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**RESEARCH ARTICLE**

# Potential of Caffeic Acid Derivatives as Antimalarial Leads.

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**Abstract:**

- Background

Malaria remains one of the deadliest infectious disease in 2021. Indeed, this infection, mostly caused by a protozoon called *Plasmodium falciparum*, is responsible annually for more than 200 million cases and around 400 000 related deaths, mainly in Africa. Despite the availability of efficient drugs, an increase of patients occurs since 2015, which could find its origin in development of resistances from the parasite but also from its vectors, Anopheles mosquitoes. Consequently, it is necessary to search for new alternative treatments.

- Objective

Polyphenols, and more precisely small phenolic acids, could represent a good starting point for new antimalarials. Indeed, these molecules, such as caffeic acid (**1**), exhibit several pharmacological activities and an interesting pharmacokinetic profile. Therefore, we have developed several small derivatives of this scaffold to define the potential pharmacophore responsible for the antiplasmodial properties.

- Results

A good to low activity on *Plasmodium falciparum* ($IC_{50}$ = 16-241 µM) was observed especially for the small ester derivatives (**2**-**6**). These molecules were thus good antiplasmodials compared to their mother compound ($IC_{50}$ = 80 µM) and showed a selectivity against human cells for the most active. These structures have such highlighted the need of the catechol and carboxyl moieties in the anti-*Plasmodium* effect.

- Conclusion

None of the synthetic caffeate derivatives reported here, seemed sufficiently effective to become a potential antimalarial ($IC_{50}$ > 1 µM). However, the significant increase of their efficacy on the malaria agent and the selectivity to human cells highlighted their potential as new leads for future developments.

**Keywords:** Malaria; *Plasmodium*; Caffeic Acid; Pharmacomodulation; Polyphenols; Antimalarials

## 1. INTRODUCTION

Among the parasites recognized as infectious for human, *Plasmodium falciparum* remains one of the deadliest in 2021. Indeed, this pathogen, transmitted thanks to mosquitoes, is responsible for one of the most problematic zoonotic illnesses: malaria. This infection is characterized by cyclic fever, chills, anaemia in most cases but could lead to death if untreated.[1] As a result, more than 200 million cases are annually reported, mostly in Africa, and among the patients, around 400 000 will die from this parasitosis. Moreover, for years now, we observe a slight increase of the cases (from 211 million in 2015 to 229 million in 2019) after ten years of constant reduction.[2–4]

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This trend could be partially explained by an increase of the testing capacity but could also find its origin in the appearance of resistances from the vectors (Anopheles)[5] to insecticides but also from the parasites to the current recommended treatment.[2, 6–8]

Therefore, the artemisinin-combined therapies which are widely used in the world could lose their efficacy in the future.[9] But there is a lack of innovations in the malaria treatment since the discovery and FDA-approval of artemisinin and its derivatives (artesunate, artemether) at the end of the 20$^{th}$ century.[10] Consequently, there is an urgent need of new antimalarials.

Regarding the statistics, most of the infectious diseases have their "cures" coming from Nature or inspired by natural scaffolds.[11] Consequently, it seemed logical to look for the next antimalarial in the field of natural compounds, isolated from plants, marine organisms or even fungi.[12–15]

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However, highly active compounds have some limitations such as poor bioavailability,[16] weak water solubility,[17] are scarcely available or simply expensive.

Thus, a compromise must be made between the activity and these other parameters. Therefore, a widely distributed molecule with good druggability parameters and a lower efficacy could be more interesting for development than a potent molecule with poor ADME properties and only present in trace in Nature.

Hence, we have investigated the potential of a widely distributed polyphenol in the plant kingdom, caffeic acid (**1**), for malaria treatment. Indeed, this molecule and its derivatives possess multiple biological activities[18] such as antimicrobial,[19–22] antiinflammatory[23–26] or anticancer[24, 27]. Moreover **1** was recognized as slightly active on *Plasmodium* (Tab. 1)[28–32] and showed interesting pharmaceutical properties such as a good hydrosolubility (0.98 g/L),[33] a weak toxicity,[34, 35] and is orally bioavailable[36, 37]…

Indeed, this monocarboxylic acid possesses an interesting ADME profile compared to other small polyphenolic acids such as gallic acid (GA). Contrary to this scaffold, 3,4-dihydroxycinnamic acid could cross the intestinal barrier thanks to a selective transporter, mono-carboxylic transporter (MCT). Consequently, the transfer of caffeic acid was 100-folder superior to 3,4,5-trihydroxybenzoic acid.[38] Moreover, due to the transport selectivity, the *in vivo* absorption of **1** was also greater than GA (70 more bioavailable).[36, 39]

On the other hand, a lot of absorption-distribution-metabolism-excretion information are available for this structure of interest.[40] This gives us valuable knowledges to hypothesize how the synthetic derivatives could be processed *in vivo*, especially for the metabolism part.

In our previous work on this lead, several caffeate derivatives have been synthesized for *in vitro* and *in vivo* experiments to explore the antimalarial potential of the caffeic/chlorogenic acid scaffolds.[32] From all these structures, methyl and ethyl caffeate (**2**-**3**) were the compounds with the best inhibitory effect on a 3D7 strain of *Pf* ($IC_{50}$ ~ 21 µM).

Consequently, they have been submitted to *in vivo* evaluation following the Peters' 4-days suppressive test.[41] Unfortunately, only a dose of 100 mg/kg of ethyl caffeate showed a significant decrease of parasitaemia (55 % reduction) with a preferential activity on the young stages of *Pf* (first 6 hours of schizogonic cycle).[32]

We have then decided to extend our library of compounds with small analogues of **1**. Indeed, this scaffold has two main moieties which could be modified, two *ortho* phenolic alcohols and one carboxylic function. Consequently, we have focused our pharmacomodulation on the design of several esters and amides structures, with various substitutions of the phenolic functions.

In conclusion, the objectives of our investigations were the enhancement of our understanding of which parts of the caffeic acid scaffold were necessary for the anti-*Plasmodium* activity. In addition, we would like to obtain an increased effect on this parasite ($IC_{50}$ < 20 µM). Moreover, we reported the impact of these modifications on some critical characteristics such as the cell toxicity.

## 2. MATERIALS AND METHOD

3,4-dihydroxycinnamic acid (mostly *trans*) was obtained from ABCR®. Celite® 545, thionyl chloride, 3,4-(methylenedioxy)cinnamic acid, 3,4-dimethoxycinnamic acid, diethylamine, ammoniac 25%, were obtained from Sigma-Aldrich® or Fluorochem®. Ethyl acetate, toluene, acetonitrile, hexane, methanol, ethanol, isopropanol, and *n*-propanol from VWR®. Melting points were determined on Stuart® SMP3 capillary apparatus and are uncorrected. Products were purified thanks to Buchi Reveleris® prep. on irregular silica cartridge 4-80g.

