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Polyhydroxybenzoic acid derivatives as potential new antimalarial agents

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Abstract

With more than 200 million cases and 400,000 related deaths, malaria remains one of the deadliest infectious diseases of 2021. Unfortunately, despite the availability of efficient treatments, we have observed an increase in people infected with malaria since 2015 (from 211 million in 2015 to 229 million in 2019). This trend could partially be due to the development of resistance to all the current drugs. Therefore, there is an urgent need for new alternatives. We have, thus, selected common natural scaffolds, polyhydroxybenzoic acids, and synthesized a library of derivatives to better understand the structure–activity relationships explaining their antiplasmodial effect. Only gallic acid derivatives showed a noticeable potential for further developments. Indeed, they showed a selective inhibitory effect on *Plasmodium* (IC_{50} ~20 µM, SI > 5) often associated with interesting water solubility. Moreover, this has confirmed the critical importance of free phenolic functions (pyrogallol moiety) for the antimalarial effect. Methyl 4-benzoxy-3,5-dihydroxybenzoate (**39**) has, for the first time, been recognized as a potential lead for future research because of its marked inhibitory activity against *Plasmodium falciparum* and its significant hydrosolubility (3.72 mM).

KEYWORDS
gallic acid, hydroxybenzoic acid, malaria, medicinal chemistry, *Plasmodium*

1 | INTRODUCTION

Among all the public health problems, the response to infectious diseases remains a major challenge, as demonstrated by the recent outbreak of SARS-Cov-2. This could be explained by a lack of innovations or satisfactory treatments and the quick development of resistance. Moreover, infections are a high burden for low- and middle-income countries with millions of cases annually and costing billions of dollars.[1,2]

Therefore, malaria, one of the deadliest infections, is a cause of concern even in 2021. It is commonly characterized by cyclic fevers, headaches, and chills, but could lead to more severe symptoms: cerebral malaria, severe anemia, and finally death, if untreated.[3] This pathology is caused by a multistage parasite belonging to *Plasmodium* spp., and transmitted to humans through mosquitoes. Five species are currently reported as human pathogens: *Plasmodium falciparum* (*Pf*), *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*.[4] Among them, *Pf* is more problematic because it is responsible for more than 95% of cases worldwide and because of its higher mortality.[5]

This parasitosis results in more than 200 million cases and 400,000 related deaths annually, mainly of children under five.[5] Unfortunately, after more than a decade of constant decreases, the last few years have been characterized by an increase in patients with malaria (211 million in 2015 to 229 million in 2019). This trend could be partially explained by the development of resistances to all the recommended active principles, for example, in the Greater Mekong subregion and even in Africa (Rwanda).[6–8] However, it could also be a result of the development of resistances for the *Plasmodium* vectors to the common insecticides (mainly pyrethroids).[5] A vaccine (RTS,S/AS01 or Mosquirix®), developed by GSK and the Walter Reed Army Institute of Research, is now in pilot trials in some African countries, and the only vaccine that significantly reduces the

incidence of malaria (30% reduction). Currently, it cannot be used extensively.[5,9]

Consequently, in terms of the available drugs, the first to be reported was quinine (**1**, Figure 1), an alkaloid from *Cinchona* sp. bark. It has been followed by the development of other 4-aminoquinoline derivatives including chloroquine, hydroxychloroquine, and amodiaquine (**2**–**4**, Figure 1).[10–12] After that, different classes of molecules, mostly inspired by natural compounds, were developed, including sulfadoxine, pyrimethamine, and lumefantrine (**5**–**7**, Figure 1).[12]

However, the last major discovery was artemisinin (**8**, Figure 1), a sesquiterpene lactone, isolated from *Artemisia annua*.[13] Because of its low solubility in water and lipids, several derivatives have been developed including artemether and artesunate (**9**–**10**, Figure 1).[13,14] Now, these agents are recommended worldwide in combination with other antimalarials (ACTs) to cure *P. falciparum* infections. Unfortunately, because of the development of resistances, there is an urgent need for innovations in the field of antimalarials. [6–8]

Therefore, we have selected gallic acid (GA) (**11**, Figure 2) as a promising candidate for optimization. Indeed, this structure is a common secondary metabolite in plants and commercially available. Moreover, it has excellent hydrosolubility (>10 mg/ml), even in acidic conditions, and has already been studied for some ADME (absorption, distribution, metabolism, and excretion) properties (t_{max} = 60 min; C_{max} = 0.71 µM).[15–17] This phenolic compound also showed some pharmacological activities, including antitrypanosome, antibacterial, and antiplasmodial, without high toxicity in various cell lines, or during in vivo studies (ED_{50} > 80 mg/kg in mice) (Table 1).[18–28] On the contrary, some derivatives from this scaffold have already been studied for anti-*Plasmodium* purposes and have shown interesting inhibitory effects (IC_{50} ~10 µM).[20,29]

We decided to work on these polyphenols, despite their known PAINS (pan-assay interference compounds) potential, for various reasons.[30,43] First, in the case of antiplasmodial research, these interferences could be easily identified and controlled by microscopy counting with the Giemsa stain.[20] Second, in our opinion, even these panacea compounds exist and are responsible for bias during in vitro assays, you cannot decently discard all the molecules from a same family for all their potential uses, just because some of them are guilty for bias during some studies.[31] Moreover, some of the mechanisms responsible for these interferences are the same as those for biological effects.[43] Thus, we have considered the PAINS

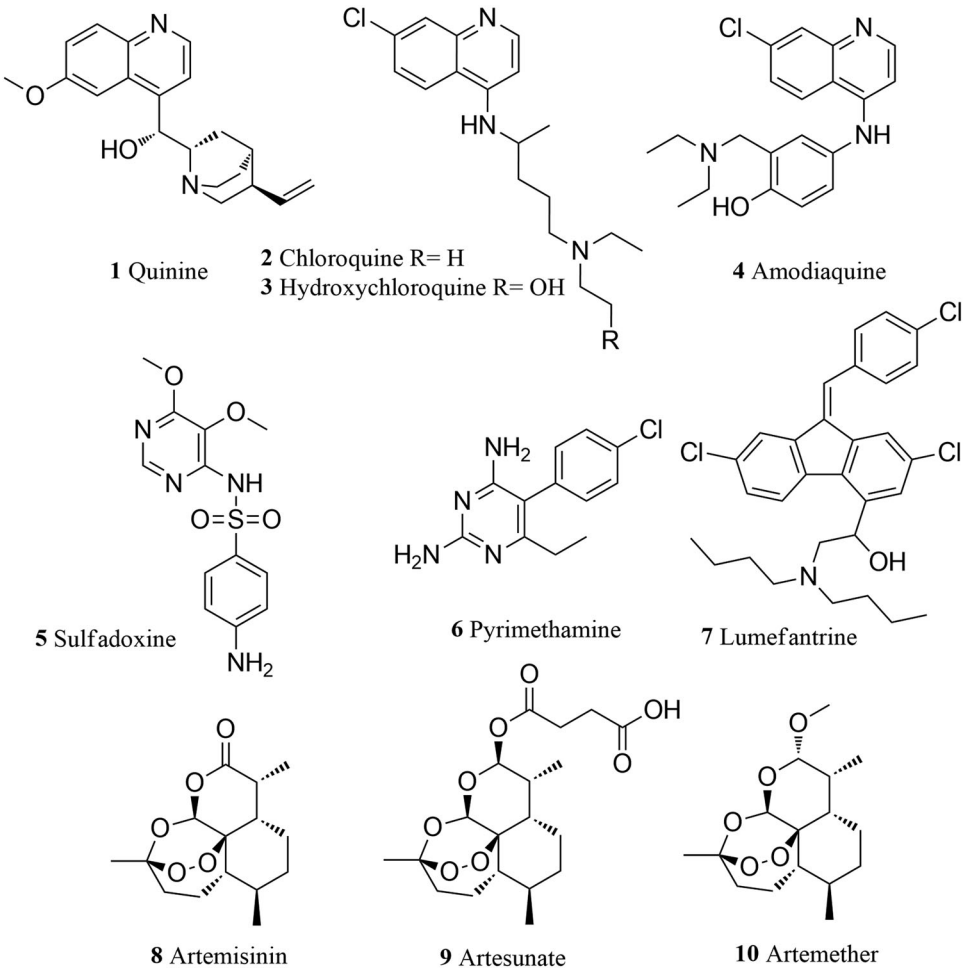


FIGURE 1 Examples of antimalarial drugs accepted for treatment against *Plasmodium falciparum*

