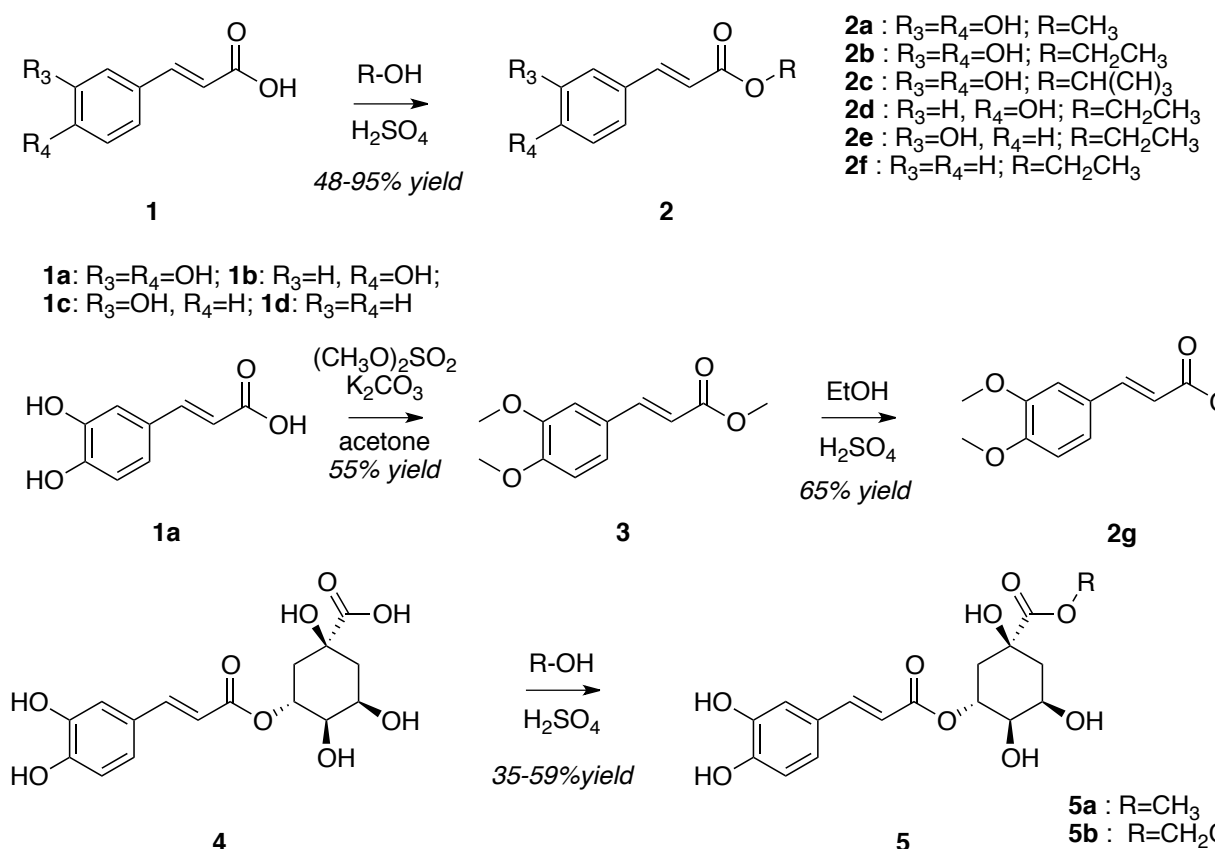


Figure 1. Schemes for obtaining compounds of interest **2a-g** and **5a-b**.

Figure 2

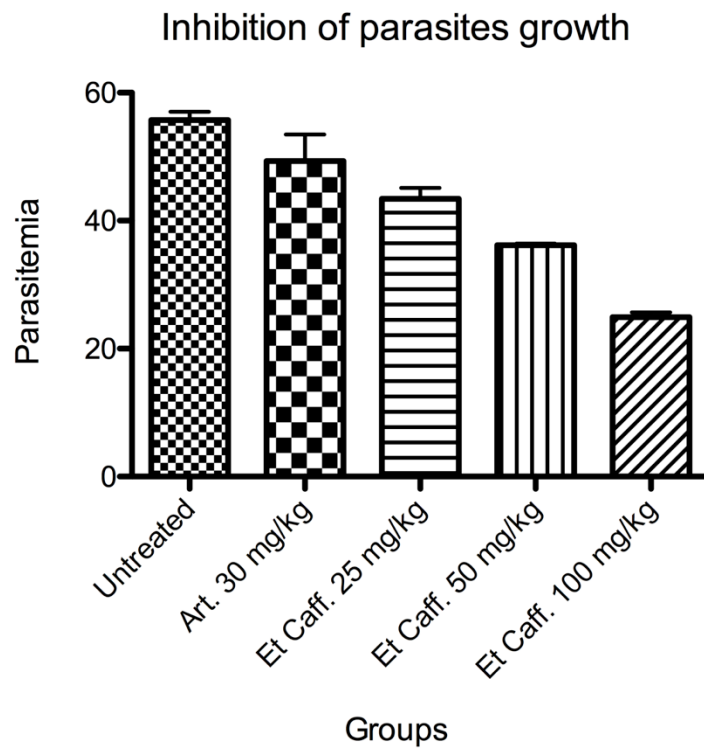


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