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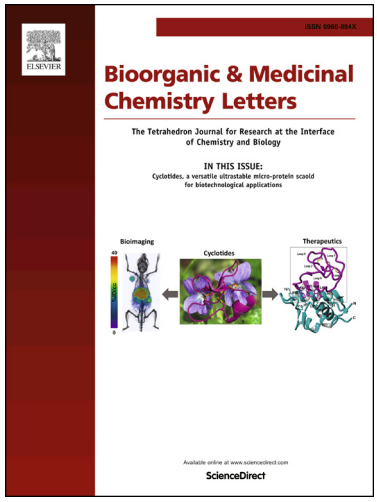

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Semisynthesis, cytotoxicity, antimalarial evaluation and structure-activity relationship of two series of triterpene derivatives

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Abstract

In this report, we describe the semisynthesis of two series of ursolic and betulinic acid derivatives through designed by modifications at the C-3 and C-28 positions and demonstrate their antimalarial activity against chloroquine-resistant *P. falciparum* (W2 strain). Structural modifications at C-3 were more advantageous to antimalarial activity than simultaneous modifications at C-3 and C-28 positions. The ester derivative, 3β-butanoyl betulinic acid (**7b**), was the most active compound (IC_{50} = 3.4 µM) and it did not exhibit cytotoxicity against VERO nor HepG2 cells (CC_{50} > 400 µM), showing selectivity towards parasites (selectivity index > 117.47). In combination with artemisinin, compound **7b** showed an additive effect (CI = 1.14). While docking analysis showed a possible interaction of **7b** with the *Plasmodium* protease PfSUB1, with an optimum binding affinity of -7.02 kcal/mol, the rather low inhibition displayed on a *Bacillus licheniformis* subtilisin A protease activity assay (IC_{50} = 93 µM) and the observed accumulation of ring forms together with a delay of appearance of trophozoites *in vitro* suggests that the main target of 3β-butanoyl betulinic acid on *Plasmodium* may be related to other molecules and processes pertaining to the ring stage. Therefore, compound **7b** is the most promising compound for further studies on antimalarial chemotherapy. The results obtained in this study provide suitable information about scaffolds to develop novel antimalarials from natural sources.

Keywords: Antimalarial; betulinic acid; *Plasmodium falciparum*; semisynthesis; ursolic acid.

Malaria is a devastating disease that remains a significant public health problem worldwide. There are more than 198 million cases of malaria and 584,000 deaths annually, mainly affecting children under 5 years of age.[1] Among the five protozoan species of *Plasmodium* that cause human malaria, *Plasmodium falciparum* is the most virulent, responsible, by far, for the greatest morbidity and mortality, with several hundred million cases of clinical malaria and deaths.[1,2]

The antimalarial drugs used for treatment depend on the species of malaria parasite causing the infection, where the infection was acquired, pregnancy status, and the severity of infection.[1,2] Chloroquine (CQ) was the antimalarial of choice for several decades due to its efficiency, safety, tolerance and low cost. Nevertheless, because of its irrational use, CQ treatment has been a failure, and the use of CQ to treat falciparum malaria is restricted to just a few countries.[1,3] Currently, the first-line treatment of uncomplicated malaria caused by *P. falciparum* is artemisinin combination therapy (ACT).[1] Combination therapy has been adopted to prevent drug-resistant parasites; however, the emergence and spread of drug-resistant malaria, mainly with artemisinin's efficacy declining, has been a major problem that hinders the control of malaria.[1,4,5]

The high morbidity and mortality caused by malaria, the lack of licensed vaccines, and the widespread resistance to artemisinin, together with the lack of drugs safely administered to children and pregnant women are, in general, the main issues that point to the real and critical requirement for discovering new antimalarial medications.[1,5,6] Despite the advances in malaria research and the great efforts towards developing new effective and safe drugs, the majority remain very limited in their mechanisms of action, and until now, no innovative drug is commercially available, proving that more studies and novel lead chemotypes are urgently needed.[5,6]

In the context of improving therapy against malaria infection, natural products could be a potential source of new classes of drugs with high activity and low toxicity, which can be further optimized by chemical modifications.[7,8] Ursolic acid (UA) and betulinic acid (BA) (Fig. 1) are pentacyclic triterpenoids

extracted from several natural sources and have been reported to possess a broad and promising spectrum of biological activities, including antimicrobial, anticancer, and anti-inflammatory characteristics.[9–11]