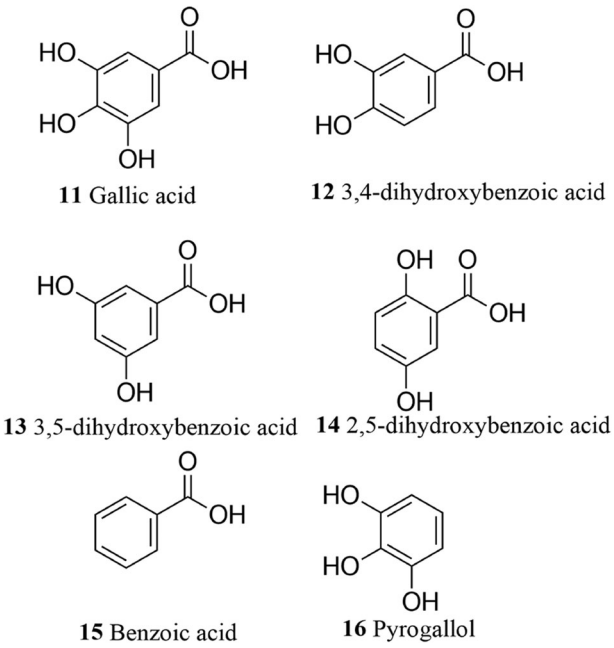


FIGURE 2 Structures of polyphenolic compounds selected for pharmacomodulation

TABLE 1 Previously reported cytotoxicity of gallic acid (**11**)

Cell lines	IC_{50} (μM)	Reference
L1210	>50	[18]
CEM	>50	[18]
HCT15	564	[19]
Lymphocytes	>588	[19]
HepG2	265	[21]
MCF-7	470	[21]
MDA-MB231	441	[21]
HT29	411	[21]
MCF10A	>3527	[21]
Caco-2	>235	[22]
L929	>235	[22]
U937	Nontoxic	[22]

character as a "Pay-Attention" flag, but not a sufficient reason to ignore this potential class of compounds.

Consequently, we have synthesized various compounds whose structures are inspired by the gallate scaffold. This focused chemical library was then mainly evaluated to identify new antiplasmodial agents. Moreover, we have also tested other hydroxybenzoic molecules (**12**–**14**, Figure 2) to better understand the features responsible for this activity.

We have also followed the impact of the pharmacomodulations on water solubility, a key parameter for drug development. In conclusion, the main objective of our study was to obtain a compound with a good to promising activity against *Pf* (IC_{50} < 15 μM), without significantly affecting the water solubility and cytotoxicity.

2 | RESULTS AND DISCUSSION

2.1 | Chemistry

A library of hydroxybenzoic structures was selected for evaluations and pharmacomodulations during this study to better understand the structure–activity relationships responsible for the antiplasmodial effect. The target compounds were mainly derivatives of **11** as well as 3,4-, 3,5-, and 2,5-dihydroxybenzoic acids (**12**–**14**, Figure 3). In addition, benzoic acid and pyrogallol (**15**–**16**, Figure 3) were also used to explore the impact of the phenolic and carboxylic functions on pharmacological activity. Therefore, as reported in Scheme 1, various synthetic pathways were used to obtain the desired polyhydroxybenzoic acid derivatives. First, we synthesized various esters of **11** (**17**–**20**, Figure 3) to observe the impact of the *O*-alkyl chain on the *Plasmodium* growth inhibition. Owing to a protocol adapted from Fischer's esterification, small esters of GA were obtained and all have shown a similar range of activity against the 3D7 strain of *Pf* (IC_{50} ~20 μM, Table 2).[32] Similarly, all the other benzoic acid derivatives were converted into their corresponding methyl esters (**21**–**23**, Figure 3).

These reactions resulted in a slight increase in the lipophilicity as could have been anticipated (LogP increased from 0.53 to 0.77 for **11**–**17**, respectively).[33] After that, we introduced two different kinds of protective groups for the phenolic functions. Indeed, they are usually reported as being essential for the biological effects, for example, the antiplasmodial activity of caffeic acid derivatives or the antibacterial effect of gallate derivatives.[27,32]

Therefore, easily reversible protective groups were inserted through an acetylation reaction (**24**–**27**, Figure 3) while phenol methylation was performed, resulting in a less-labile protective group (**31**–**34**, Figure 3).[32,34] Moreover, to show if these *O*-alkyl substituents were sufficient to increase the antiplasmodial activity of our hit compounds, we also prepared their corresponding acid derivatives (**28**–**30** and **35**–**37**, Figure 3). Consequently, acetylated prodrugs were obtained after reaction with acetic anhydride (Ac_2O) and sulfuric acid, following the process described by Gokcen et al.,[34] except for 1,2,3-triacetoxybenzene (**27**), which required the action of Ac_2O and pyridine.[34,35]

In terms of the methoxylated analogs, the reaction of the hydroxybenzoic acids (**31**–**34**) with dimethylsulfate (DMS) was followed by basic saponification by means of LiOH to cleave the methyl ester (**35**–**37**, Figure 3).[32,36]

Finally, as **11** was the most active among the selected hydroxybenzoic acids, we decided to explore this scaffold with some bigger substituents to increase its lipophilicity in a more marked way. Therefore, we followed the protocol of *para*-substitution described by

