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Correlation study on methoxylation pattern of flavonoids and their heme-targeted antiplasmodial activity

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Abstract

A library of 33 polymethoxylated flavones (PMF) was evaluated for heme-binding affinity by biomimetic MS assay and *in vitro* antiplasmodial activity on two strains of *P. falciparum*. Stability of heme adducts was discussed using the dissociation voltage at 50% (DV_{50}). No correlation was observed between the methoxylation pattern and the antiparasitic activity, either for the 3D7 chloroquine-sensitive or for the W2 chloroquine-resistant *P. falciparum* strains. However, in each PMF family an increased DV_{50} was observed for the derivatives methoxylated in position 5. Measurement of intra-erythrocytic hemozoin formation of selected derivatives was performed and hemozoin concentration was inversely correlated with heme-binding affinity. Kaempferol showed no influence on hemozoin formation, reinforcing the hypothesis that this compound may exert *in vitro* antiplasmodial activity mostly through other pathways. Pentamethoxyquercetin has simultaneously demonstrated a significant biological activity and a strong interaction with heme, suggesting that inhibition of hemozoin formation is totally or partially responsible for its antiparasitic effect.

Keywords

Polymethoxyflavone, *Plasmodium falciparum*, Heme-binding, Hemozoin.

1. Introduction

Malaria is one of the major parasitic disease affecting humans [1]. In 2018, an estimated 228 million cases occurred worldwide and US\$ 2.7 billion from international efforts were invested for its control and eradication [2]. It is transmitted by the bite of female mosquitoes of *Anopheles* genus infected by *Plasmodium* spp. [3]. Malaria infection starts with a mosquito bite introducing *Plasmodium* sporozoites, which invade the host's liver cells. During the intraerythrocytic trophozoite stage of the life cycle, the parasite internalizes up to 80% of the host cell hemoglobin in a phagocytic organelle called cytosome [4]. The cytosome then transports hemoglobin into an acidic digestive vacuole (pH 5.2) where it is digested by proteolytic enzymes into small peptides used as source of amino acids for the parasite [5]. During this process, four molecules of heme (ferriprotoporphyrin IX), toxic to the parasite, are released for each hemoglobin unit digested. The main heme-detoxication pathway developed by *Plasmodium* spp. consists in the biocrystallization of heme into hemozoin, which is accumulated in the vacuole in an inert, solid form. This detoxication pathway has been the most attractive target in the search for new antimalarial drugs for decades [6–8]. It can be assessed *in vitro* by measuring crystallization of a solution of heme into the synthetic analog of hemozoin, referred to as β -hematin.

Several antimalarial drugs including 4-aminoquinolines [9] and artemisinin [10] are able to form stable adducts with heme, thus impeding the detoxification process and contributing to the antiparasitic activity. We previously assessed the ability of polymethoxyflavones (PMFs) isolated from *Pentzia monodiana* to form adducts with heme in a mass spectrometry-based assay [11]. This first report of heme affinity for this class of compounds was intriguing as antimalarial activity was greatly variable, from low to significant [11,12]. In order to explore the structure-activity relationship within this chemical series and more precisely the influence of methoxylation/hydroxylation pattern, a library of 33 PMFs was generated and evaluated for heme-binding affinity by MS assay and *in vitro* antiplasmodial activity on two strains of *P. falciparum* (sensitive and resistant). MS-based collision induced dissociation allowed to establish the dissociation voltage at 50% (DV_{50}) for each PMF-heme adduct. Calculation of physico-chemical parameters such as partition coefficient (logP) and topological polar surface area (tPSA) was carried out to provide indications about compounds lipophilicity. In addition, in order to correlate the heme-binding affinity with the *in vitro* antiplasmodial activity of PMFs, the intra-erythrocytic hemozoin formation of selected derivatives was measured.