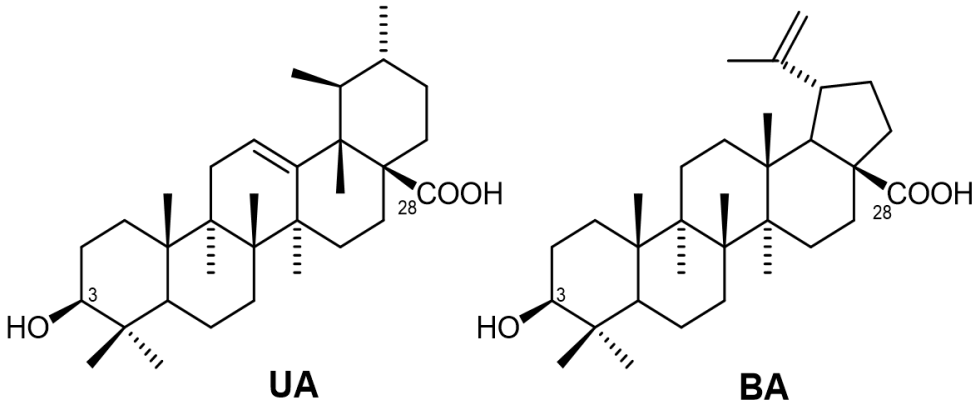


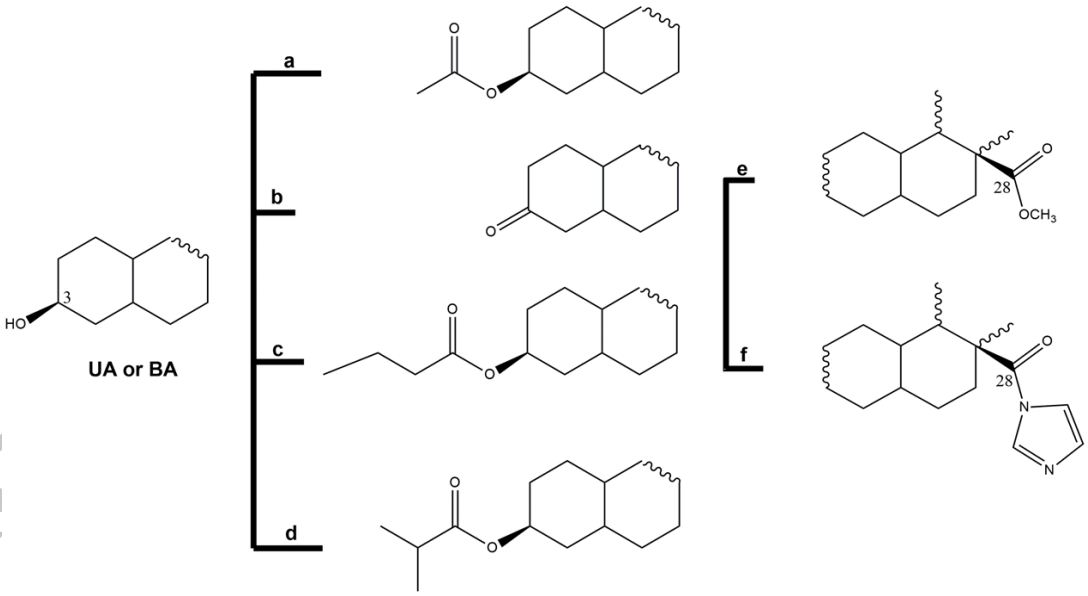
Figure 1 - Chemical structures of ursolic acid (UA) and betulinic acid (BA).

(Source: ChemDraw Ultra® Software)

The antimalarial activity of these compounds and their derivatives was previously assessed by our group, and they demonstrated to be promising lead compounds. The UA and BA analogues bearing an acetyl group at C-3 and piperazine moiety at C-28 were active on CQ-resistant (FcB1) and CQ-sensitive (3D7) *P. falciparum* strains.[12,13] Afterwards, two series of UA and BA derivatives possessing several ester substituents at C-3 were evaluated against CQ-sensitive (3D7) *P. falciparum*, and the derivatives with shorter side-chains were more active.[14] Two inactive BA-group compounds were selected to verify the importance of C-3 groups on cytotoxicity when C-28 is linked to piperazine. In both compounds, the piperazine moiety significantly increased the antiplasmodial activity, showing satisfactory *in vitro* selectivity.[15] However, these compounds presented some *in vivo* toxicity attributed to the piperazine moiety (unpublished data).