The $^{1}H$ and $^{13}C$-NMR spectra were recorded on a Bruker® Advance (500 MHz for $^{1}H$; 125 MHz for $^{13}C$) instrument using deuterated dimethyl sulfoxide (DMSO-$d_6$) or deuterated chloroform ($CDCl_3$) as solvents with tetramethylsilane (TMS) as internal standard; chemical shifts are reported in δ values (ppm) relative to that of internal TMS. The abbreviations s = singlet, d =doublet, t = triplet, q = quadruplet, m = multiplet, dd = doublet of doublet, qd = quadruplet of doublet, dt = doublet of triplet, tt = triplet of triplet, and bs = broad singlet are used throughout.

Elemental analyses (C, H, N, S) were realized on a Thermo Scientific® Flash EA1112 elemental analyzer and were within ± 0.4 % of the theoretical values for carbon, hydrogen, and nitrogen. This analytical method certified a purity of ≥ 95% for each tested compound. All reactions were routinely checked by TLC on silica gel Merck® 60 F254.

### 2.1. Literature screening

To obtain all the caffeic acid derivatives already characterized and tested against *Plasmodium sp.*, Scifinder® database has been selected. Indeed, this database supplies the references from PubMed, CAB abstract and Patents. The terms Malaria OR *Plasmodium* have then been combined with several concepts related to **1**: caffeic acid OR caffeate OR caffeoyl OR 3,4-dihydroxycinnamic acid OR 3,4-dihydroxycinnamate.

From the results, we have selected all the reviews and original articles in English or French until September 2020 containing one or more caffeate structures tested against a malaria agent.

### 2.2. Antiplasmodial *in vitro* activity

The selected strain was obtained through BEI Resources, NIAID, NIH: *Plasmodium falciparum*, Strain 3D7, MRA-102, contributed by Daniel J. Carucci. *Plasmodium falciparum* was continuously grown *in vitro* as previously described.[42, 43] Indeed, this chloroquine-sensitive strain is maintained with human red blood cells (A+ or O+) and a

culture medium mainly composed by RPMI 1640 (Gibco, Fisher Scientific®, UK) with 1.76 g/L of glucose (Sigma-Aldrich®, Belgium), 44 mg/mL of hypoxanthine (Sigma-Aldrich®, Belgium), 100 mg/L of gentamycin (Gibco, Fisher Scientific®, UK), and 10% human pooled serum.

Pure products were dissolved in DMSO to reach 10 mg/mL and were compared to artemisinin (Sigma-Aldrich®, Belgium), a positive standard. As DMSO is toxic for *Pf*, the maximal amount during the assay was 1 % for the initial concentrations (100 µg/mL in RPMI). Finally, each sample was evaluated in a series of eight 2-fold dilutions and tested in triplicate (n = 3), as previously described.[44]

After 2-day incubation, plates were frozen at - 20°C for min. 12h and parasite growth was quantified according the measurement of Plasmodium lactate dehydrogenase activity.[45] As parasitized and healthy erythrocytes are used as positive (100% growth) and negative controls (0% growth), the comparison of their corresponding absorbance at 650 nm allows to estimate the growth inhibition. Consequently, $IC_{50}$ values were calculated from linear regression.

### 2.3. Hemolytic activity

Hemolytic potential was evaluated for the most active compounds as previously reported.[46] A 10% red blood cells suspension in PBS (v/v) (A+ or O+) was incubated with compounds in duplicate. The primary solutions were diluted in PBS to reach 100 µg/mL as final concentration (DMSO < 1%). After agitation at room temperature for 1 h, the mixtures were centrifuged for 5 min at 2000 rpm, and 150 µL of supernatant was transferred to a 96-wells plate.

The absorbance was evaluated at 550 nm with a microplate reader (OD). The positive control was Triton X-100 1% (v/v) (corresponding to 100% lysis) and PBS as the negative control (corresponding to 0% lysis). The percentage of red blood cell lysis (H) was calculated as follows: $H = (OD_{550}$ nm sample − $OD_{550}$ nm PBS)/ ($OD_{550}$ nm Triton X-100 1% (v/v) − $OD_{550}$ nm PBS)*100.[47]

### 2.4. *In vitro* cytotoxicity

Some compounds were submitted to evaluation of their cytotoxicity on healthy cells. Therefore, Human Umbilical Vein Endothelial Cells, furnished by Lonza were finally selected. These cells were maintained *in vitro* thanks to EBM-2

medium, supplemented with FBS, hydrocortisone, hFGF-B, VEGF, R3-IGF-1, ascorbic acid, hEGF, GA-1000 and heparin, known as EGM-2 SingleQuots® from Lonza. As for antiplasmodial evaluation, samples were first dissolved in DMSO to reach 10 mg/mL.

As DMSO is also toxic for cells, the maximal concentration was 0.5 %. Thus, a first dilution in culture medium was performed. The compounds were tested between 50 and 0.5 µg/mL. For the assay, 100 µL of cell culture ($10^4$ cells) was seeded on all wells of the 96-wells plate and let 24h for adhesion at 37°C.

After that, the supernatant was replaced by the test solution (3 wells for each concentration) and let for a 2-days incubation. Finally, the supernatant was removed and replaced by 10-fold diluted PrestoBlue® in EBM-2. Therefore, the plate was incubated at 37°C for minimum 2h before reading in fluorescence mode (560-590 nm). The growth is compared between treated and untreated cells. Each compound was tested in triplicate.

### 2.5. Synthesis

#### *2.5.1. General procedure for esterification*[32]

3,4-Dihydroxycinnamic acid (1 g) was dissolved in 50 mL of the appropriate alcohol and a few drops of concentrated $H_2SO_4$ were cautiously added. The mixture was heated under reflux until the end of the reaction (monitored by TLC, around 6 h). The reaction mixture was cooled before a part of the organic solvent was eliminated by evaporation under vacuo. After that, water (25 mL) was added, and the solution was extracted three times with ethyl acetate (50 mL). Then, the organic phase was washed with brine (50 mL) and dried over anhydrous magnesium sulfate ($MgSO_4$). The suspension was filtered, and the solvent was removed by evaporation under vacuo. The solid was placed overnight in a stove (30°C).