2. Results and discussion

2.1. PMFs library

Sixteen PMFs were obtained by isolation from plant extracts (4 compounds from *Pentzia monodiana*, 8 compounds from *Gardenia oudiepe*) or as commercial standards (4 compounds). Then, methoxylation pattern was modified by two semisynthetic pathways with MeI or Me₂SO₄ as methyl donor in basic conditions (DBU or K₂CO₃), obtaining 17 derivatives [12,13]. The resulting library of 33 compounds, comprising 8 flavones (**1-5**, **23-25**) and 25 flavonols (**6-22**, **26-33**) can be clustered into 9 sub-families depending on their substitution in positions 3, 5, 6, 7, 3', 4' and 5' by hydroxyl or methoxyl groups: derivatives of chrysin (**1-2**), apigenin (**3-5**), kaempferol (**6-10**), viscosine (**11-14**), quercetin (**15-19**), chrysopenetin (**20-22**), eupatilin (**23-25**), myricetin (**26-29**) and 5'-hydroxyquercetagenin (**30-33**) derivatives (**Figure 1**, **Table 1**).

2.2 Physicochemical characteristics

Calculation of partition coefficient (logP) and topological polar surface area (tPSA) was carried out to provide indications about lipophilicity of PMFs. Within each sub-family of derivatives, higher degree of methoxylation is positively correlated with increasing logP and decreasing tPSA calculated values. This is due to the loss of the capacity of hydroxyl/phenol groups, once methoxylated, to form hydrogen bonds. This expected result is in accordance with previous reports [14].

However methoxylation of position 5 decreases the estimated logP. This drop of hydrophobicity for the methoxylated analogues is well known for flavonoids: the 5-OH is engaged in a strong intramolecular hydrogen bond with the carbonyl in position 4 [15]. This bond decreases the partial negative charge on the carbonyl oxygen atom and reduces the ability of the carbonyl group to form dipolar interactions with other polar molecules. If position 5 is bearing a methoxy group, the hydrogen binding ability of the carbonyl is restored and overrides the overall increase of hydrophobicity caused by the addition of a methoxy group, leading to a reduced LogP. This effect is obvious when comparing the following pairs of compounds: **9/10**, **13/14**, **21/22**, **28/29** and **32/33**. Apigenin derivative **5** does not conform to this hypothesis, without any explanation being found for that exception. An experimental measurement of the LogP should be made for this compounds to rule out a simulation software defect before concluding to a peculiar behavior of this compound. As a side observation, compound **18** shows that a phenol on position R3 can also establish an hydrogen bond with the carbonyl.

2.3 Correlation between methoxylation degree and heme affinity

A similar behavior is observed concerning the effect of methoxylation on the stability of heme-flavonoids adducts. Heme is hydrophobic, and hydrophobic interactions are involved in the formation of such adducts [16]. As methoxylation increases hydrophobicity of the flavonoids, it is expected to have an impact on the ability of the flavonoid to bind to heme. Such an effect is clearly observed for almost every family (derivatives of chrysin, apigenin, viscosine, quercetin, eupatilin, myricetin and 5'-hydroxyquercetagenin) with an increase of DV₅₀ along with methoxylation degree. Methoxylation in position 5 is responsible for a slight decrease of cLogP, therefore it was expected to slightly decrease DV₅₀. Instead, an unexpected, significant increase of DV₅₀ by a factor 2 or more was systematically observed upon methoxylation in position 5. Taking viscosine family as an example, DV₅₀ values of derivatives **11-13** are in a range of 7.85 to 8.16 eV, whereas methoxylation increases DV₅₀ up to 15.83, an effect observed by a right shift of the sigmoid for compound **14** (**Figure 2**). These data suggest that hydrophobicity is not the only factor influencing binding, and that position 5 has a pivotal effect on heme binding. This hypothesis is currently being explored by docking experiments, based on a quantum model of free heme.