The triterpenes UA and BA were obtained by our group from natural sources from southern Brazil, apple pomace (*Malus domestica*) and *Platanus acerifolia* bark, with good yields (3.5% and 1.5%, respectively) using simple and low-cost sources and methodologies.[13,16] A large number of structural modifications in the triterpenes are possible. Here, the semisynthesis

processes were focused at the C-3 and C-28 positions, which are available sites for feasible chemical modifications in these structures. In both triterpenes, the hydroxyl group at the C-3 position was acylated using acetic (**1a, 1b**)[13], butyric (**7a, 7b**)[14] and isobutyric anhydrides (**10a, 10b**)[14]. Two other derivatives were obtained through the oxidation of this hydroxyl group (**4a, 4b**)[17], totaling eight derivatives with a substitution at the C-3 position alone. Diverse natural species have antimalarial and concomitant anticancer activity. Knowing that they are unlikely to be biologically closely related because malaria involves protozoa parasite and cancer involves aberrant mammalian cells, it is equally unlikely that they share the same disease target mechanisms.[21] Due to the cytotoxic profile of methyl and imidazole groups on different cancer cells lines[18–20], starting with C-3 modified triterpene derivatives (esters or ketone groups) the C-28 carboxylic acid was replaced by methyl (**2a, 2b; 5a, 5b; 8a, 8b; 11a,11b**) and imidazole moieties (**3a, 2b; 6a, 6b; 9a, 9b; 12a, 12b**). Therefore, twenty-four compounds in two groups of derivatives were successfully prepared using triterpene skeletons (Scheme 1).



Scheme 1 - Synthesis of derivatives 1a - 12b. Reagents and conditions: (a) dichloromethane, acetic anhydride, pyridine, rt., 24hs; (b) acetone, Jones Reagent at 0 °C, → rt., 3hs; (c) dichloromethane, butyric anhydride, DMAP, rt., 24hs; (d) dichloromethane, isobutyric anhydride, DMAP, rt., 24hs (e)

dichloromethane, K_2CO_3, iodomethane, N_2 atmosphere, 55 °C, 48h; (f) dichloromethane, oxalyl chloride, N_2 atmosphere, 0 °C, 3h. - trimethylamine, 0 °C - imidazole, rt., 24hs. (Source: ChemDraw Ultra® Software)

P. falciparum CQ-resistant (W2 strain - IC_{50} CQ = 0.156 µM) was utilized in the *in vitro* evaluation. The parasite culture was performed as previously described by Trager and Jansen,[22] and was maintained in fresh human erythrocytes (O^+ blood) suspended at a 2% hematocrit in complete medium at 37 °C. The use of human blood was approved by the UFRGS Research Ethics Committee, under protocol number 1.242.369. An anti-*P. falciparum* assay was performed,[23] and the half-maximal drug inhibitory response (IC_{50}) was estimated by curve fivefold dilution, ranging from 100 – 3.12 µM, using software from OriginLab Corporation®. The results were compared with drug-free control wells (100% parasite viability), and artemisinin was tested in parallel as an antimalarial control. In addition, the effects of **7b** on the growth of W2 *P. falciparum* were assessed in order to verify the plasmodial stages affected by the compound. The test was performed with some modifications that were described by Moneriz et al. (2011)[24], using a parasitaemia of 2%. As mentioned, the current treatment regimen for malaria relies on the concept of artemisinin-based combination therapy (ACT). Therefore, given the consensus that combination therapy is essential to the optimal control of malaria and the attractiveness of further development of combinations,[25] the *in vitro* antimalarial efficacy of the combinations of artemisinin and triterpene compounds (**7b** and **7a**) was assessed and the combination index (CI), which is the sum of the two fractional inhibitory concentrations (FIC), was calculated.[26] The *in vitro* cytotoxicity was determined for 24, 48 and 72h, using VERO (African Green Monkey Kidney, ATCC CCL-81) and HepG2 (Human Hepatocellular Carcinoma, ATCC HB-8065) cells,[27] and the concentration that inhibited cell growth by 50% (CC_{50}) was determined by curve eightfold dilution, ranging from 400 – 1.56 µM. The selectivity indexes (SI) were calculated in relation to HepG2 cells ($SI = CC_{50}/IC_{50}$). The structure-activity relationship (SAR) study of these two series of compounds was then evaluated. All the experiments were performed in triplicate and with at least three independent cultures (n = 3).