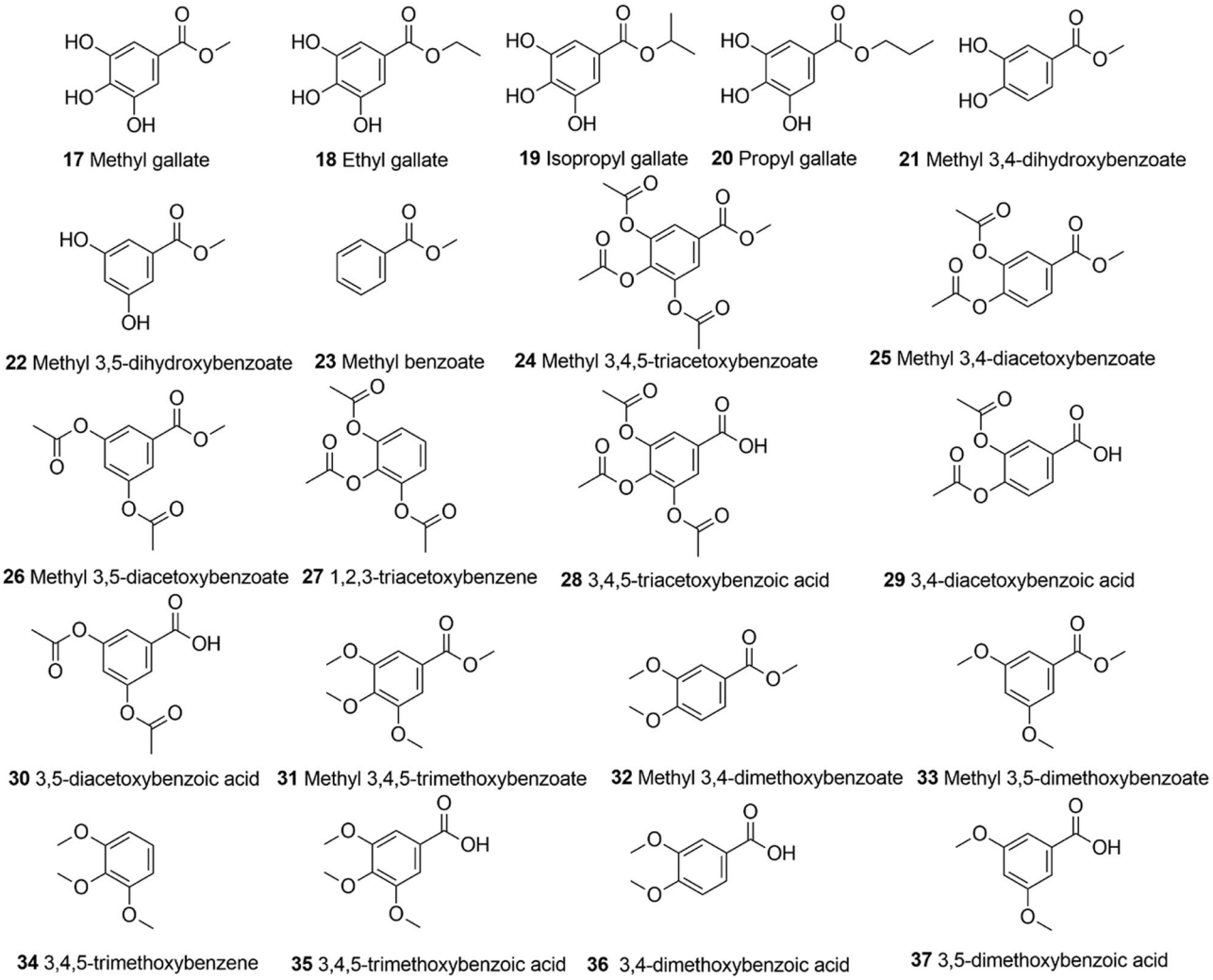


FIGURE 3 Structures of the derivatives from polyhydroxybenzoic acids screened for antiplasmodial activities

Pearson et al.[35] to obtain methyl 4-benzyloxy-3,5-diacetoxybenzoate (**38**) and then methyl 4-benzyloxy-3,5-dihydroxybenzoate (**39**) after saponification (Scheme 2). A superior homolog of **39**, with a naphthylmethyl substituent (**40**, Figure 4), was obtained using another protocol.[36] The steric hindrance of the naphthylmethoxy substituent could explain why this second protocol could not be applied for the introduction of benzyl. Indeed, the substitution could occur on the other phenols (3- and 5-OH) with benzyl bromide and not so easily for the 2-(bromomethyl)naphthalene.

2.2 | Pharmacology/biology

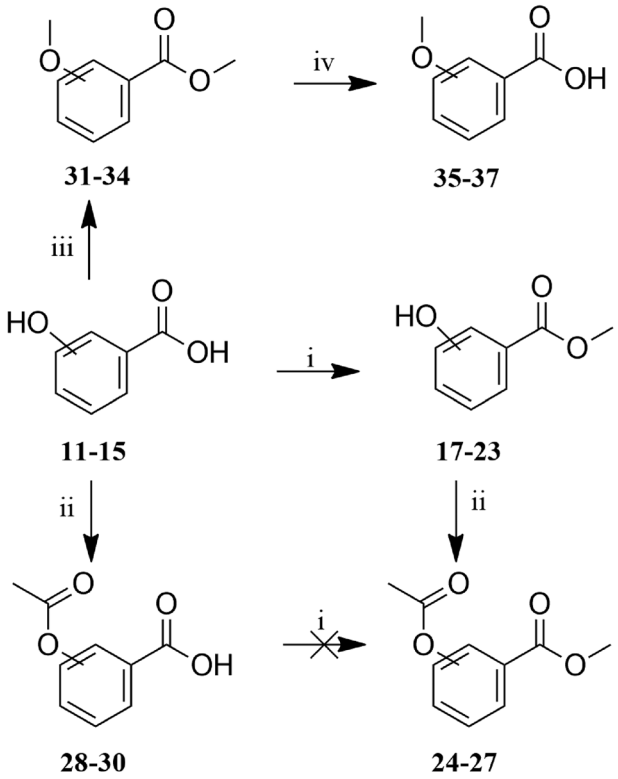
2.2.1 | In vitro antiplasmodial assay

Plasmodium lactate dehydrogenase

All the compounds were then assessed for their antiplasmodial potential. However, only the active molecules on *Pf* are reported in Table 2 and further explored. First, we evaluated the different hydroxybenzoic acid scaffolds on the selected 3D7 strain of *P. falciparum* (chloroquine-sensitive). Their effect was compared to the activity shown by two reference antimalarial drugs: quinine (**1**) and artemisinin (**8**). These standards were used to confirm the validity of the assays. Indeed, their IC_{50} values were similar to the values reported previously: 600 ± 12 nM and 9.2 ± 5.2 nM, respectively.[37,38]

We observed that **11** showed a modest inhibitory effect against the malaria parasite, with an IC_{50} value of around 70 µM, and a quite similar activity for **16**, which has an IC_{50} value of 58 ± 38 µM. The activity obtained with **11** was similar to previously reported data, where the IC_{50} value was found to be 71.53 ± 8.96 µM.[28]

On the contrary, **12**–**15** were not active up to 100 µg/ml (IC_{50} > 700 µM). These results were different from that reported by Ndjonka et al.,[28] for example, **14**, for which the IC_{50} value was quantified as 49.76 ± 0.32 µM. The explanation for these differences may be the revelation method, which consisted of a [^{3}H]-hypoxanthine incorporation assay in that paper, in the test protocol, as they employed a 24h-exposure, compared to 48h in our case. Similarly, Aldulaimi et al. [39]



SCHEME 1 Synthetic pathways used on hydroxybenzoic acid scaffolds. Reagents and conditions: (i) MeOH, H_2SO_4; (ii) Ac_2O, H_2SO_4; (iii) DMS, K_2CO_3; and (iv) LiOH, MeOH/H_2O

demonstrated greater activity of **11** and **14** on *P. falciparum*, with IC_{50} values of around 27 and 84 μM, respectively. These significant differences in the concentration range could be due to the Dd2 strain used, known to be resistant compared to the sensitive 3D7 strain that we used. In addition, the protocol used was also slightly different, with *Plasmodium* DNA detection.

As highlighted by previous studies with caffeic acid derivatives, esters are known to be more efficient than their corresponding acids in antiplasmodial assays.[32] This trend could be partially explained by higher lipophilicity, which could increase the ability of the compounds to reach the cytoplasm of red blood cells (RBC) and then inhibit the growth of the asexual stages of *Pf*.[40] This was highlighted in the study of Zahrani et al.,[41] who summarized the observations made about alkyl gallates. Indeed, several studies on anticancer properties have shown more favorable activities of the esters compared to their corresponding acid. This was attributed to the higher lipophilicity caused by a long alkyl chain and responsible for improved cancer cell permeability. However, this increase in lipophilicity was not always linear with the activity. Indeed, this enhancement of the anticancer effect seemed limited to a specific range of LogP values. Therefore, the optimum compounds had a partition coefficient between 3.32 and 6.09 against leukemia cells.[18] In contrast, an acidic molecule is not often recognized as a promising structure to cross the intestinal barrier, even if some transporters exist on enterocytes, for example, the monocarboxylic acid transporter.[17]