2.4 In vitro antiplasmodial activity of PMFs on 3D7 chloroquine-sensitive and W2 chloroquine-resistant strains

Regarding *in vitro* antiplasmodial activity on *P. falciparum*, no correlation between methoxylation pattern and antiparasitic activity was observed, neither for the 3D7 chloroquine-sensitive strain nor for the W2 chloroquine-resistant strain. However, some observations can be discussed within groups, considering 10 μ M as an efficacy cut-off value as recommended by

OMS [17]. On 3D7 chloroquine-sensitive strain, all the quercetin derivatives tested (**15** to **19**) were active within a 3-9 μM range, regardless of their methoxylation degree. Within their own families, kaempferol **6** ($\text{IC}_{50} = 3.78 \mu\text{M}$), viscosine **11** ($\text{IC}_{50} = 6.06 \mu\text{M}$) and eupatilin ($\text{IC}_{50} = 29.31 \mu\text{M}$) were the least methoxylated but the most active derivatives, other derivatives being mostly inactive. In these series, methoxylation of any phenol or hydroxyl group leads to a loss of activity. Inversely, for apigenin, chrysoplenetin, myricetin and 5'-hydroxyquercetagenin sub-families, methoxylation would in some cases increase activity (**4**, **22**, **28**, **32**). Compounds from chrysin, eupatilin and myricetin sub-families were mostly inactive.

On W2 chloroquine-resistant strain, activities were for most compounds following the same pattern as on 3D7, with a slight decrease of IC_{50} . This is well illustrated by the apigenin, viscosine, quercetin and 5'-hydroxyquercetagenin families. In some families, an increase of activity was observed on W2 when compared with 3D7, like kaempferol **6**, compounds **22**, **23**, **25** and **28**. As with 3D7 results, no structure-activity relationships could be drawn, as within each family some derivatives showing either low or high degree of methoxylation were active. Kaempferol can be highlighted as one of the most active compound on both strains, and compounds **11**, **15**, **19**, **22** and **32** were also active against both chloroquine-sensitive and resistant strains, without any obvious influence of the methoxylation degree or pattern.

Unfortunately, these data altogether do not allow to draw any predictive correlation between DV_{50} values and antiplasmodial activity. The most striking example is kaempferol **6**, showing good antiparasitic activity but no binding at all to heme in the MS assay. These results highlight that affinity of PMFs for heme cannot be predictive of an antiplasmodial activity and that other mechanisms and targets may be implied in this effect. This hypothesis can be supported by the fact that flavonoids are known to be multi-target compounds, especially on protozoal organisms [18].

2.5. *In vitro* hemozoin formation

Hemozoin crystallization is not mediated by any enzyme, therefore an antiplasmodial mode of action based on its disruption cannot trigger resistance *per se*. Resistance against molecules showing this mode of action is mediated by modification of transport and efflux through the parasite membranes [19,20].

To further decipher the biological impact of the observed heme-binding affinity in the MS assay, hemozoin was quantified after *in vitro* incubation of parasites in erythrocytes infected by the chloroquine-sensitive 3D7 strain together with 3 PMFs: kaempferol **6**, active against 3D7 and W2 strains but not forming adduct with heme; tetramethoxykaempferol **10**, inactive against 3D7 but forming very stable heme-adduct ($\text{DV}_{50} = 15.77 \text{ eV}$); and pentamethoxyquercetin **19**,

with low IC_{50} against 3D7 (3.96 μM) and W2 (9.51 μM) strains and strongly binding to heme ($DV_{50} = 15.89 \text{ eV}$) (**Figure 3**).

In accordance with the results of the biomimetic MS binding assay, kaempferol **6** showed no influence in the quantity of hemozoin formation, reinforcing the hypothesis that this compound may exert its *in vitro* antiplasmodial activity mostly through other pathways. Indeed, kaempferol **6** and quercetin **15** are known to be inhibitors of FabG, FabZ and FabI enzymes, involved in type II fatty acid biosynthesis (FAS-II) of *P. falciparum*, essential to the life cycle of the parasites [21]. In addition, PMFs from kaempferol sub-family are reported as inhibitors of human serine-threonine kinase and thioredoxin reductase, which show high homology with their plasmodial equivalents, fundamental for parasite growth and survival [22–24].