*Methyl caffeate* (**2**): slightly brown solid, Yield 95 %, MP = 158.9°C/ Lit. 158°C[48], $^1$H-RMN (in DMSO) δ 7.48 (1H, d), 7.04 (1H, d), 7.0 (1H, dd), 6.76 (1H, d), 6.27 (1H, d), 3.69 (3H, s); $^{13}$C (in DMSO) δ 167.48 (s), 149.02 (s), 146.08 (s), 145.67 (s), 125.86 (s), 121.97 (s), 116.17 (s), 115.20 (s), 114.07 (s), 51.68 (s) EA Th: C, 61.85 %; H, 5.19 %; Found: C, 61.97 %; H, 5.32 %

*Ethyl caffeate* (**3**): white solid, Yield 84 %, MP =143.1°C/ Lit. 147-149°C[49], $^1$H-RMN (in DMSO) δ 7.47 (1H, d), 7.04 (1H, d), 7.0 (1H, dd), 6.76 (1H, d), 6.25 (1H, d), 4.15 (2H, d), 1.24 (3H, t); $^{13}$C (in DMSO) δ 167.02 (s), 148.91 (s), 146.05 (s), 145.48 (s), 125.93 (s), 121.82 (s), 116.18 (s), 115.25 (s), 114.47 (s), 60.15 (s), 14.74 (s) EA Th: C, 63.45 %; H, 5.81 %; Found: C, 63.79 %; H, 5.91 %

*Propyl caffeate* **(4):** white solid, Yield 66 % after DCVC (DCM/MeOH (1/0 to 95/5), MP =128°C/ Lit. 127 °C[50], $^1$H-RMN (in DMSO) δ 7.47 (1H, d), 7.04 (1H, d), 7.01 (1H, dd), 6.75 (1H, d), 6.26 (1H, d), 4.07 (2H, t), 1.64 (2H, sex), 0.92 (3H, t); $^{13}$C (in DMSO) δ 167.09 (s), 148.86 (s), 146.03 (s), 145.48 (s), 125.96 (s), 121.81 (s), 116.19 (s), 115.28 (s), 114.46 (s), 65.65 (s), 22.15 (s), 10.86 (s) EA Th: C, 64.85 %; H, 6.35 %; Found: C, 64.84 %; H, 6.39 %

*Isopropyl caffeate* **(5):** white solid, Yield 61.2 %, MP =146°C/ Lit. 129.8-135°C[32], $^1$H-RMN (in DMSO) δ 7.45 (1H, d), 7.03 (1H, d), 6.99 (1H, dd), 6.75 (1H, d), 6.23 (1H, d), 4.98 (1H, h), 1.24 (6H, d); $^{13}$C (in DMSO) δ 166.55 (s), 148.81 (s), 146.02 (s), 145.30 (s), 125.98 (s), 121.72 (s), 116.19 (s), 115.29 (s), 114.91 (s), 67.35 (s,22.23 (s)) EA Th: C, 64.85 %; H, 6.35 %; Found: C, 64.78 %; H, 6.38 %

*Butyl caffeate* **(6):** white solid, Yield 14 % after DCVC (DCM/MeOH (1/0 to 95/5), MP =112.1°C/ Lit. 110 °C[50], $^1$H-RMN (in DMSO) δ 7.46 (1H, d), 7.03 (1H, d), 6.99 (1H,

dd), 6.75 (1H, d), 6.25 (1H, d), 4.11 (2H, t), 1.61 (2H, m),1.37 (2H, m) 0.91 (3H, t); $^{13}$C (in DMSO) δ 167.11 (s), 149.08 (s), 146.09 (s), 145.51 (s), 125.80 (s), 121.88 (s), 116.14 (s), 115.17 (s), 114.32 (s), 63.86 (s), 30.83 (s), 19.15 (s), 14.08 (s) EA Th: C, 64.85 %; H, 6.35 %; Found: C, 64.84 %; H, 6.39 %

*Ethyl 3-(3,4-methylenedioxyphenyl)-2-propenoate* **(13):** white solid, Yield 77 %, MP = 66.8°C/ Lit. 68°C[51], $^{1}$H-RMN (in DMSO) δ 7.56 (1H, d), 7.41 (1H, d), 7.19 (1H, dd), 6.95 (1H, d), 6.50 (1H, d), 6.08 (2H, s), 4.17 (2H, q), 1.25 (3H, t); $^{13}$C (in DMSO) δ 168.91 (s), 149.80 (s), 148.51 (s), 144.75 (s), 128.96 (s), 125.45 (s), 116.48 (s), 108.94 (s), 107.17 (s), 102.05 (s), 60.31 (s), 14.71 (s) EA Th: C, 65.45 %; H, 5.49 %; Found: C, 65.19 %; H, 5.58 %

### *2.5.2 General procedure for acetylated derivatives*[52]

The ester (20 mmol) was mixed in acetic anhydride (120 mmol) before a few drops of concentrated $H_2SO_4$ were added to the solution. A fast temperature rise was observed and after all the solid was dissolved, the stirring was continued for 1-2 hours. Then, water (100 mL) was added, and the solution was stirred for further 1 h to remove any excess acetic anhydride left. The solid precipitate was filtered on $G_3$, washed with water (3x50 mL), and dried under vacuo.

*3,4-diacetoxy-trans-cinnamic acid* **(7):** white solid, Yield 48 %, MP = 197°C, $^{1}$H-RMN (in DMSO) δ 7.66 (1H, d), 7.63 (1H, dd), 7.56 (1H, d) 7.31 (1H, d), 6.53 (1H, d), 2.29 (6H, d); $^{13}$C (in DMSO) δ 168.67 (s), 168.58 (s), 167.88 (s), 143.73 (s), 142.80 (s), 142.44 (s), 133.65 (s), 127.20 (s), 124.56 (s), 123.43 (s), 20.85 (s), 20.79 (s) EA Th: C, 59.09 %; H, 4.58 %; Found: C, 59.68 %; H, 4.66 %

*Methyl 3,4-diacetoxy-trans-cinnamate* **(8):** white solid, Yield 48 %, MP = 84°C, $^{1}$H-RMN (in $CDCl_3$) δ 7.64 (1H, d), 7.40 (1H, dd), 7.35 (1H, d) 7.22 (1H, d), 6.39 (1H, d), 3.80 (3H, s) 2.31 (6H, d); $^{13}$C (in $CDCl_3$) δ 168.08 (s), 167.99 (s), 167.03 (s), 143.51 (s), 142.93 (s), 142.45 (s), 133.28 (s), 126.42 (s), 123.95 (s), 122.72 (s), 119 (s), 51.82 (s), 20.62 (s) EA Th: C, 60.43 %; H, 5.07 %; Found: C, 60.43 %; H, 5.09 %

*Ethyl 3,4-diacetoxy-trans-cinnamate* **(9):** white solid, Yield 42 %, MP = 73.6°C/Lit. 73-74°C[53], $^{1}$H-RMN (in DMSO) δ 7.71 (1H, d), 7.67 (1H, dd), 7.64 (1H, d) 7.32 (1H, d), 6.65 (1H, d), 4.20 (2H, q) 2.29 (6H, d), 1.26 (3H, t); $^{13}$C (in DMSO) δ 168.66 (s), 1678.57 (s), 166.49 (s), 143.95 (s), 143.12 (s), 142.81 (s), 133.38 (s), 127.42 (s), 124.60 (s), 123.65 (s), 119.72 (s), 60.62 (s), 20.85 (s), 20.78 (s), 14.66 (s); EA Th: C, 61.64 %; H, 5.37 %; Found: C, 61.83 %; H, 5.48 %