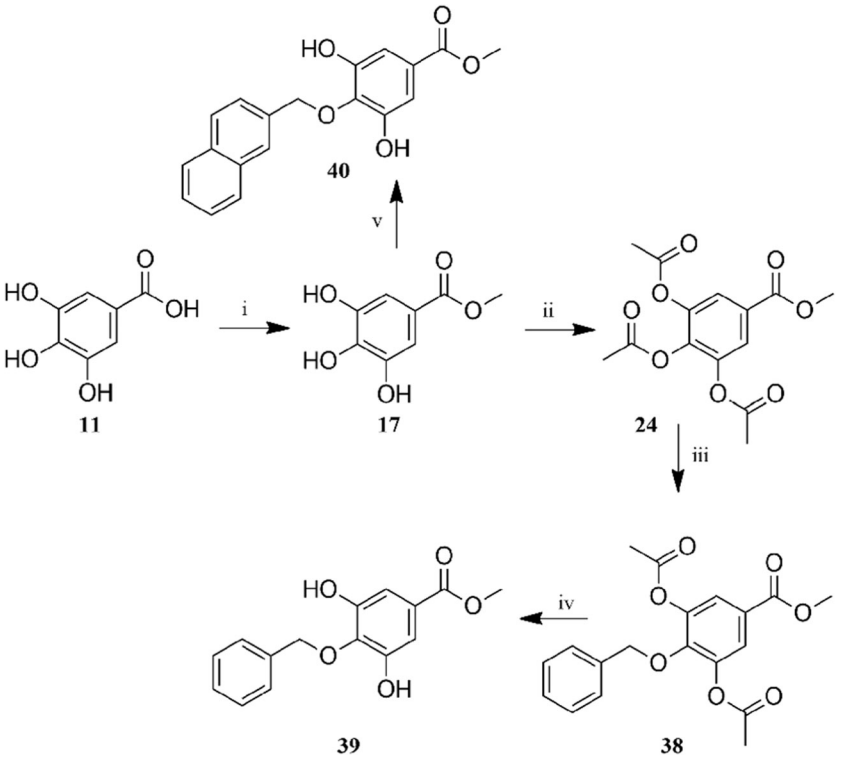
TABLE 2 Biological activities of the reported structures

Products	*Plasmodium falciparum*[a]	HUVEC (SI)[b]	Solubility (mM)	cLogP[c]
1	0.60 ± 0.12	-	-	2.48
8	0.0092 ± 0.0052	-	-	3.17
11	68 ± 20	>294 (4)	70.2 ± 8.2	0.53
16	58 ± 38	-	-	0.06
17	27 ± 1.8	171 ± 43 (6)	74.3 ± 7.7	0.77[33]
18	22 ± 0.7	-	205 ± 26	1.27
19	34 ± 11	-	130 ± 23	1.78
20	29 ± 13	-	121 ± 7.4	1.62
21	99 ± 22	-	-	1.36
24	9.3 ± 3.0	>161 (17)	0.59 ± 0.75	1.20
25	113 ± 22	-	-	1.54
27	24 ± 3.0	>198 (8)	-	0.204
28	203 ± 34	-	-	0.94
38	56 ± 2.2	>140 (3)	0.46 ± 0.22	3.62
39	33 ± 5.4	>182 (6)	3.72 ± 0.79	2.32
40	17 ± 4	140 ± 4.3 (8)	0.005 ± 0.001	3.50

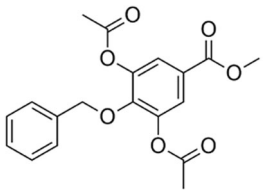
[a]IC_{50} value in μM of the malaria agent *Plasmodium falciparum* 3D7-strain.

[b]IC_{50} values in μM of human umbilical vein endothelial cells (HUVEC) (selectivity index).

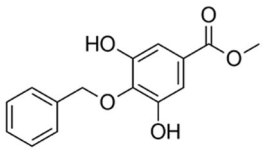
[c]Calculated LogP using ChemDraw 12.0.



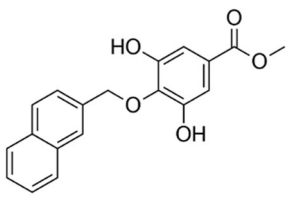
SCHEME 2 Synthetic pathways for *para*-substituted gallate derivatives. Reagents and conditions: (i) MeOH, H_2SO_4; (ii) Ac_2O, H_2SO_4; (iii) BnBr, K_2CO_3, KI; (iv) K_2CO_3, MeOH/H_2O; and (v) 2-(bromomethyl)naphthalene, K_2CO_3



38 Methyl 4-benzyloxy-3,5-diacetoxybenzoate



39 Methyl 4-benzyloxy-3,5-dihydroxybenzoate



40 Methyl 3,5-dihydroxy-4-(naphthalen-2-ylmethoxy)benzoate

FIGURE 4 Structures of the *para*-substituted gallate

This trend prompted us to prepare esters of the selected benzoic scaffolds **11**–**15** and to explore the impact of the alkyl chain on the in vitro activity against 3D7 (**17**–**23**). Surprisingly, we observed a similar range of activity for all the structures related to **11** (IC_{50} ~28 μM, Table 2). In contrast, all the esters of the other hydroxybenzoic molecules (**21**–**23**) were mostly inactive.

The effect of **18** was similar to that reported previously in Jansen et al.,[29] where the IC_{50} value was found to be 32.3 ± 5.0 μM. In contrast, in the studies of Arsianti et al.,[20] as well as Aldulaimi et al.,[39] multiple esters of **11** were synthesized for antiplasmodial purposes and showed a greater effect and even reached the micromolar range of activity. The difference in our results could again be partially explained by the different experimental protocols used. Therefore, as only **17**–**20** were quite active and as the methyl ester is known to be more resistant to hydrolysis, we chose **17** as the lead compound to continue our pharmacomodulation.

As mentioned before, phenolic functions are often considered essential for the pharmacological activities of polyphenols.[27,32] Therefore, we protected the phenolic groups with methoxy or acetoxy substituents to observe the impact of hiding the former on the growth-inhibitory effect of our compounds.

The antiplasmodial assay led to two totally different observations. First, none of the methoxy derivatives (**31**–**37**) were efficient against the malarial agent, even for the 3,4,5- or the 3,4-dihydroxybenzoic scaffolds, whose esters were previously active.

In contrast, some of the acetylated derivatives (**24**–**25** and **28**–**29**) showed, respectively, a low to good inhibitory effect (IC_{50} = 9.3–203 μM), especially **24**, which was the most active compound identified in this paper (IC_{50} = 9.3 μM). In addition, **27** was also a marked inhibitor of the 3D7 strain of *Pf*, with IC_{50} = 24.0 ± 3.0 μM. Thus, the increase in the activity of our phenolic scaffolds, especially **11** and **12**, may be explained by the increase in lipophilicity, as previously described for caffeic acid derivatives.[32]

On the contrary, the introduction of a stable protective group, methyl ether, impeded the antiplasmodial effect. This decrease in efficiency could be explained by the loss of the free phenol groups. In contrast, a labile acetoxy substituent seemed mostly beneficial for the anti-infectious effect. Indeed, the acetyl moiety was often used in drug development to increase the lipophilicity in a reversible manner, for example, acetylsalicylic acid. Consequently, acetylated structures such as **24** are often considered prodrugs, and not responsible for the observed efficacy. In our case, these results highlighted the need for the phenolic moieties for the antiplasmodial effect.

Finally, as among the polyhydroxybenzoic acids (**11**–**15**), the gallate moiety was the most active one, with an IC_{50} value of around 28 μM (**16**–**20**); we attempted to increase this activity to reach the micromolar range (IC_{50} < 5 μM).

For this purpose and guided by the increase in activity linked to higher lipophilicity, we introduced some bulky hydrophobic chains on the phenolic functions, and more precisely, on the *para*-position (Scheme 2). Therefore, a benzyl or a naphthyl substituent was grafted on the 4-position of the trihydroxy scaffold (**38**–**40**, Figure 4) and tested against the 3D7 strain. Unfortunately, none of them reached the desired range of activity (<5 μM) and were often less active than **17** or **24** (IC_{50} > 25 μM), except for **40** (IC_{50} = 17 ± 4.0 μM).