Tetramethoxykaempferol **10** is able to form stable adduct in the MS assay but does not modify the *in vitro* hemozoin formation in 3D7 strain. Along with the absence of antiplasmodial activity, these data suggest that this compound may not reach its target *in vitro*. Explanations for such a biological inefficacy can be active efflux, rapid metabolization, poor solubility, poor membrane-crossing ability or binding to other non-vital targets. Some PMFs such as tangeretin and wogonin have been previously reported to show poor membrane permeability in several *in vitro* assays on biological membrane models [25]. These properties could hamper the penetration of methoxyflavonoids first into the erythrocyte, then through the parasite membrane. This suggests that some compounds may never reach the parasite vacuole, where hemozoin formation takes place. Noteworthy, absence of antiplasmodial activity is observed for almost all of the 5-methoxy derivatives (**5**, **10**, **14**, **29**, **33**) and in a lower extent for the 5-hydroxy derivatives **20**, **21** and **25**. Methoxylation in R5 also systematically resulted in absence of cytotoxicity. It can therefore be interpreted as a pattern favoring a poor biological relevance.

Yet compound **19** is an exception to this hypothesis. Pentamethoxyquercetin **19** ($IC_{50} = 3.96 \mu\text{M}$ and 9.51 μM on 3D7 and W2 strain, respectively and $DV_{50} = 15.89 \text{ eV}$) decreased hemozoin formation of 44 % after 14 h of incubation. A similar effect was observed for chloroquine ($DV_{50} = 15.86 \text{ eV}$), which reduced hemozoin formation by 63 %. This result allows to correlate the heme-binding ability exerted by this compound in the MS assay with the antiplasmodial effect. In agreement with the IC_{50} values against both *P. falciparum* strains, we can hypothesize that one of the mechanisms of antiplasmodial activity of pentamethoxyquercetin **19** is the obstruction of the main detoxification mechanism of heme, by impeding hemozoin formation.

2.6. Cytotoxic activity

Eupatilin **23** was the strongest cytotoxic flavone when tested on HUVEC cells. Six other derivatives **12**, **16**, **17**, **20**, **21** and **26** have demonstrated an IC_{50} below 20 μM . They all possess

a phenol group on position 5 and are methoxylated on R3 and R4', excepted for **23** and **16** which bear a hydrogen on R3 and a phenol group on R4', respectively.

3. Conclusions

Impairment of hemozoin formation is the main mechanism of action of major antimalarial drugs such as artemisinin and chloroquine. The thorough assessment of a series of PMFs for *in vitro* antiplasmodial activity, heme-binding affinity and physicochemical characteristics has provided valuable data about the influence of the substitution pattern on heme binding, principally methoxylation in position R5. However, no correlation between PMFs' heme affinity and antiplasmodial activity could be clearly drawn. Some flavonoids with significant biological activity, such as kaempferol, are already known to interfere with other parasitic targets. Pentamethoxyquercetin **19** has simultaneously demonstrated a significant biological activity and a strong interaction with heme, highlighting that inhibition of hemozoin formation is totally or partially responsible for its antiparasitic effect. In an intermediate case, a high affinity for heme associated to a weak biological activity may result from several physicochemical parameters affecting *in vitro* and *in vivo* biological activity (solubility of compounds, pH stability or membrane permeability). This highlights one of the limitations of the MS assay, which is a cell-free methodology, giving false positive cases. Further studies on this PMFs library regarding membrane permeability, molecular docking on heme and other biological and physicochemical properties are required to better understand the antiplasmodial activity of these compounds.

4. Material and methods

4.1. Chemical and reagents

Mass spectrometry analyses of compounds were performed on a Waters ZQ 2000 mass spectrometer (Saint-Quentin, France). Silica gel 60 25-40 μm (Macherey-Nagel GmbH & Co, Düren, Germany) was used for column chromatography (CC). ^1H and ^{13}C NMR experiments were performed in deuterated chloroform on a Bruker AC 300 MHz or a Bruker 400 MHz spectrometer (Wissembourg, France) using Bruker pulse programs. All the reagents and solvents were purchased from Carlo Erba-SDS (Chaussée du Vexin, France). Cyclohexane and ethyl acetate were distilled before use. Deionized water was used for all experiments.