*Propyl 3,4-diacetoxy-trans-cinnamate* **(10):** white solid, Yield 71 %, MP = 103°C, $^{1}$H-RMN (in $CDCl_3$) δ 7.62 (1H, d), 7.41 (1H, dd), 7.36 (1H, d) 7.22 (1H, d), 6.39 (1H, d), 4.16 (2H, t), 2.31 (6H, d), 1.73(2H, sex), 0.99 (3H, t); $^{13}$C (in $CDCl_3$) δ 168.09 (s), 168.02 (s), 166.70 (s), 142.61 (s), 142.61 (s), 142.59 (s), 133.40 (s), 126.37 (s), 123.92 (s), 122.71 (s), 119.51 (s), 66.30 (s), 22.06 (s), 20.63 (s), 10.44 (s) EA Th: C, 62.74 %; H, 5.82 %; Found: C, 62.64 %; H, 5.96 %

*Isopropyl 3,4-diacetoxy-trans-cinnamate* **(11):** white solid, Yield 72 %, MP = 73°C, $^{1}$H-RMN (in DMSO) δ 7.71 (1H, d), 7.67 (1H, dd), 7.62 (1H, d) 7.32 (1H, d), 6.62 (1H, d), 5.03(1H, h), 2.29 (6H, d), 1.26 (6H, d); $^{13}$C (in DMSO) δ 168.65 (s), 168.56(s), 166 (s), 143.91 (s), 142.95 (s), 142.80 (s), 133.40 (s), 127.35 (s), 124.58 (s), 123.67 (s), 120.12 (s), 67.94 (s), 22.16 (s), 20.78 (s) EA Th: C, 62.74 %; H, 5.92 %; Found: C, 62.84 %; H, 5.94 %

*Butyl 3,4-diacetoxy-trans-cinnamate* **(12):** white solid, Yield 70 %, MP = 74°C, $^{1}$H-RMN (in $CDCl_3$) δ 7.62 (1H, d), 7.39 (1H, dd), 7.36 (1H, d) 7.22 (1H, d), 6.39 (1H, d), 4.21 (2H, t) 2.31 (6H, d), 1.69 (2H , q), 1.44 (2H, sex), 0.96 (3H, t); $^{13}$C (in $CDCl_3$) δ 168.09 (s), 168.01 (s), 166.70 (s), 143.42 (s), 142.59 (s), 142.43 (s), 133.40 (s), 128.13 (s), 126.37 (s), 123.92 (s), 122.71 (s), 119.52 (s), 64.59 (s), 30.74 (s), 20.67 (s), 19.20 (s), 13.75 (s) EA Th: C, 63.74 %; H, 6.29 %; Found: C, 63.72 %; H, 6.31 %

### *2.5.3. N-ethyl-3-(3,4-dihydroxyphenyl)-2-propenamide*

Caffeic acid (0.54 g, 3 mmol) was dissolved in 5 mL of DMF at 0°C. Then, triethylamine (0.4 mL, 3.15 mmol) et ethylamine (2.3 mL, 4.55 mmol) were added to the solution. After that, solution of BOP in DCM (1.4 g in 5 mL) was poured in the mixture. After 1-day agitation at RT, water was added to the reaction and extracted with EtOAc. The organic phase was washed with HCl 1 N and brine. After general drying method ($MgSO_4$), the solvent was removed by evaporation under vacuo. The resulting product was purified through DCVC with *n*-hexane/EtOAc (1/0 to 0/1).

*N-ethyl-3-(3,4-dihydroxyphenyl)-2-propenamide* **(14):** white solid, Yield 6.3 %, MP =207.6-209.1°C/Lit. 117-118°C[21], $^{1}$H-RMN (in DMSO) δ 7.96 (1H, t), 7.22 (1H, d), 6.93 (1H, d), 6.82 (1H, dd) 6.73 (1H, d), 6.30 (1H, d), 3.17 (2H, 5h), 1.06 (3H , t); $^{13}$C (in DMSO) δ 165.58 (s), 147.73 (s), 146.01 (s), 139.30 (s), 126.85 (s), 120.79 (s), 119.07 (s), 116.19 (s), 114.20 (s), 33.90 (s), 15.31 (s), EA Th: C, 63.76 %; H, 6.32 %, N, 6.76 % ; Found: C, 63.30 %; H, 6.32 %, N, 6.74 %

### *2.5.4. N,N-diethyl-3-(3,4-methylenedioxyphenyl)-2-propenamide*

The acid (0.54 g, 2.8 mmol) was dissolved in 5 mL DMF and heated to 60°C. Then, CDI (0.6 mmol) and diethylamine (0.29 mL, 2.81 mmol) were added to the mixture and stirred overnight. After that, 5 mL HCl 1 N were added to the solution which was extracted with EtOAc. The organic phase was washed with brine and dried with $MgSO_4$ before evaporation under vacuo. The product was purified through DCVC *n*-hexane/EtOAc (3/1 to 0/1).

*N,N-diethyl-3-(3,4-methylenedioxyphenyl)-2-propenamide* **(15):** white solid, Yield 35 %, MP = 67°C/Lit. 62-64°C[54], $^{1}$H-RMN (in DMSO) δ 7.45 (1H, d), 7.41 (1H, d), 7.13 (1H, dd), 6.96 (1H, d), 6.93 (1H, d), 6.06 (2H, s), 3.51 (2H, q), 3.36 (2H, q), 1.14 (3H, t), 1.06 (3H, t); $^{13}$C (in DMSO) δ 165.21 (s), 148.89 (s), 148.39 (s), 141.38 (s), 130.17 (s), 124.47 (s), 117.18 (s), 108.82 (s), 107.06 (s), 101.84 (s), 41.85 (s), 15.73 (s), 15.72 (s) EA Th: C, 68.00 %; H, 6.93 %, N, 5.66 % ; Found: C, 67.66 %; H, 7.06 %, N, 5.87 %

#### *2.5.5. 3-(3,4-dimethoxyphenyl)-2-propenamide*

3,4-dimethoxycinnamic acid (2,4 mmol) was dissolved in thionyl chloride (55 mmol). The mixture was stirred under reflux during 3h before removing the excess of $SOCl_2$ under vacuo. Then, toluene was poured on the residue and evaporated 3 times. After, the resulting product reacted with an excess of ammonia 25 % in dioxane for 3h. The final product was filtrated, washed, and then dried under vacuo.