This trend confirmed again the importance of the phenolic functions (a catechol moiety) because the increase in lipophilicity was not followed by a similar enhancement of the activity. This could be due to the phenoxy groups, which could remain partially hidden in comparison with **24**. However, as opposed to the methoxy scaffolds, the graft of these bulky and nonversatile substituents did not lead to a complete loss of activity. This could be partially explained by the fact that some phenols could remain free but also by the establishment of new interactions with the biological target.[42]

In conclusion, we can consider that the most essential part of the hydroxybenzoic structure for the antiplasmodial effect was the pyrogallol moiety (**16**), considering the low efficacy of the other benzoic acid scaffolds, as highlighted by previous studies.[39] Therefore, the pyrogallol moiety was essential for the effect but not only, as **12** or **21** had no significant inhibition of *Plasmodium*.

Microscopy

In addition, we used a second revelation method to confirm the absence of PAINS behavior during our in vitro assays on *Pf*. Indeed, as mentioned before, polyphenols are often reported as panacea compounds.[43,44] Thus, it was necessary to confirm that the reduction in parasitemia was not the result of interference between the revelation reagents and our products.

Consequently, a microscopy measure was performed for **24**, **38**, and **40**. Thus, after the 48-h incubation, the culture was centrifuged at 2000 rpm to concentrate the erythrocytes. Then, a thin blood smear was prepared and stained with Giemsa as reported before.[20] Finally, 1000 RBCs were counted to establish the percentage of parasitemia at each concentration.

Fortunately, all the test samples showed a significant concentration-dependent reduction in parasite growth. Moreover, the estimated IC_{50} values were in the same range as the values obtained using the main revelation method.[45] This confirmed the efficacy of our structures on the malaria agent and their potential as antiplasmodial lead compounds.

2.2.2 | Hemolysis

However, to establish whether the antiplasmodial effect could be due to a factor other than an inhibitory effect on the malarial agent, we also tested our molecules in a hemolysis assay. Indeed, as *Pf* is mostly an intracellular parasite, the destruction of RBCs can impede its multiplication. Therefore, a hemolytic product could bias the evaluation of antiplasmodial effects by a toxic effect on RBC.

None of our compounds showed any hemolytic potential. Indeed, at 100 μg/ml, none reached a percentage of hemolysis superior to 1%. Therefore, the observed effect on *Pf* (3D7) could not be explained by the destruction of erythrocytes.

2.2.3 | Cytotoxicity

As some of our compounds were quite toxic against the protozoon *P. falciparum* (3D7), we performed an evaluation of cytotoxicity to confirm if this effect was selective. Therefore, we assessed the most active ones on a healthy cell line. As the action site of antimalarial agents is mainly blood vessels, we selected human umbilical vein endothelial cells (HUVEC) as the most interesting model to evaluate the selectivity of our derivatives (Table 2).

Finally, none of the selected molecules was highly toxic on the HUVEC. Therefore, it seemed that our compounds showed good selectivity for *Plasmodium* 3D7 (SI > 3). This selectivity was previously described for alkyl derivatives on HepG2. Indeed, most of the compounds were slightly toxic on this cell line despite significant antiplasmodial effects on Dd2 strains.[39] However, evaluations on other cell lines could be beneficial to confirm this lack of cytotoxicity.

Interestingly, the increase in the lipophilicity that has been previously reported to increase the cancerous cell toxicity or the anti-*Plasmodium* activity by higher membrane permeability did not lead to increased cytotoxicity on the chosen healthy cell line.[18,39]

2.2.4 | Solubility

One of the main problems with highly active molecules in vitro was often their low water solubility, which could negatively impact the ADME profile, for example, low oral bioavailability. Thus, it was important to follow the impact of our pharmacomodulation on this critical property. Considering the results from the in vitro assays, we have decided to quantify the maximal water solubility at room temperature (RT; C_{max}, Table 2) for some derivatives owing to a protocol adapted from Bala et al., in a kind of ADME-early vision.[46] Therefore, as reported before, **11** was a highly soluble scaffold, with a C_{max}

value of around 10 mg/ml at RT~25°C.[15,16] This was confirmed by our UV spectrophotometric method, with a C_{max} value of 11.9 ± 1.4 mg/ml. Moreover, **17**–**20** showed similar behavior in water, with a high C_{max} value, for example, of 13.7 ± 1.4 mg/ml for **17**. Thus, the esterification of **11** with a small alkyl chain, like methanol, did not impact the water solubility of this scaffold negatively. However, the solubility of the ethyl and (iso)propyl esters (**18**–**20**) was up to 3-fold higher than that of **11** or **17**, despite increased lipophilicity (cLogP). This could be explained by the experimental conditions, especially the temperature, which has a major influence on this parameter as demonstrated before.[15] However, it could also be a result of how the molecules interact with each other in the solvents. Indeed, ellagic acid, a GA dimer, is a highly hydrophilic structure (cLogP 1.05), but scarcely soluble in water (C_{max} = 9.7 µg/ml) because of the strong inter- and intramolecular bonds, enabled by its flat structure.[46,47] Therefore, a similar phenomenon could explain why **18** was more soluble than its corresponding acid, despite its higher LogP value (1.27 vs. 0.53, respectively).

However, the substitution of the phenol functions with moieties like acetyl (**24**) or the *para*-substitution (**38**–**40**) has a greater impact on this parameter. Indeed, we observed a marked reduction in the C_{max} value (<1 mM), which was consistent with the reduction of the H-bond capacity and the increase of the lipophilicity (cLogP).

In contrast, the deprotection of some phenol groups, as observed with **38** and **39**, led to an increase (eightfold) in the C_{max} value, for example, 165 ± 80 µg/ml for **38** to 1.02 ± 0.22 mg/ml for **39**. Surprisingly, **40** did not show a similar behavior. Indeed, the water solubility of the naphthyl derivative was even lower (95- to 120-fold) than that measured for **24** or **38**. This trend could be partially explained by the steric hindrance of the substituent and the high lipophilicity of this compound (cLogP = 3.5). In conclusion, **39** seems to be the best of the substituted derivatives in terms of the lipophilicity–hydrosolubility balance, with C_{max} = 3.72 mM and cLogP = 2.32. Indeed, it is necessary to control both parameters because of their major influence on the bioavailability and thus, the pharmacokinetic component, in addition to the influence on the pharmacodynamics.[41,48]

3 | CONCLUSIONS

We selected various hydroxybenzoic acids, based on the activity of **11** on *Pf* combined with its interesting ADME profile. Our pharmacomodulation aimed to gain a better understanding of the structure–activity relationships and the synthesis of potential new antimalarials.

The in vitro assay against *Pf* showed that the important functions were served by the phenolic groups, especially a trihydroxy scaffold. Moreover, the increase in lipophilicity led to a greater effect, certainly as a result of a higher penetration rate in the erythrocytes. Thus, the introduction of a strong and bulky chain on the 4-position (**39**–**40**) improved the activity without higher cell toxicity (SI > 3). Indeed, these derivatives were selective as none demonstrated any toxicity against healthy cells in humans. Moreover, they demonstrated various ranges of hydrosolubility (5 µM to 205 mM), which was considered a critical parameter here for the pharmacokinetic profile.

In conclusion, **39** seemed to be the most promising compound from our library as it showed good activity, with no signs of cytotoxicity (SI > 5.6) or hemolysis (<1%). Moreover, it showed an interesting solubility (3.72 mM) in contrast to other highly active compounds. Consequently, for the first time, this study highlighted the potential of *para*-benzyl gallates as promising antiplasmodials and the next avenue for future antimalarial research.