The derivatives were evaluated against two cells lines: mammalian cells (VERO) and hepatocellular cancer cells (HepG2). HepG2 cells show similarity to human liver cells, and can be useful as an alternative model in the screening for cytochrome P450 (CYP450) enzyme inducers early in the drug discovery process.[28] For this reason, it is very important to assess a drug's cytotoxicity not just in mammalian, but also in HepG2 cells. The CC_{50} concentration of the compounds, excluding UA and **4b**, was > 400 µM for both cells lines, presenting, therefore, low toxicity in the tested conditions. UA presented inexpressive antimalarial activity (IC_{50} = 50.64 µM) and cytotoxicity against VERO (CC_{50} 24h = 98.08 µM; CC_{50} 48h = 101.64; CC_{50} 72h = 99.91), and HepG2 cell lines (CC_{50} 24h = 101.32 µM; CC_{50} 48h = 100.44 µM; CC_{50} 72h = 113.62 µM), thus being nonselective (SI = 2.24). The oxidized derivative, **4b,** presented cytotoxicity on the HepG2 cell line alone after 72h (CC_{50} 72h = 150.50 µM). Therefore, considering its antimalarial activity, it was selective towards the parasites (SI = 19.12) (Table 1).

The IC_{50} values of the derivatives against *P. falciparum* (W2) were in the range of 3.4 and > 100 µM (Table 1). The compound modified at the C-3 position alone, 3β-butanoyl betulinic acid (**7b**), was the most active compound (IC_{50} = 3.4 µM), corroborating with that previously reported on a CQ-sensitive strain (IC_{50} = 5.0 µM).[14] Compound **7b** was the most active against CQ-resistant *P. falciparum* (W2), and was also the most active against the sensitive strain (3D7), which elucidates its effectiveness as an antimalarial agent and suggests low levels of cross-resistance to CQ. Taking into account the resistance index, calculated from the ratio of the IC_{50} value of the sensitive and resistant strains of *P. falciparum* (W2/3D7), the ratio found for compound **7b** was 0.68, lower than that observed for CQ (4.01) (data not shown).

The butyric and isobutyric ester derivatives (**7a, 7b; 10a, 10b**) possess the same number of carbons in the side-chain at the C-3 position (four-carbons); nevertheless their spatial conformations are completely different, reflecting in different fits for the molecular targets, and consequently, different profiles of antimalarial activity (Table1). This fact, however, did not affect their cytotoxicity towards VERO and HepG2 cells.

A ketone moiety at the C-3 position was also active, especially oxidized BA (**4b**) (IC_{50} = 7.87 µM). Interestingly, the oxidation, which exchanges a donor grouping for a hydrogen bond acceptor, did not demonstrate a strong difference in antimalarial activity, but after 72 h, showed some cytotoxic activity against HepG2 cells. On the other hand, a functional group at C-28 that is capable of acting as a hydrogen bond donor (COOH) was most important for the antimalarial activity, while the presence of a group that cannot donate a hydrogen bond (methyl group - $COOCH_3$) was not favorable at this position, annulling the activity (IC_{50} > 100 µM). For these compounds, the cytotoxic potential was not affected. Although the imidazole ring did not affect the cytotoxicity, it showed interesting behavior in its antiplasmodial activity, sometimes increasing or decreasing the IC_{50} value of the initial scaffold (Table 1). The methyl group is acyclic, while imidazole is an aromatic heterocycle. The spatial structures of the compounds are, therefore, different, and could influence their biological activity.

It is generally accepted that biological efficacy is not due to *in vitro* cytotoxicity when SI ≥ 10.[29] Twelve compounds showed selectivity indexes ≥ 10 (Table 1), which indicated high selectivity indexes for these compounds towards parasites. It is worth mentioning that compound **7b** presents the best selectivity index (> 117.47). Combination interactions of triterpene derivatives (**7a**, **7b**) and artemisinin were done using the "1 point" Chou – Talalay approach, which is used to investigate the possible synergy at a minimum of one combination ratio.[26] A combination index (CI) ≤ 1 was considered to indicate synergy, 1 < CI ≤ 2 additivity and CI > 2 antagonism.[26,30] The effect of **7a** and **7b** with artemisinin was additive, with CI = 1.05 and CI = 1.34, respectively (Fig. 2). Moreover, the dose reduction indexes (DRI) were favorable,[31] almost 7.0 and 4.0 for **7a** and **7b**, respectively, and approximately 1.0 for artemisinin. Artemisinin's DRI indicates that **7a** and **7b** did not affect its antimalarial activity, but artemisinin decreased the dose of **7b** by almost 7 and 4-fold in order to achieve the same therapeutic effect.