4.2 Flavonoids library

Flavonoids library is composed of either isolated, commercial or semi-synthetic derivatives. Compounds **7**, **11**, **12**, **23**, **26**, **27**, **30** and **31** were isolated from bud exudates of *Gardenia*

oudiepe (Rubiaceae); flavones **20**, **21**, **24** and **25** were isolated from aerial parts of *Pentzia monodiana* (Asteraceae) [11,12]. Compounds **1**, **3**, **6** and **15** were purchased from Sarsyntex (Merignac, France) or Sigma Aldrich (St. Louis, MO, USA). Compounds **7-8**, **13-14** and **32-33** were previously semisynthesized [12]. Compound **2** was obtained by methylation of **1**; likewise, compound **4** and **5** were obtained from **3**; **9** and **10** from **6**; **16-19** from **15**; **22** from **20** and **28-29** from **27** (see section 4.5).

4.3 Plant material

G. oudiepe (Rubiaceae) buds were collected in October 2008 in Forêt Plate, North Province of New Caledonia. Voucher specimens (POU-0290 respectively) are kept at the Herbarium of the Botanical and Tropical Ecology Department of the IRD Center, Noumea, New Caledonia.

Aerial parts of *P. monodiana* (Asteraceae) were collected in the Algerian Southern Sahara, in Tassili Ahaggar National Park nearby the Tahat mountain (23° 8'39.95", 5°43'46.28", alt.: 2019 m), Wilaya of Tamanrasset, Algeria, in September 2013. The collection agreement was issued by the director of the Ahaggar National Park Office (OPNA), n°965/DPLBCVPC/MC/13 and 111/SDV/MC/13. Identification was done under supervision of M. Belghoul, head of Department of Study of Natural Resources from the Ahaggar National Park. A voucher specimen is stored at the Herbarium of the Department of Pharmacognosy, Paris Descartes University, France (voucher specimen MKOSB2013KDHPM).

4.4 Extraction and isolation

Fifty-five grams of *G. oudiepe* exudate were dissolved in 1 L of CH₂Cl₂ and paper-filtered in order to remove vegetal pieces. The residual gum was subjected to several steps of silica gel chromatographic separation using various mixtures of solvents of increasing polarity as eluent (cyclohexane/CH₂Cl₂, EtOAc/CH₂Cl₂ and CH₂Cl₂/MeOH) to provide compounds **7**, **11**, **12**, **23**, **26**, **27**, **30** and **31**.

Identification of isolated compounds was confirmed by NMR and MS analysis and by comparison with literature data [26,27].

4.5 Semisynthesis of compounds **2**, **4-5**, **9-10**, **16-19**, **22**, **28** and **29**.

Semisynthesis of compound **8** from **7**; **13** and **14** from **12**; **32** and **33** from **31** were previously reported, using dimethyl sulfate (Me₂SO₄, 4 equiv.) and 1,8-diazobicyclo[5.4.0]undec-7-en (DBU, 2 equiv.) in dried acetone at room temperature [12]. Methylation of isolated compounds **20** and **27** to provide **22**, **28** and **29** and methylation of commercially available compounds **1**, **3**, **6** and **15** to provide compounds **2**, **4-5**, **9-10**, **16-19**, respectively, were performed by stirring the substrate with methyl iodide (MeI, 13.5 equiv.) and potassium carbonate (K₂CO₃, 6.6 equiv.) in

dried acetone at room temperature for 1 h. The crude reaction mixtures were precipitated and washed with ice-cold water. The resulting residues were solubilized with EtOAc (10 mL) and treated with 1N HCl solution (3 mL). The final products were extracted with EtOAc (3x10 mL) and the organic phases were washed with saturated NaCl solution and dried over Na₂SO₄. After filtration, and solvent evaporation, methylated compounds were purified by silica gel chromatography, using a mixture of CH₂Cl₂/MeOH (99.1/0.1 to 95/5 v/v) as eluent. Identification of resulting pure compounds was confirmed by comparison of ¹H, ¹³C-NMR and MS analysis with literature data.

Methylation of 50 mg (0.20 mmol) of 5,7-dihydroxyflavone (chrysin, **1**) afforded **2** (48.5 mg, yield 92 %) [28].