*3-(3,4-dimethoxyphenyl)-2-propenamide* **(16):** white solid, Yield 15 %, MP =161°C/Lit. 168°C[55], $^{1}$H-RMN (in DMSO) δ 7.35 (1H, d), 7.15 (1H, d), 7.10 (1H, dd), 6.99 (1H, d), 6.49 (1H, d), 3.79 (6H, d); $^{13}$C (in DMSO) δ 167.44 (s), 150.56 (s), 149.36 (s), 139.71 (s), 128.13 (s), 121.91 (s), 120.46 (s), 112.19 (s), 110.41 (s), 56.01 (s), EA Th: C, 63.76 %; H, 6.32 %, N, 6.76 % ; Found: C, 63.33 %; H, 6.39 %, N, 6.55 %

## 3. RESULTS AND DISCUSSION

### 3.1. Literature screening

To guide our synthesis, we have screened the literature (Scifinder®) to report all the previously described caffeate derivatives tested on *Plasmodium* species. The results of these investigations are summarized in Tab. 1-2 and the corresponding structures could be found in the Supplementary Materials (Appendix A).

This screening has first confirmed the few numbers of studies about the potential of the caffeate scaffold in malaria treatment (~ 20 articles). But it has highlighted the great structural diversity of this structure, mainly isolated from natural sources. Moreover, we have noticed than a large concentration range of antiplasmodial activity (between 0.6 and 280 µM) have been obtained for these caffeate with some highly active compounds ($IC_{50}$ < 5 µM). Unfortunately, this potent antiplasmodial effect was often combined with a weak selectivity against human cell (SI around 1).

However, we have retrieved some important trends from these investigations. First, the necessary decrease of the ionization capacity for a good antiplasmodial effect. Indeed, free carboxylic acid functions are often detrimental for the *P. falciparum* growth inhibition. In addition, a reduction of free hydroxyls (aromatic or aliphatic) was also beneficial. Therefore, an increased lipophilicity of the structure (higher logP value) was often observed for the most active compounds.

However, this high logP did not guarantee a great antiplasmodial effect. Consequently, other parameters seemed to modulate the efficiency, for example the 3D conformation. Indeed, we have noticed that enantiomers did not exhibit a similar activity e.g., 3-*O*-*E/Z*-caffeoyl lupeol (logP = 10). The *trans* was inactive and *cis* had an $IC_{50}$ = 14.6 µM.[56]

Consequently, we have focused our pharmacomodulation on the reduction of the ionization potential by the modification of the carboxylic acid moiety, with esters, which are commonly reported, but also with some amides which are scarcely studied. Moreover, we have an increase of the lipophilicity (logP) as **1** has a clogP quantified at 1.15.

### 3.2. Synthesis

As an increase of the *O*-alkyl chain could be beneficial to the antiplasmodial effect, we have first decided to extend our library of small caffeate esters. Therefore, we have synthesized methyl, ethyl, propyl, isopropyl and butyl caffeate to confirm than a higher lipophilicity led to a greater inhibitory effect on *Plasmodium.*

For this, we have used a modified Fischer's esterification between the corresponding alcohol and 3,4-dihydroxycinnamic acid (Scheme 1).[32]

In addition, even if a protection of the free hydroxyls seemed beneficial for the effect, the phenolic functions are often reported as essentials. Consequently, we have produced ethyl 3-(3,4-methylenedioxyphenyl)-2-propenoate. In this case, the catechol moiety was hidden with a strong substituent which could not be easily removed in physiological conditions.

We have employed the Fischer's esterification protocol with 3,4-(methylenedioxy)cinnamic acid as start compound. On the other hand, the acetoxy-derivatives of our esters could be defined as prodrugs because acetyls were labile substituents in vivo. Therefore, as previously described by Gokcen et al. for gallic acid, acetic anhydride and sulfuric acid were employed to protect the phenolic alcohols of the structure.[52]

Another way to reduce the ionization potential of the carboxylic acid moiety was the amide formation. Moreover, these structures are often considered as more metabolically stable than esters which could be easily cleaved by various esterases. Therefore, we have produced small, substituted amides of 3,4-hydroxycinnamic acid, 3,4-dimethoxycinnamic, and 3,4-(methylenedioxy)cinnamic acid to observe the impact on the antiplasmodial activity. For that, we have used several protocols but only few derivatives have been obtained with a sufficient degree of purity for *in vitro* assays.

### 3.3. Antiplasmodial assay

All the compounds involved in this paper have been then submitted to the evaluation of their antiplasmodial potential (Tab. 3). Their effect was compared to artemisinin as reference compound, with $IC_{50}$ = 3.98 ± 2.07 nM, on a sensitive strain of *Plasmodium falciparum* (3D7).

Unsurprisingly, none of the tested molecules were equivalent to artemisinin. But we could highlight the significant reduction of $IC_{50}$ between caffeic acid ($IC_{50}$ = 80.2 µM) and some of its derivatives. Indeed, all the esters showed a lower range of concentration ($IC_{50}$ ~ 20 µM).

This increment of the anti-*Plasmodium* activity could be explained by the reduction of the ionization potential and the increase of the lipophilicity (LogP).[57–59]

Consequently, as proposed before, the molecules could reach more easily their site of action, inside the red blood cells (RBC).[32]

These results were consistent with previous studies in which small esters of caffeic acid were found to be 2- to 4-fold more active than the corresponding acid.[32] However, we could notice than butyl caffeate was slightly more efficient than the other esters ($IC_{50}$ = 16.2 µM).

Thus, a bigger *O*-alkyl chain on the carboxylic function could lead to a better effect on *Plasmodium.* This can be confirmed by the effect of octadecyl caffeate, reported before with $IC_{50}$ = 11.2 µM.[60]

Similarly, all the acetylated derivatives (**7-12**, Chart. 2) showed a greater inhibitory effect than **1** ($IC_{50}$ ~ 25 µM). Thus, this could also find its origin in the increase of the hydrophobicity linked to a greater permeation rate in the erythrocytes.[57–59]

But they were not more efficient than their corresponding esters contrary to what has already been demonstrated with other polyphenolic structures such as gallic acid (unpublished results). This could perhaps be explained by a reduced hydrosolubility which impeded the maximal concentration in the culture media or a saturation of the active sites.

However, this could also find its origin in another phenomenon. In fact, as already suggested for other biological activities of caffeate derivatives (e.g. antiinflammatory),[59, 61] the inhibitory activity toward *Pf* could only increase with the hydrophobicity until an optimum logP value. Consequently, a higher lipophilicity did not enhance the efficiency and could even cause a loss of activity.[61–63]

In contrast, the labile character of this substitution was important for the effect. Indeed, the methylenedioxy-derivative of ethyl caffeate (**13**, Chart 3) was much less active ($IC_{50}$ = 97 µM) than the corresponding ester (**3**) or acetoxy- structure (**9**).