Table 1 – Antiplasmodial and cytotoxic activity of compounds **UA**, **1a-12a**; **BA**, **1b-12b**. The results represent three independent experiments (n=3).

	Compounds		*P. falciparum*	Cells		
			W2	VERO – 72h	HepG2 – 72h	SI[a]
	R^1	R^2	(IC_{50}, µM)	(CC_{50}, µM)	(CC_{50}, µM)	
UA	OH	COOH	50.64 ± 0.96	99.91± 0.22	113.62 ± 5.33	2.24
1a	OAc	COOH	9.08 ± 0.06	> 400	> 400	> 44.03
2a	OAc	COOMe	> 100	> 400	> 400	-
3a	OAc		50.64 ± 0.63	> 400	> 400	> 7.90
4a	=O	COOH	24.26 ± 3.03	> 400	> 400	> 16.48
5a	=O	COOMe	> 100	> 400	> 400	-
6a	=O		9.07 ± 2.27	> 400	> 400	> 44.08
7a		COOH	13.32 ± 0.50	> 400	> 400	> 30.04
8a		COOMe	> 100	> 400	> 400	-
9a			10.39 ± 0.97	> 400	> 400	> 38.50
10a		COOH	> 100	> 400	> 400	-
11a		COOMe	> 100	> 400	> 400	-
12a			13.59 ± 3.28	> 400	> 400	> 29.42
BA	OH	COOH	12.78 ± 1.46	> 400	> 400	> 31.35
1b	OAc	COOH	65.50 ± 4.54	> 400	> 400	> 6.1
2b	OAc	COOMe	> 100	> 400	> 400	-
3b	OAc		8.90 ± 0.49	> 400	> 400	> 44.94
4b	=O	COOH	7.87 ± 0.96	> 400	150.50 ± 2.78	19.12
5b	=O	COOMe	> 100	> 400	> 400	-
6b	=O		5.20 ± 1.77	> 400	> 400	> 78.85
7b		COOH	3.40 ± 1.05	> 400	> 400	> 117.47
8b		COOMe	> 100	> 400	> 400	-
9b			57.62 ± 0.28	> 400	> 400	> 6.94
10b		COOH	12.11 ± 0.84	> 400	> 400	> 33.03
11b		COOMe	> 100	> 400	> 400	-
12b			29.81 ± 3.0	> 400	> 400	> 13.51
Artemisinin			0.0163	NT	NT	
Chloroquine			0.158	NT	NT	

a = Selectivity Index (SI);
NT = Not tested

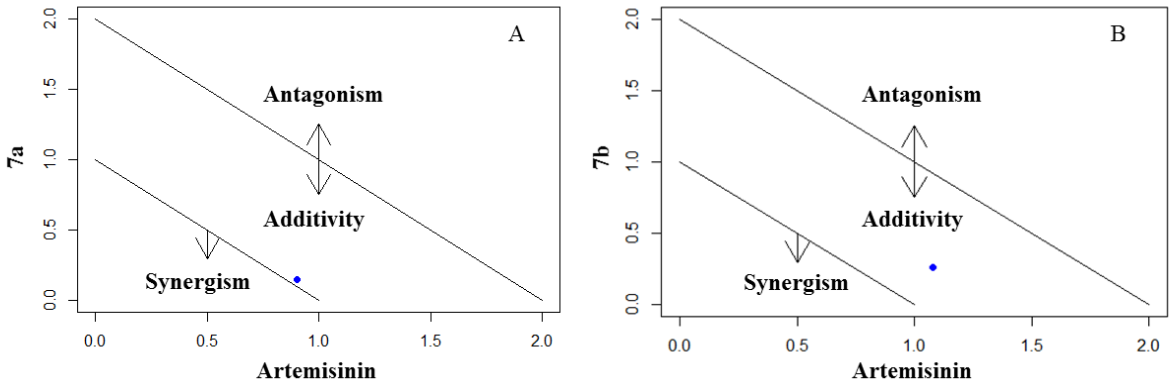


Figure 2 - Normalized isobologram of triterpene derivatives. (A) Isobologram of **7a**; (B) Isobologram of **7b**. (Source: Rstudio® Program)