Methylation of 50 mg (0.19 mmol) of 5,7,4'-trihydroxyflavone (apigenin, **3**) gave **4** (31.6 mg, 60 %) and **5** (8.3 mg, 15 %) with a ratio of 4/1 [29].

Methylation of 100 mg (0.35 mmol) of 5,7,3',4'-tetrahydroxyflavone (kaempferol, **6**) gave **9** (71.1 mg, 62 %) and **10** (14.3 mg, 12 %) with a ratio of 5/1 [30].

Methylation of 150 mg (0.5 mmol) of 3,5,7,3',4'-pentahydroxyflavone (quercetin, **15**) gave **16** (15.6 mg, 10 %), **17** (15.6 mg, 9.5 %), **18** (97.8 mg, 55 %) and **19** (17.0, 9.2 %) with a ratio of 1/1/6/1 [31–33].

Methylation of 6 mg (0.017 mmol) of **20** gave **22** (5.5 mg, 85 %) [34].

Methylation of 12 mg (0.032 mmol) of **27** gave **28** (7.5 mg, 61 %) and **29** (3.9 mg, 28 %) with a ratio of 2/1 [35].

4.6 Biomimetic heme-binding assay by MS

Hemin (iron-containing porphyrin with chlorine counterion) and compounds were dissolved in DMSO at 5 mM. Citric buffer saturated octanol (CBSO) [36] was prepared by mixing 5 mL of citric acid 50 mM pH 5.2 and 15 mL of n-octanol (anhydrous, ≥99%) and letting it to settle at 23 °C for 30 min. Upper phase of CBSO was used. An auto sampler (G1367E 1260 Hip ALS, Agilent Technologies, Les Ulis, France) was used to realize the automatic mixing in 384 well-plates of: test compound 5 μL + heme 5 μL + tween 20 (10 μM in water) 5 μL + CBSO 85 μL. Five microliters of the sample were injected in infusion mode (direct injection) in a 6530 Accurate-Mass QToF LC/MS instrument (Agilent Technologies) with the following settings: positive ESI mode, 2 GHz acquisition rate. Ionization source conditions were: drying gas temperature 325 °C, drying gas flow rate 11 L/min, nebulizer 35 psig, fragmentor 175 V, skimmer 65 V. Range of *m/z* was 200-1700. In the positive-ion mode, purine C₅H₄N₄ [M + H]⁺ ion (*m/z* 121.050873) and the hexakis (1H,1H,3H-tetrafluoropropoxy)-phosphazene

$C_{18}H_{18}F_{24}N_3O_6P_3$ $[M+H]^+$ ion (m/z 922.009 798) were used as internal lock masses. Full scans were acquired at a resolution of 11 000 (at m/z 922). On the same spectrum (spectral windows) appears heme at m/z 616.16; adducts at m/z 616.16 + mass of ligand and 2heme + mass of ligand; heme + DMSO at m/z 694.19; heme dimer at m/z 1231.32 $[2\text{heme} - H]^+$. Chloroquine 5 mM in water (chloroquine diphosphate, Sigma-Aldrich) was used as positive control [37].

4.7 Collision induced dissociation of non-covalent adducts

Dissociation voltage at 50 % (DV_{50}) was determined by applying increasing collision energy from 0 to 32 eV. MSMS targeted mode, with isolation width of ~ 1.3 m/z and flow injection analysis mode (FIA) were used. MSMS source parameters were the same as described above. Results were plotted and a sigmoidal regression curve was calculated using GraphPad Prism software. DV_{50} is the collision energy dissociating 50% of the adduct [37,38].

4.8 Culture of *Plasmodium falciparum*

The chloroquine-sensitive 3D7 and chloroquine-resistant W2 *P. falciparum* strains were obtained from Malaria French National Reference Center (CNR Paludisme, Hôpital Bichat Claude Bernard, Paris). The strains were maintained in O+ human erythrocytes in albumin-supplemented RPMI medium under continuous culture using the candle-jar method. The parasites were synchronized to the ring stage by repeated sorbitol treatment.