Thus, as often demonstrated for polyphenols, the phenolic *ortho* alcohols (catechol moiety) were required for their pharmacological effects.[32, 64–67]

This could find its explanation in the antioxidant potential of this structure which could play a role in the antiplasmodial activity. Therefore, a stable substitution on the phenolic functions could impede their activities.

In addition, the carboxylic function, even involved in ester formation, seemed also necessary for a good inhibition of *Plasmodium falciparum.* In fact, the amide derivatives (**14-16**, Chart 3) showed a decreased effect compared to other scaffolds ($IC_{50}$ > 90 µM). This could partially find its origin in a reduced logP (1.08) compared to **1** (1.15).

Thus, despite the decrease of the ionization potential, amides could not enter more easily the RBC. Another hypothesis could be the loss of some beneficial interactions with the target, due to the modification of hydrogen bond donor/acceptor balance (2/4 for **2-6** against 3/3 for **14**).[68]Interestingly, this balance was often considered as beneficial for the antioxidant profile. Indeed, amide derivatives of caffeic acid are often reported as more antioxidant than their corresponding esters.[69–71]

In conclusion, antioxidant properties activity could not be the only characteristic responsible for the antiplasmodial effect. But it must be further explored to identify the exact mechanism of action.

### 3.4. Cytotoxicity

As *Plasmodium falciparum* has mostly an intracellular development in Human,[3, 72] it was important to consider the potential toxicity of our molecules against normal cells.

First, we have tested the toxic effect on erythrocytes (Tab. 3). Indeed, the hemolysis induction by our derivatives could be deleterious to the parasitic development. Consequently, it could bias the previous *in vitro* assay as the *Plasmodium* could not grow due to the erythrocytic membrane rupture. Fortunately, none of our structures have shown a significant hemolytic activity (< 1 %) at 100 µg/mL.

On the other hand, to confirm the selectivity of the caffeate described here, we have chosen to test some of the most active ones against HUVEC (Human Vein Endothelial Cells). In fact, these cells are primary culture-like and could be malaria related because they are at the site of action of potential antimalarial drugs, in the blood vessels. Therefore, we have tested concentrations between 50 and 0.5 µg/mL.

Fortunately, none of the tested molecules have exhibited a significant toxicity against the selected cells. Moreover, the selectivity index value was superior or equal to 3 for each compound. Consequently, the antiplasmodial effect seemed selective to *Plasmodium*. However, it could be necessary to employ other cell lines or a zebrafish model to further explore the selectivity of action and eventual toxicity.

## CONCLUSION

In our research of new alternative treatments for malaria, we have focused our efforts on well-known and widely distributed natural polyphenols. Indeed, these molecules have several advantages such as a good water solubility and are often reported for interesting pharmacological activities. Among them, 3,4-dihydroxycinnamic acid (**1**) seemed a good starting point for investigations.

As demonstrated before for **1** this scaffold[32] or 3,4,5-trihydrohybenzoic acid scaffolds (unpublished results), small ester derivatives were often more active on *Plasmodium falciparum*. This could be explained due to the increase of their lipophilicity (cLogP), and then of their permeation rate in the RBC.[57–59]

Consequently, we have focused our pharmacomodulation on the reduction of the hydrophilicity. In addition, our library of compounds could give us the information needed to establish the pharmacophore responsible for the antiplasmodial effect.

It was found that the elements necessary for the activity seemed to be the catechol and the carboxyl moieties. Unfortunately, none of the designed compounds were highly promising because none of them reached the micromolar range ($IC_{50}$ = 16-241 µM). However, this antiparasitic effect seemed selective since no toxicity was observed on the selected human cells (RBC or HUVEC, SI > 3).

In conclusion, based on the classical guidelines ($IC_{50}$ < 1 µM)[73], none of our derivatives could be considered as potent antimalarial agents at this point. However, we have demonstrated that the increase of the lipophilicity was beneficial for the antiplasmodial effect. Therefore, an additional enhancement of this parameter needs to be explored and could lead to a better inhibitory effect. In addition, it will be necessary to study the impact of the water solubility on the pharmacokinetic profile, since it is well known that such parameter could impede the bioavailability of the structure, especially for an oral medicinal use.

## LIST OF ABBREVIATIONS

If abbreviations are used in the text either they should be defined in the text where first used, or a list of abbreviations can be provided.

## CONFLICT OF INTEREST

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## SUPPORTIVE/SUPPLEMENTARY MATERIAL

The supplementary materials could be obtained from the site of the journal. They are constituted by the structures of the antiplasmodial caffeate derivatives from the literature screening and the NMR spectra of the synthetic compounds.

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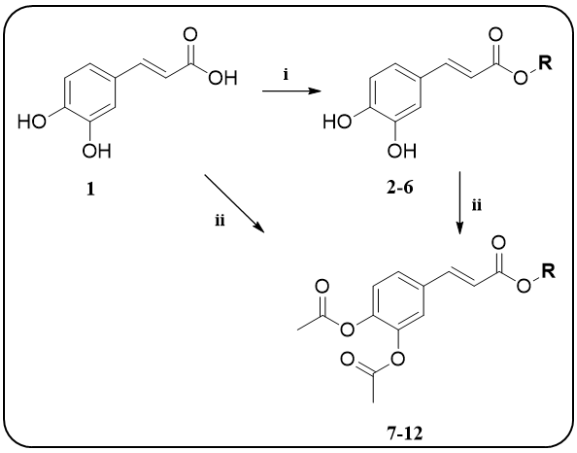
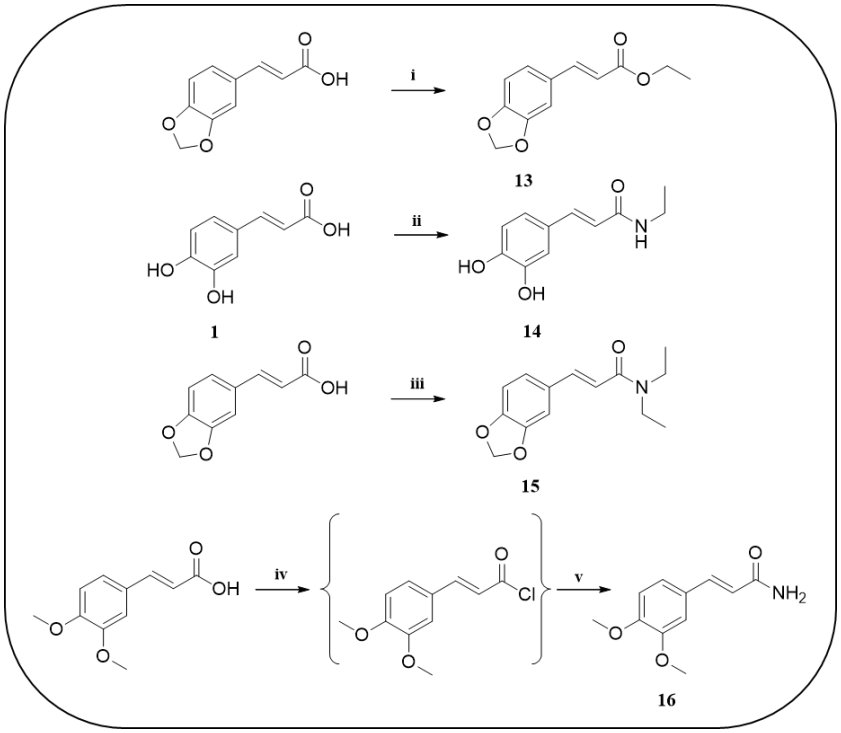
## FIGURES/ILLUSTRATIONS