The incubation of synchronized cultures of *P. falciparum* (W2) with **7b** at 100 µM prevents an increase in parasitaemia, leading to parasite cell death if the compound is present for up to 72 hours (Fig. 3). The addition of **7b** at ring-stage results in the accumulation of ring forms and the delayed appearance of trophozoites, suggesting that its inhibition occurs as early as the ring stage. However, the compound, as well as other compounds of the terpene class, may display multiple-target activity, affecting sequential processes occurring at ring-trophozoite and schizont stages.[32]

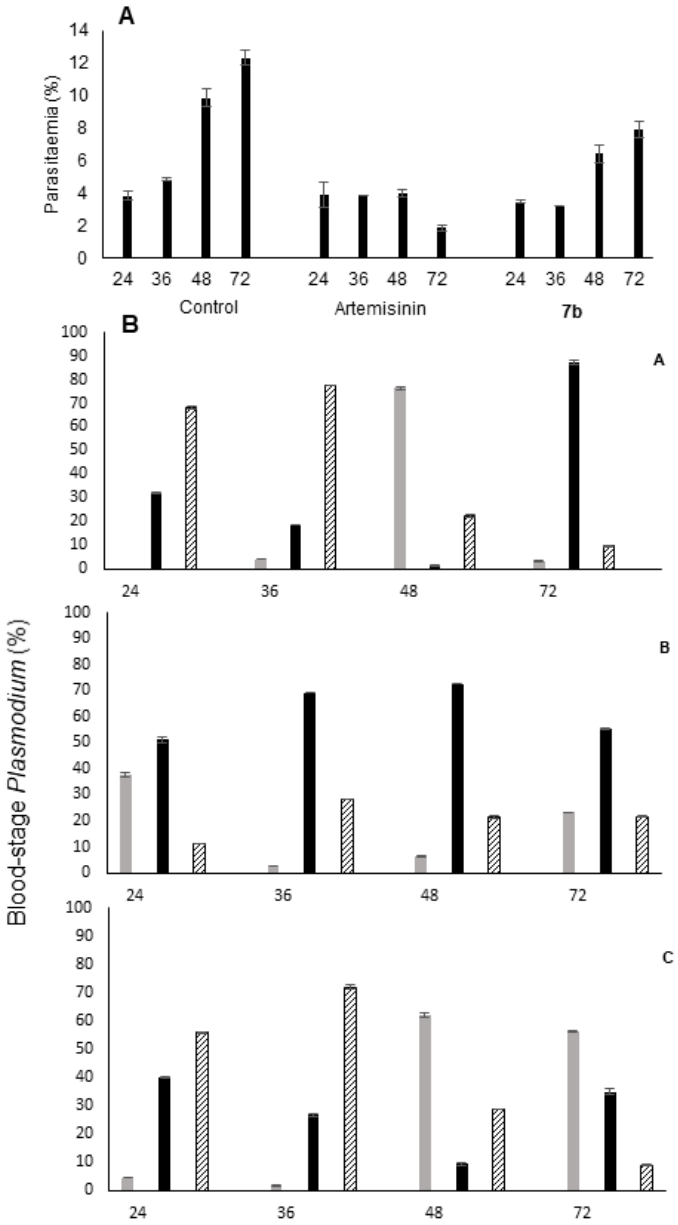


Figure 3 - A. Total parasitaemia reduction after 24, 36, 48 and 72 hours of treatment with compound **7b**; **B.** Effect of **7b** treatment on different *P. falciparum* erythrocytic stages: (A) Control; (B) Artemisinin; (C) Compound **7b** (100 μM). Bars show the percentage of rings (grey); trophozoites (black) and schizonts (hatched) forms observed after 24, 36, 48 and 72 hours of treatment with compound **7b**. Data collected by microscopic inspection of smears accounts for a total of 1,000 erythrocytes.