4.9 *In vitro* antiplasmodial activity

A 2.5% (V/V) erythrocytes suspension with 1% parasitemia was incubated with the compounds. Compound concentrations obtained by serial dilution ranged between 48.5 nM and 1 μ M. Assays were made in duplicates. After 44 h of incubation at 37 °C, plates were subjected to 3 freeze-thaw cycles to achieve complete hemolysis. The parasite lysis suspension was diluted by 1:5 in lysis buffer. Monitoring of *in vitro* susceptibility uses the concentration that inhibits 50 % of the parasite growth (IC_{50}). Parasite growth was determined by using SYBR[®] Green I, a dye showing strong fluorescence enhancement upon contact with parasite DNA. Incorporation of SYBR[®] Green I (Applied Biosystems, Strasbourg, France) in parasite DNA was measured using the Mastercycler epRealplex (Eppendorf, France) according to the following program to increase the SYBR[®] Green I incorporation: 90 °C (1 min), decrease in temperature from 90 °C to 10 °C (during 5 min), followed by fluorescence reading. Untreated infected and uninfected erythrocytes were used as controls and chloroquine diphosphate (Sigma, France) as reference drug [39,40]. IC_{50} were calculated by IC-estimator online software (<http://www.antimalarial-icestimator.net>).

4.10 Cytotoxic activity

For determination of cytotoxicity, human umbilical vein endothelial cells (HUVECs) were cultured in Dulbecco's Modified Eagle medium/nutrient mixture F-12 (DMEM-F12) medium in the presence of 10 % fetal bovine serum (FBS) plus 1 % streptomycin and incubated in 5 % CO₂ at 37 °C. Cytotoxicity of compounds (starting concentration of 10 mM) was evaluated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric method [40]. HUVEC cells were seeded in a 96-well plate at 15,000 cells per well and incubated for 24 h until cells reached > 80% confluence. After discarding the old medium, the cells were incubated in the medium containing test compound at eight concentrations ranging from 0.78 to 50 μM. After 24 h incubation, 20 μL of MTT (5 mg/mL) were added to each well and cells were incubated for another 3 h. Finally, the culture medium containing MTT solution was removed and 100 μL DMSO were added to dissolve formazan crystals. Absorbance was read with an Eppendorf plate reader at 546 nm. IC₅₀, defined as compound concentration that reduces the number of viable cells by 50 %, was calculated using GraphPad Prism Software (Version 5.0, San Diego, CA, USA) [39].

4.11 ADMET Prediction

FAF-Drugs4 [42] and ChemSketch (ACD/Labs Products) software were used to screen the flavonoids library in order to predict their ADMET proprieties. For FAF-Drugs4 online service, the input structures (as a previously generated SDF files) were formatted according to FAF-Drugs4's requirements using Bank-Formatter. XLOGP3 was chosen as the clogP computation program to estimate the lipophilicity and the derived ADMET descriptors. For ChemSketch software, the input files were previously generated as SK2 files and prediction of clogP were estimated for all compounds [41].

4.12 Hemozoin *in vitro* formation

Hemozoin *in vitro* formation was evaluated by Heller and Roepe methodology [42] with slight modifications. Briefly, 8 mL of synchronized plasmodial culture of 3D7 *P. falciparum* strain at 2.5 % (v/v) erythrocytes suspension with 3 % of parasitemia were incubated with test compounds at IC₉₀ concentration in duplicates and cultivated as previously described conditions. After 9, 14 and 17 h of incubation, the suspension cultures were centrifuged, and parasite were lysed with 0.1 % saponin solution (w/v). Samples were then washed four times with ice-cold PBS buffer to yield purified parasites pellets. Then, pellets were washed once with 150 μL of PBS, 50 μL of H₂O and 50 μL of 1 M HEPES to remove any final traces of hemoglobin. Four hundred μL of 4 % SDS in 0.1 M NaHCO₃ were added to the pellet, and the mixture was shaken and incubated at 37°C for 2.5 h and then centrifuged at 1300 rpm. The pellet containing hemozoin was washed three times with bicarbonate buffer and finally

dissolved in 500 μ L of 3 N NaOH solution. One hundred microliters were placed into 96-well plate and UV absorption was read at 405 nm.

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