**Scheme 1.** Synthetic pathways employed to obtain various caffeate derivatives.




i alcohol, $H_2SO_4$; ii $Ac_2O$, $H_2SO_4$

**Scheme 2.** Synthesis of amides molecules derived from 3,4-dihydroxycinnamic acid.




i: EtOH, $H_2SO_4$ (77%); ii: triethylamine, ethylamine, BOP, DMF, DCM (7 %); iii CDI, diethylamine, DMF (35 %); iv $SOCl_2$; v ammonia, dioxane (15 %)

## CHEMICAL STRUCTURES

**Chart 1.** Small esters of caffeic acid synthetized for antiplasmodial evaluation.

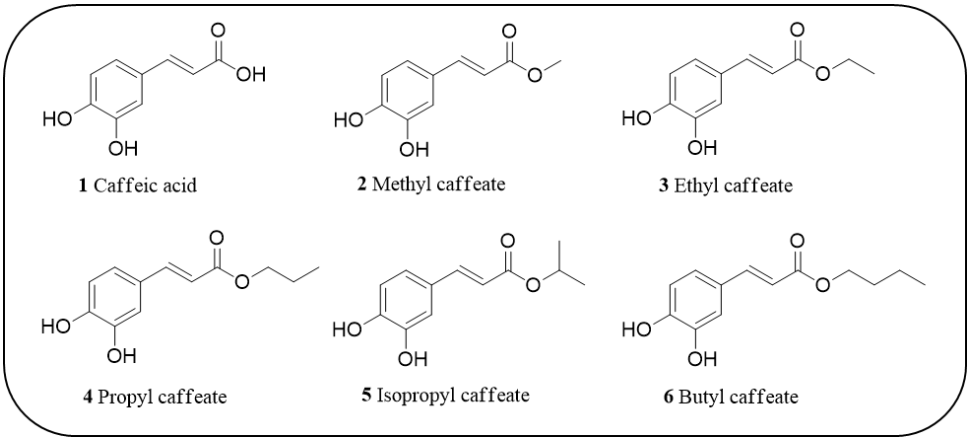



**Chart 2.** Acetylated caffeate derivatives screened for antiplasmodial activity.

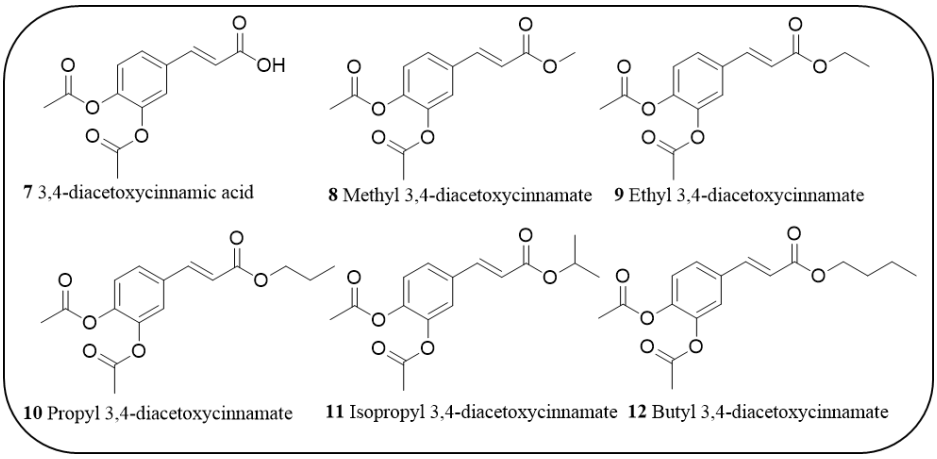
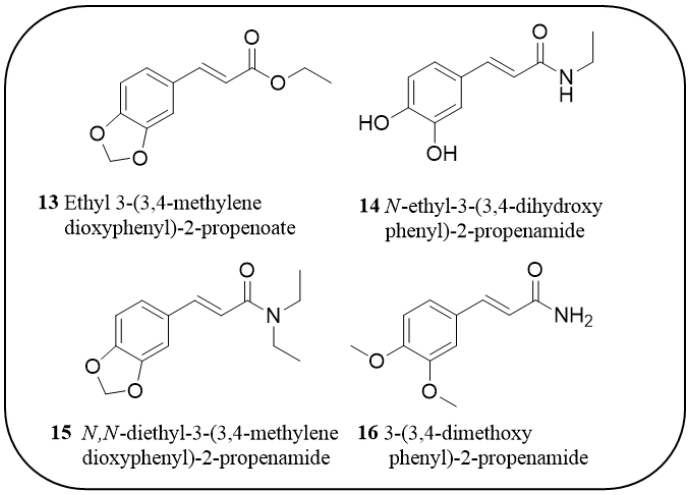