In order to identify another possible parasite target of the derivatives, *in silico* docking analysis of two selected compounds (**7b** and **9b**) in *Plasmodium falciparum* subtilisin-like serine protease-1 (PfSUB1) were performed using AutoDock 4.2.[33] This enzyme has emerged as a promising antimalarial drug target, since it is a key enzyme in both erythrocytes egress and invasion processes[15] and it has been reported as a target for other pentacyclic triterpenoids.[32] **7b** showed optimum binding affinity with a molecular target, with a binding energy of –7.02 kcal/mol as compared to MRT12113 (-6.32 kcal/mol), a highly selective inhibitor of PfSUB1[34] and the carboxylic acid and ester group of seems to be important for the activity in this target. Since the *in vitro* antimalarial activity of **7b** was seventeen-fold higher than the activity of the compound with an imidazole ring (**9b**), we expected and found a worse binding energy (-5.87 kcal/mol). Therefore, **7b** has more affinity for PfSUB1 than **9b** (Fig. 4). To analyze the possible inhibition of **7b** on PfSUB1 activity, an *in vitro* assay was performed using the closely related *Bacillus licheniformis* subtilisin A. The inhibition kinetics yielded a rather high IC_{50} = 93 µM (Fig. 5) suggesting that either **7b** targets other proteins and processes of the intraerithrocytic cycle of *Plasmodium*, or the conditions *in vivo* differ from the assay with a higher binding affinity. Docking studies simulate the binding of a flexible ligand to a rigid biological receptor and not foresee all other factors that are involved.[35] It may also not be discarded that **7b** may show higher specificity for PfSUB1 than for subtilisin A.

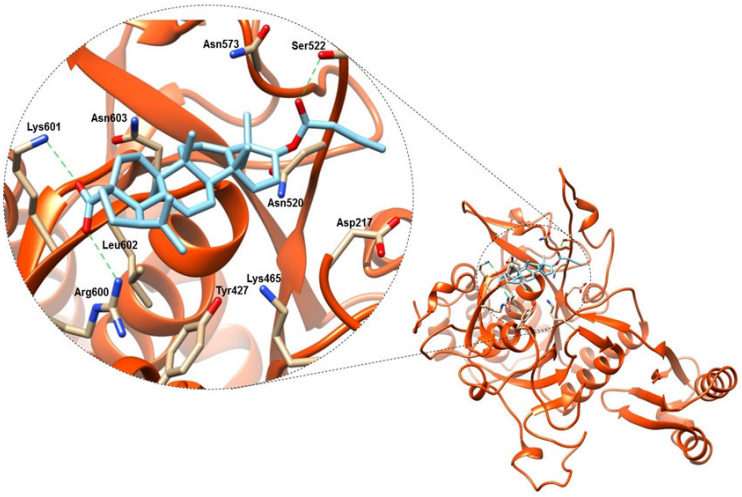


Figure 4 - Result of docking between PfSUB1 from *P. falciparum* (PDB code 4LVN) (orange-red ribbons) and compound **7b** (predominantly light blue and stick representation). The closest amino acids were highlighted. The dashed green lines are hydrogen bonds between the target and **7b**. (Source: UCSF Chimera®)

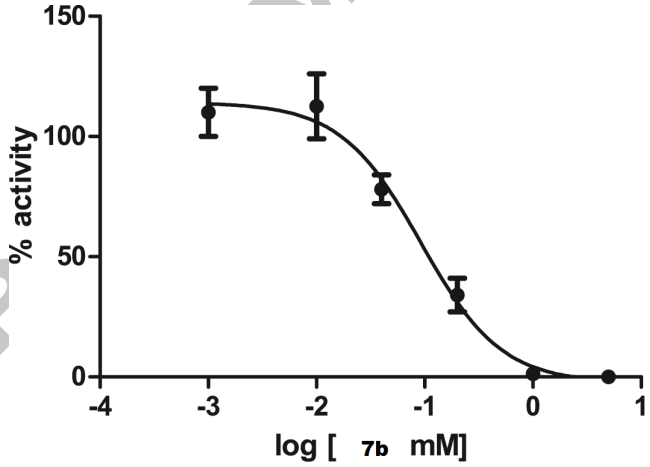


Figure 5 - Dose-response curve of compound **7b** on *B. licheniformis* subtilisin A (Sigma-Aldrich). The protease inhibition assay was performed by using EnzCheck Peptidase/protease assay kit form Molecular Probes as indicated by manufacturer, with concentration of **7b** ranging from 5 to 0.01 mM. The resulting data was plotted as fluorescence vs. log **7b** concentration. (Source: GraphPad® software)