4 | EXPERIMENTAL

4.1 | Chemistry

4.1.1 | General

All the chemicals were purchased from Fluorochem, Sigma-Aldrich, FisherScience, or VWR. MilliQ water was obtained from the Milli-Q Reference A+ system. Melting points were determined on the Stuart SMP3 capillary apparatus and were uncorrected. Products were purified by the Buchi Reveleris® prep on an irregular silica cartridge, 4-80 g. The ^{1}H and ^{13}C NMR spectra (see the Supporting Information) 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 a solvent with tetramethylsilane (TMS) as the internal standard; chemical shifts are reported in δ values (ppm) relative to that of internal TMS. Elemental analyses (C, H, N, and S) were carried out 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 confirmed a purity of 95% or above for each tested compound. All reactions were routinely checked by thin-layer chromatography (TLC) on a silica gel Merck 60 F254. The UV spectra were obtained using a Hitachi U-3010 UV/Vis spectrophotometer model.

The InChI codes of the investigated compounds, together with some biological activity data, are provided as the Supporting Information.

4.1.2 | General procedure for the synthesis of ester derivatives

Hydroxybenzoic acid (1 g) was dissolved in 50 ml of the appropriate alcohol (mostly MeOH) 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, ~6 h). The reaction mixture was cooled before a part of the organic solvent was eliminated by evaporation under vacuum. 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 vacuum. The solid was placed overnight in a stove (30°C).

*Methyl gallate (**17**)*

White solid, yield 88%, melting point (MP): 257°C (decomp.)/Lit. 240–242°C[49]; UV: 271 nm, ^{1}H-NMR (in DMSO) δ 9.26 (2H, s), 8.93 (1H, s), 6.94 (2H, s), and 3.75 (3H, s); ^{13}C (in DMSO) δ 166.78 (s), 146.04 (s), 138.87 (s), 119.75 (s), 108.95 (s), and 52.05 (s); theorical elemental analysis (EA Th): C, 52.18%; H, 4.38%; Found: C, 52.30%; H, 4.38%.

*Ethyl gallate (**18**)*

White solid, yield 77%, MP: 145°C (decomp.)/Lit. 148–150°C[48]; ^{1}H-NMR (in DMSO) δ 6.94 (2H, s), 4.19 (2H, q), and 1.27 (3H, t); ^{13}C (in DMSO) δ 166.28 (s), 146.01 (s), 138.79 (s), 120.03 (s), 108.91 (s), 60.45 (s), and 14.73 (s); EA Th: C, 54.55%; H, 5.09%; Found: C, 53.95%; H, 5.07%.

*Isopropyl gallate (**19**)*

White solid, yield 93%, MP: 124.5°C/Lit. 126–128°C[50]; ^{1}H-NMR (in DMSO) δ 9.22 (2H, s), 8.89 (1H, s), 6.93 (2H, s), 5.03 (1H, m), and 1.27 (6H, d); ^{13}C (in DMSO) δ 165.77 (s), 145.98 (s), 138.71 (s), 120.39 (s), 108.89 (s), 67.59 (s), and 22.25 (s); EA Th: C, 56.60%; H, 5.70%; Found: C, 56.20%; H, 5.90%.

*Propyl gallate (**20**)*

White solid, yield 86%, MP: 145°C/Lit. 145–146°C[48]; ^{1}H-NMR (in DMSO) δ 6.95 (2H, s), 4.12 (2H, t), 1.68 (2H, m), and 0.95 (3H, t); ^{13}C (in DMSO) δ 166.33 (s), 146.02 (s), 138.81 (s), 120.02 (s), 108.91 (s), 65.89 (s), 22.16 (s), and 10.86 (s); EA Th: C, 56.60%; H, 5.70%; Found: C, 56.93%; H, 6.10%.

*Methyl 3,4-dihydroxybenzoate (**21**)*

White solid, yield 93%, MP: 137°C/Lit. 137–139°C[51]; ^{1}H-NMR (in DMSO) δ 9.77 (1H, s), 9.36 (1H, s), 7.35 (1H, s), 7.30 (1H, s), 6.79 (1H, s), and 3.76 (3H, s); ^{13}C (in DMSO) δ 166.62 (s), 150.88 (s), 145.54 (s), 122.22 (s), 120.95 (s),116.72 (s), 115.78 (s), and 52.06 (s); EA Th: C, 57.14%; H, 4.80%; Found: C, 57.05%; H, 4.87%.

*Methyl 3,5-dihydroxybenzoate (**22**)*

White solid, yield 98%, MP: 166°C/Lit. 169–170°C[52]; ^{1}H-NMR (in DMSO) δ 9.62 (2H, s), 6.81 (2H, s), 6.44 (1H, s), and 3.79 (3H, s); ^{13}C (in DMSO) δ 166.73 (s), 159.01 (s), 131.75 (s), 107.63 (s), and 52.47 (s); EA Th: C, 57.14%; H, 4.80%; Found: C, 57.21%; H, 4.91%.

*Methyl benzoate (**23**)*

Colorless oil, yield 76%, ^{1}H-NMR (in $CDCl_3$) δ 8.04 (2H, d), 7.56 (1H, t), 7.44 (2H, t), and 3.92 (3H, s); ^{13}C (in $CDCl_3$) δ 166.13 (s), 132.92 (s), 130.18 (s), 129.58 (s), 128.37 (s), and 52.11 (s).

4.1.3 | General procedure for acetylated derivatives

To a stirred solution of hydroxybenzoic acid or the corresponding ester (20 mmol) in acetic anhydride (120 mmol), a few drops of concentrated H_2SO_4 were added. A fast increase in temperature was observed and after the entire solid dissolved, the stirring was continued for 1 or 2 h. Then, water (100 ml) was added and the solution was stirred for another 1 h to remove any excess acetic anhydride left. The solid precipitate was filtered on G_3, washed with water (3 × 50 ml), and dried under vacuum.

*Methyl 3,4,5-triacetoxybenzoate (**24**)*

White solid, yield 92%, MP: 126°C/Lit. 126–128°C[35]; UV: 232 nm, ^{1}H-NMR (in DMSO) δ 7.80 (2H, s), 3.88 (3H, s), 2.34 (3H, s), and 2.30 (6H, s); ^{13}C (in DMSO) δ 168.48 (s), 167.39 (s), 164.86 (s), 143.83 (s), 139.15 (s), 128.05 (s), 122.40 (s), 53.17 (s), 20.86 (s), and 20.32 (s); EA Th: C, 54.20%; H, 4.55%; Found: C, 54.16%; H, 4.69%.

*Methyl 3,4-diacetoxybenzoate (**25**)*

Colorless oil, yield 81% (after flash chromatography *n*-hexane/EtOAc: 1:0–3:2), ^{1}H-NMR (in $CDCl_3$) δ 7.95 (1H, dd), 7.87 (1H, d), 7.28 (1H, d), 3.91 (3H, s), and 2.31 (6H, s); ^{13}C (in $CDCl_3$) δ 168.01 (s), 167.71 (s), 165.54 (s), 145.95 (s), 141.99 (s), 128.73 (s), 128.12 (s), 125.08 (s), 123.50 (s), 52.40 (s), 20.69 (s), and 20.58 (s).

*Methyl 3,5-diacetoxybenzoate (**26**)*

Colorless oil, yield 66% (after flash chromatography *n*-hexane/EtOAc: 1:0–3:2), ^{1}H-NMR (in $CDCl_3$) δ 7.66 (2H, s), 7.14 (1H, s), 3.91 (3H, s), and 2.31 (6H, s); ^{13}C (in $CDCl_3$) δ 168.77 (s), 165.36 (s), 150.95 (s), 132.23 (s), 120.29 (s), 120.09 (s), 52.51 (s), and 21.02 (s).