**Chart 3.** Caffeic acid inspired molecules tested on Plasmodium falciparum.



## TABLES

**Table 1.** Natural or Synthetic Caffeate derivatives previously screened against *Plasmodium* sp.

| Products | *Pf* (µM)[a] | Cells (µM)[b] | Cell line[c] | SI[d] | Ref. |
|---|---|---|---|---|---|
| Caffeic acid (**1**) | 60.4 ± 4.3<br>47.5 ± 8.8 | / | / | / | 28 |
| | >278 | / | / | / | 29 |
| | 50 ± 2 | 300 ± 50<br>180 ± 20<br>560 ± 60 | HL-60<br>FLK<br>MH22a | 6<br>3.6<br>11 | 30,31 |
| | 80.5 ± 22.8 | / | / | / | 32 |
| Methyl caffeate (**2**) | >103 | 2.2 ± 0.5<br>>103<br>45.3 ± 4.5<br>89.6 | NCIH187<br>KB<br>BC<br>Vero | 0.02<br>1<br>0.4<br>0.9 | 22 |
| | 27.3 ± 3.4 | / | / | / | 32 |
| Ethyl caffeate (**3**) | / | / | / | / | 42 |
| | 21.9 ± 9.4 | / | / | / | 32 |
| Isopropyl caffeate (**5**) | 21.9 ± 19.8 | / | / | / | 32 |
| Octadecyl caffeate | 11.2 | / | / | / | 43 |
| Ethyl 3',4'-dimethoxycinnamate | 196.6 | / | / | / | 32 |
| Chlorogenic acid or 3-caffeoylquinic acid | >141 | >254 | L6 | 1.8 | 44 |
| | 69.4 ± 6.4<br>61.4 ± 4.3 | / | / | / | 28 |
| | 39 ± 3.8 | >2000<br>1000 ± 80<br>/ | HL-60<br>FLK<br>MH22a | 51<br>26<br>/ | 30,31 |
| | >280 | / | / | / | 32 |
| 3-*O*-caffeoylquinic methyl ester | >136 | >244 | L6 | 1.8 | 44 |
| | >136 | / | / | / | 29 |
| | 173.8 ± 87.7 | / | / | / | 32 |
| Ethyl chlorogenate | >131 | / | / | / | 29 |
| | 168.9 ± 61.3 | / | / | / | 32 |
| 5-*O*-Caffeoylshikimic acid | 127 | 250 | L6 | 2 | 44 |
| 28-*O*-acetyl-3*β*-*O*-*trans*-caffeoylbetulin | NA | NA<br>31.5<br>75.7 | KB<br>NCIH187<br>Vero | / | 45 |
| 3*β*-*O*-*trans*-caffeoylbetulin | NA | 28.4<br>16.2<br>8.9 | KB<br>NCIH187<br>Vero | / | 45 |
| 3-*O*-*trans*-caffeoylbetulinic acid | 4.5 | 36.5<br>4.0<br>5.0 | KB<br>NCIH187<br>Vero | 8<br>0.9<br>1.1 | 45 |
| | 1.4 ± 0.02 (D6)<br>0.98 ± 0.014 (W2) | 4.0 | KB | 2.9<br>4.0 | 46 |
| 3-*O*-*trans*-caffeoyllupeol | NA | NA<br>NA<br>NA | KB<br>NCIH187<br>Vero | / | 45 |
| | NA | / | / | / | 47 |
| 3-*O*-*cis*-caffeoyllupeol | 14.6 | / | / | / | 47 |

a = $IC_{50}$ value in µM on *Plasmodium falciparum* (*Pf*); b = $IC_{50}$ value in µM in cell toxicity assays; c = tested cell lines; d = Selectivity index (SI); NA= Non active

**Table 2.** Natural or Synthetic Caffeate derivatives previously screened against *Plasmodium sp*.

| Products | *Pf* (µM)[a] | Cells (µM)[b] | Cell line[c] | SI[d] | Ref. |
|---|---|---|---|---|---|
| 3*α*-*O*-*trans*-caffeoyl-22-hydroxyhopane | NA | 34.6<br>28.5<br>16.6 | KB<br>NCIH187<br>Vero | / | [45] |
| Quercetagetin-6-*O*-(6-*O*-caffeoyl-*β*-D-glucopyranoside) | NA | / | / | / | [48] |
| Kelampayoside B | NA | / | / | / | [49] |
| Rosmarinic acid | 65.1 ± 5.0<br>65.0 ± 7.0 | / | / | / | [28] |
| 4-caffeoylquinic acid | 61.4 ± 4.3<br>53.6 ± 5.0 | / | / | / | [28] |
| 5-caffeoylquinic acid | 84.8 ± 6.4<br>85.3 ± 4.2 | / | / | / | [28] |
| 3,4-di-caffeoylquinic acid | 36.2 ± 1.0<br>49.0 ± 6.8 | / | / | / | [28] |
| 3,4-di-caffeoylquinic acid methyl ester | 2.90 | NA | KB<br>NCI-H87<br>MCF7 | / | [50] |
| 3,5-di-caffeoylquinic acid methyl ester | 2.4 | NA | KB<br>NCI-H87<br>MCF7 | / | [50] |
| 4,5-di-caffeoylquinic acid | 29.3 ± 2.4<br>43.2 ± 4.2 | / | / | / | [28] |
| 3,4,5-tri-caffeoylquinic acid | 181.4 ± 2.1<br>65.0 ± 7.0 | / | / | / | [28] |
| Betulinic acid 3-diacetylcaffeate | 0.7 ± 0.04<br>0.6 ± 0.04 | 3.0 | KB | 4.4<br>4.8 | [46] |
| *ent*-18-(E)-caffeoyloxy-7b-hydroxy-3-cleroden-15-oic acid | 14.6 ± 1.6 | / | / | / | [51] |
| *ent*-18-(E)-caffeoyloxy-8(17)-labden-15-oic acid | 42 ± 3.7 | / | / | / | [51] |
| *ent*-15-(E)-caffeoyloxy-8(17)-labden-18-oic acid | 32 ± 1.7 | / | / | / | [51] |

a = $IC_{50}$ value in µM on *Plasmodium falciparum* (*Pf*); b = $IC_{50}$ value in µM in cell toxicity assays; c = tested cell lines; d = Selectivity index (SI); NA= Non active

**Table 3.** Biological activities of caffeate derivatives.

| Products | 3D7[a] | Hemolysis (%) | HUVEC[b] (SI) | cLog P[c] |
|---|---|---|---|---|
| Artemisinin | 0.004 ± 0.002 | ~ | ~ | ~ |
| **1** | 80.2 ± 22.9 | < 1 % | ~ | 1.15 |
| **2** | 27 ± 3.4 | < 1 % | ~ | 1.42 |
| **3** | 21.9 ± 9.4 | < 1 % | ~ | 1.75 |
| **4** | 22.8 ± 2.6 | < 1 % | >235 (>10) | 2.24 |
| **5** | 21.9 ± 9.8 | < 1 % | ~ | 2.07 |
| **6** | 16.2 ± 2.4 | < 1 % | 135 ± 26 (8) | 2.66 |
| **7** | 36.8 ± 10 | < 1 % | ~ | 1.10 |
| **8** | 22.3 ± 8.3 | < 1 % | >180 (>8) | 1.37 |
| **9** | 27.3 ± 6.2 | < 1 % | ~ | 1.71 |
| **10** | 46.7 ± 8.2 | < 1 % | >139 (>3) | 2.19 |
| **11** | 27.8 ± 3.1 | < 1 % | 148 ± 3.8 (5) | 2.02 |
| **12** | 20.2 ± 5.4 | < 1 % | >156 (>8) | 2.61 |
| **13** | 97.3 ± 19.9 | < 1 % | ~ | 2.31 |
| **14** | 94.7 ± 22.5 | < 1 % | ~ | 1.08 |
| **15** | 165.2 ± 64.1 | < 1 % | ~ | 2.21 |
| **16** | >241 | < 1 % | ~ | 1.03 |

a $IC_{50}$ values in µM against the 3D7 strain of *P. falciparum*; b $IC_{50}$ values in µM calculated on Human Umbilical Vein Endothelial Cells; c Calculated with Chemdraw Version 12.0