Two series of UA and BA derivatives were successfully prepared with the purpose of investigating the structure-activity relationship of the compounds. It is possible to conclude that compounds that were acylated and oxidized at C-3 presented relevant activity, and the insertion of an imidazole ring at C-28 seems to be not favorable to activity, increasing or not increasing the IC_{50} value. In contrast, the insertion of a methylester group at C-28 abolished the antimalarial activity. None of the compounds presented significant cytotoxic activity against mammalian and hepatic cells. The BA derivative modified at the C-3 position alone, 3β-butanoyl betulinic acid (**7b**), presented the best *in vitro* antimalarial activity against CQ-resistant *P. falciparum* (W2), being 3.76-fold more effective than its natural precursor. Similarly, this potent activity was previously demonstrated effective against a strain of CQ-sensitive *P. falciparum* (3D7), suggesting that the action is not affected by the CQ resistance mechanism. Moreover, **7b** was featured in the *in vitro* activity, showing high selectivity towards parasites (SI > 117.47), and presenting an additive effect when associated with artemisinin. Compound **7b** probably has multiple-target activity, leading to the accumulation of ring forms and the delayed appearance of trophozoites *in vitro*, and *in silico*, suggesting interacting with PfSUB1; however, it was not able to inhibit subtilisin's activity *in vitro*. Therefore, the results obtained in this study could provide suitable information for the development of new antimalarial compounds derived from natural sources.

Acknowledgements

S.T.C. and A.F.S. performed the reactions and analysis of the compounds; S.T.C, D.M.S., A.P.A.S., C.B.G.T. and A.P. carried out the biological assays; F.B.Z performed the docking analysis; P.S.M., G.G. and S.B.G. coordinated the study and provide financial support; and S.T.C. and S.B.G. drafted and edited the manuscript.

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Figure Captions

Figure 1 - Chemical structures of ursolic acid (UA) and betulinic acid (BA). (Source: ChemDraw Ultra® Software)

Figure 2 - Normalized isobologram of triterpene derivatives. (A) Isobologram of **7a**; (B) Isobologram of **7b**. (Source: Rstudio® Program)

Figure 3 - A. Total parasitaemia reduction after 24, 36, 48 and 72 hours of treatment with compound **7b**; **B.** Effect of **7b** treatment on different *P. falciparum* erythrocytic stages: (A) Control; (B) Artemisinin; (C) Compound **7b** (100 μM). Bars show the percentage of rings (grey); trophozoites (black) and schizonts (hatched) forms observed after 24, 36, 48 and 72 hours of treatment with compound **7b**. Data collected by microscopic inspection of smears accounts for a total of 1,000 erythrocytes.

Figure 4 - Result of docking between PfSUB1 from *P. falciparum* (PDB code 4LVN) (orange-red ribbons) and compound **7b** (predominantly light blue and stick representation). The closest amino acids were highlighted. The dashed green lines are hydrogen bonds between the target and **7b**. (Source: UCSF Chimera®)

Figure 5 - Dose-response curve of compound **7b** on *B. licheniformis* subtilisin A (Sigma-Aldrich). The protease inhibition assay was performed by using EnzCheck Peptidase/protease assay kit form Molecular Probes as indicated by manufacturer, with concentration of **7b** ranging from 5 to 0.01 mM. The resulting data was plotted as fluorescence vs. log **7b** concentration. (Source: GraphPad® software)

Scheme 1 - Synthesis of derivatives 1a - 12b. Reagents and conditions: (a) dichloromethane, acetic anhydride, pyridine, rt., 24hs; (b) acetone, Jones Reagent at 0 °C, → rt., 3hs; (c) dichloromethane, butyric anhydride, DMAP, rt., 24hs; (d) dichloromethane, isobutyric anhydride, DMAP, rt., 24hs (e) dichloromethane, K_2CO_3, iodomethane, N_2 atmosphere, 55 °C, 48h; (f) dichloromethane, oxalyl chloride, N_2 atmosphere, 0 °C, 3h. - trimethylamine, 0 °C - imidazole, rt., 24hs. (Source: ChemDraw Ultra® Software)

Table Captions

Table 1 – Antiplasmodial and cytotoxic activity of compounds **UA**, **1a-12a**; **BA**, **1b-12b**. The results represent three independent experiments (n=3).

Graphical abstract