*3,4,5-Triacetoxybenzene (**27**)*

White solid, yield 24%, MP: 167°C; ^{1}H-NMR (in $CDCl_3$) δ 7.25 (1H, t), 7.12 (1H, s), 7.10 (1H, s), 2.29 (3H, s), and 2.28 (6H, s); ^{13}C (in $CDCl_3$) δ 167.90 (s), 167.01 (s), 143.55 (s), 134.69 (s), 125.98 (s), 120.74 (s), 20.68 (s), and 20.20 (s); EA Th: C, 57.14%; H, 4.80%; Found: C, 56.94%; H, 4.96%. This solid was obtained through the acetylation protocol proposed by Pearson et al.[35]

*3,4,5-Triacetoxybenzoic acid (**28**)*

White solid, yield 99%, MP: 159°C/Lit. 166–168°C[53]; ^{1}H-NMR (in $CDCl_3$): δ 7.86 (2H, s) and 2.31 (9H, s); ^{13}C (in $CDCl_3$) δ 168.78 (s), 167.58 (s), 166.38 (s), 143.52 (s), 139.33 (s), 127.32 (s), 122.84 (s), 20.60 (s), and 20.20 (s); EA Th: C, 52.71%; H, 4.08%; Found: C, 52.74%; H, 4.14%.

*3,4-Diacetoxybenzoic acid (**29**)*

White solid, yield 59%, MP: 150°C/Lit. 157–158°C[54]; ^{1}H-NMR (in $CDCl_3$): δ 8.03 (1H, dd), 7.94 (1H, d), 7.33 (1H, d), and 2.33 (6H, s); ^{13}C (in $CDCl_3$) δ 169.74 (s), 167.98 (s), 167.65 (s), 146.70 (s), 142.10 (s), 128.78 (s), 127.74 (s), 125.71 (s), 123.69 (s), 20.70 (s), and 20.58 (s); EA Th: C, 55.47%; H, 4.23%; Found: C, 55.21%; H, 4.28%.

*3,5-Diacetoxybenzoic acid (**30**)*

White solid, yield 76%, MP: 156°C/Lit. 161°C[55]; ^{1}H-NMR (in $CDCl_3$): δ 7.72 (2H, d), 7.21 (1H, t), and 2.32 (6H, s); ^{13}C (in $CDCl_3$) δ

168.86 (s), 168.73 (s), 151.04 (s), 131.17 (s), 120.84 (s), and 21.04 (s); EA Th: C, 55.47%; H, 4.23%; Found: C, 55.30%; H, 4.19%.

4.1.4 | General procedure for methoxy derivatives

The appropriate hydroxybenzoic acid (6.5 mmol) was dissolved in acetonitrile (60 ml) and supplemented with potassium carbonate (K_2CO_3, 19.5 mmol). The mixture was stirred at 40°C for 30 min and then, dimethylsulfate (DMS; 19.5 mmol) was added. The stirred solution was heated under reflux for 5 h. Potassium carbonate was removed by filtration; the organic solvents and DMS were distilled under vacuum and the resulting solid was dried under vacuum overnight.

*Methyl 3,4,5-trimethoxybenzoate (**31**)*

White solid, yield 54 (%), MP: 81°C/Lit. 81–83°C[56]; ^{1}H-NMR (in $CDCl_3$): δ 7.30 (2H, s) and 3.91 (12H, s); ^{13}C (in $CDCl_3$) δ 166.74 (s), 152.95 (s), 142.17 (s), 125.16 (s), 106.80 (s), 60.94 (s), 56.25 (s), and 52.25 (s); EA Th: C, 58.40%; H, 6.24%; Found: C, 58.45%; H, 6.32%.

*Methyl 3,4-dimethoxybenzoate (**32**)*

White solid, yield 88% (after recrystallization in EtOH/H_2O on ice), MP: 58°C/Lit. 57–58°C[57]; ^{1}H-NMR (in $CDCl_3$): δ 7.69 (1H, dd), 7.55 (1H, d), 6.89 (1H, d), 3.94 (6H, s), and 3.90 (3H, s); ^{13}C (in $CDCl_3$) δ 166.91 (s), 152.96 (s), 148.61 (s), 123.59 (s), 122.70 (s), 111.97 (s), 110.26 (s), 56.02 (s), and 52.01 (s); EA Th: C, 61.22%; H, 6.17%; Found: C, 61.27%; H, 6.18%.

*Methyl 3,5-dimethoxybenzoate (**33**)*

White solid, yield 84%, MP: 43°C/Lit. 42–44°C[58]: ^{1}H-NMR (in $CDCl_3$): δ 7.19 (2H, d), 6.65 (1H, t), 3.91 (3H, s), and 3.83 (6H, s); ^{13}C (in $CDCl_3$) δ 166.88 (s), 160.65 (s), 130.02 (s), 107.13 (s), 105.80 (s), 55.59 (s), and 52.26 (s); EA Th: C, 61.22%; H, 6.17%; Found: C, 61.34%; H, 6.26%.

*3,4,5-Trimethoxybenzene (**34**)*

A white solid was obtained after 3 days of agitation at RT before treatment, yield 23% (after flash chromatography *n*-hexane/EtOAc), MP: 45.5°C/Lit. 43–45°C[59]; ^{1}H-NMR (in $CDCl_3$): δ 6.99 (1H, t), 6.59 (2H, d), 3.87 (6H, s), and 3.86 (3H, s); ^{13}C (in $CDCl_3$) δ 153.55 (s), 138.15 (s), 123.65 (s), 105.23 (s), 60.85 (s), and 56.09 (s); EA Th: C, 64.27%; H, 7.19%; Found: C, 64.36%; H, 7.34%.

4.1.5 | General procedure for saponification

To the stirred solution of the methoxy derivatives (1 g) in methanol (30 ml) was added a solution of lithium hydroxide in demineralized water (0.61 g/4 ml). The mixture was heated under reflux until the end of the reaction (monitored by TLC, ~2 h) and then acidified with 6 N HCl. Methanol was partially removed under vacuum and the aqueous phase was extracted with ethyl acetate (3 × 30 ml). The resulting organic phase was washed with brine (25 ml) and dried over anhydrous $MgSO_4$. The solid was filtered and the solvents were removed under vacuum. The resulting solid compound was kept under vacuum overnight.

*3,4,5-Trimethoxybenzoic acid (**35**)*

White solid, yield 81%, MP: 169°C/Lit. 166–167°C[60]; ^{1}H-NMR (in $CDCl_3$) δ 7.37 (2H, s), 3.94 (3H, s), and 3.93 (6H, s); ^{13}C (in $CDCl_3$) δ 170.49 (s), 153.01 (s), 142.97 (s), 123.96 (s), 107.43 (s), 60.98 (s), and 56.28 (s); EA Th: C, 56.60%; H, 5.70%; Found: C, 56.51%; H, 5.81%.

*3,4-Dimethoxybenzoic acid (**36**)*

White solid, yield 98%, MP: 181°C/176–178°C[61]; ^{1}H-NMR (in $CDCl_3$) δ 7.78 (1H, dd), 7.61 (1H, d), 6.93 (1H, d), and 3.96 (6H, d); ^{13}C (in $CDCl_3$) δ 171.19 (s), 153.68 (s), 148.69 (s), 124.56 (s), 121.74 (s), 112.32 (s), 110.33 (s), 56.09 (s), and 56.03; EA Th: C, 59.34%; H, 5.53%; Found: C, 59.22%; H, 5.65%.

*3,5-Dimethoxybenzoic acid (**37**)*

White solid, yield 93%, MP: 185°C; ^{1}H-NMR (in $CDCl_3$) δ 7.26 (2H, d), 6.70 (1H, t), and 3.85 (6H, d); ^{13}C (in $CDCl_3$) δ 170.58 (s), 160.73 (s), 130.92 (s), 107.68 (s), 106.65 (s), and 55.62 (s); EA Th: C, 59.34%; H, 5.53%; Found: C, 59.54%; H, 5.72%.

4.1.6 | General procedure for *para*-substitution

Methyl 3,4,5-triacetoxybenzoate (2.00 g, 6.45 mmol) was dissolved in dry acetone (100 ml) with potassium carbonate (2.70 g, 19.35 mmol) and potassium iodide (0.165 g, 0.97 mmol). Benzyl chloride was cautiously added (1.63 g, 12.90 mmol), and the mixture was heated under reflux overnight. After that, K_2CO_3 was removed by filtration and acetone was distilled under vacuum. The product was recrystallized with EtOH at 0°C, filtered on G_3, and washed thoroughly with EtOH. The resulting white solid was dried under vacuum overnight.[35]

*Methyl 4-benzoxy-3,5-diacetoxybenzoate (**38**)*

White product, yield 85%, MP: 106°C/Lit. 94–96°C[35]; UV; 254 nm, ^{1}H-NMR (in DMSO) δ 7.69 (2H, s), 7.38 (5H, m), 5.03 (2H, s), 3.85 (3H, s), and 2.25 (6H, s); ^{13}C (in DMSO) δ 169.04 (s), 165.05 (s), 147.50 (s), 144.34 (s), 136.85 (s), 128.92 (s), 128.82 (s), 128.49 (s), 125.20 (s), 122.78 (s), 75.71 (s), 52.96 (s), and 20.97 (s); EA Th: C, 63.68%; H, 5.06%; Found: C, 63.75%; H, 5.19%.

4.1.7 | General procedure for deacetylation

The acetylated product (4.5 mmol) was dissolved in methanol (80 ml) at raw temperature. A solution of potassium carbonate in water (4.07 g, 40 ml) was added to the stirred mixture. After that, the solution was acidified to pH 3 with 12 N hydrochloric acid (HCl). The aqueous solution was extracted with ethyl acetate

(3 × 100 ml). The organic phase was washed with 75 ml of water and brine and then dried over anhydrous magnesium sulfate. The drying agent was removed by filtration and the solvents were evaporated under vacuum.[35] The resulting solid was dried under vacuum overnight.

*Methyl 4-benzoxy-3,5-dihydroxybenzoate (**39**)*

White solid, yield 96%, MP: 129°C/Lit. 133–134°C[35]; UV: 263 nm, ^{1}H-NMR (in DMSO) δ 9.56 (2H,s), 7.50 (2H, d), 7.35 (3H, m), 6.97 (2H,s), 5.05 (2H, s), and 3.77 (3H, s); ^{13}C (in DMSO) δ 166.49 (s), 151.35 (s), 138.91 (s), 138.28 (s), 128.54 (s), 128.50 (s), 128.18 (s), 124.84 (s), 109.02 (s), 73.54 (s), and 52.37 (s); EA Th: C, 65.69%; H, 5.15%; Found: C, 65.94%; H, 5.31%.

4.1.8 | General procedure for the synthesis of compound **40**

Methyl gallate (17, 1.09 mmol) was dissolved in DMF (30 ml) with potassium carbonate (2.2 mmol), potassium iodide (6.52 µmol), and (2-bromomethyl)naphtalene (1.30 mmol) under vacuum. The mixture was stirred for 24 h at RT. Then, the solids were removed by filtering on Celite° and water was added to the filtrate (20 ml). The solution was extracted with EtOAc (3 × 50 ml). The organic phase was treated with aq. NaOH 1 N. The aqueous layer was acidified with HCl 1 N to pH 5 and extracted with EtOAc (3 × 50 ml). Ethyl acetate was washed with water and brine. After the general drying procedure ($MgSO_4$), the solvent was evaporated under vacuum. The crude product was dissolved in acetone and recrystallized with *n*-hexane. After that, the acetone was evaporated and purified with DCVC (*n*-hexane/EtOAc, 1:0 to 3:7) to obtain a white solid.[36]

*Methyl 4-naphtyl-3,5-dihydroxybenzoate (**40**)*

White solid, yield 70%, MP: 199°C/Lit. 203–205°C[36] UV: 225 nm, ^{1}H-NMR (in DMSO) δ 7.97 (1H,s), 7.90 (3H, d), 7.70 (1H, dd), 7.51 (2H, m), 6.97 (2H,s), 5.22 (2H, s), and 3.76 (3H, s); ^{13}C (in DMSO) δ 166.48 (s), 151.42 (s), 138.90 (s), 135.95 (s), 133.12 (s), 133.04 (s), 128.27 (s), 128.03 (s), 127.05 (s), 126.79 (s), 126.60 (s), 126.46 (s), 124.90 (s), 109.01 (s), 73.64 (s), and 52.38 (s); EA Th: C, 70.36%; H, 4.97%; Found: C, 69.96%; H, 5.06%.

4.2 | Pharmacological/biological assays

4.2.1 | In vitro antiplasmodial activity

The tested strain was obtained through BEI Resources, NIAID, NIH: *Pf*, strain 3D7, MRA-102, contributed by Daniel J. Carucci. The intraerythrocytic forms of *Pf* were continuously grown in vitro using a modified method of Trager and Jensen.[37] Tested products were dissolved in DMSO at 10 mg/ml; artemisinin and quinine (Sigma-Aldrich) were used as positive standards.

DMSO is known to be toxic for *Pf*. Thus, the maximal amount during the in vitro assay was 1% for the initial concentrations. Finally, each sample was evaluated in a series of eight twofold dilutions in triplicate ($n = 3$). The assay was performed as previously described.[32]

The main method used was the measure of *Plasmodium* lactate dehydrogenase as reported by Makler et al.[45] In addition, a visual evaluation of parasitemia reduction was also performed with a Giemsa stain.[20]

4.2.2 | Hemolysis induction

Hemolytic potential was evaluated for all the tested compounds based on a reported procedure.[62] Consequently, a 10% red blood cell suspension in phosphate-buffered saline (PBS) (v/v) (A+ or O+) was incubated with compounds at 100 µg/ml in duplicate. The positive control was Triton X-100 1% (v/v) (corresponding to 100% lysis) and PBS was used as the negative control (corresponding to 0% lysis).

4.2.3 | Maximal water solubility (C_{max})

The maximum concentration of the compounds in H_2O was determined using the classical shaking flask method using an ultraviolet (UV) spectrophotometric method in milliQ water.[46] Therefore, the maximal wavelength was determined by a UV scan between 190 and 400 nm to obtain the UV-absorption spectra in a 1-cm quartz cuvette. After that, a calibration curve was constructed by three dilutions of a standard solution prepared by dissolving an accurately weighed quantity of products in milliQ water. Linearity was evaluated by linear regression analysis ($R^2 > 0.95$).

Next, the saturated solutions were prepared by overnight agitation of an excess of the product in milliQ water at raw temperature (around 25°C). After that, it was filtered on a 0.45 µM filter (Chromafil XTra PVDF-45/25; Filter Service) to remove the nondissolved product and diluted with milliQ water. The absorbance was measured at the selected wavelength. This experiment was performed for three consecutive days. Then, the maximal water concentration was calculated by the calibration curve.

4.2.4 | In vitro cytotoxicity

The tested compounds were evaluated on HUVECs, supplied by Lonza®. These cells were maintained in vitro in EBM-2 medium, supplemented with EGM-2 SingleQuots® from Lonza®.

The samples were dissolved in DMSO at 10 mg/ml. As DMSO is known to be toxic to cells, the maximal tested concentration was 0.5% in EBM-2. For the assay, 100 µl (10^4 cells) was seeded in a 96-well plate and allowed to stand for 24 h at 37°C. After that, 50 µl of the test solutions were added (three wells for every six concentrations) before the incubation for 48 h. Finally, the

supernatant was removed and replaced by 10-fold diluted Presto Blue®. The plate was incubated at 37°C for 2 h before reading in fluorescence mode (560–590 nm). The growth was compared between treated and untreated cells. Each compound was tested in triplicate.

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interests.

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