Bioorganic & Medicinal Chemistry Letters 22 (2012) 5455–5459

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis and in vitro evaluation of new chloroquine-chalcone hybrids against chloroquine-resistant strain of *Plasmodium falciparum* ☆

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ARTICLE INFO

Article history:
Received 14 May 2012
Revised 27 June 2012
Accepted 9 July 2012
Available online 14 July 2012

Keywords:
Synthesis
Quinoline
Chalcones
Antimalarial agents

ABSTRACT

The control of malaria has been complicated with increasing resistance of malarial parasite against existing antimalarials. Herein, we report the synthesis of a new series of chloroquine-chalcone based hybrids (**8**–**22**) and their antimalarial efficacy against both chloroquine-susceptible (3D7) and chloroquine-resistant (K1) strains of *Plasmodium falciparum*. Most of the compounds showed enhanced antimalarial activity as compared to chloroquine in chloroquine-resistant (K1) strain of *Plasmodium falciparum*. Furthermore, to unfold the mechanism of action of these synthesized hybrid molecules, we carried out hemin dependent studies, in which three compounds were found to be active.

The magnitude of human suffering and fatalities associated with malaria are well known.[1] The causal agent of the most lethal form of malaria, *Plasmodium falciparum*, has developed resistance to a multitude of drugs including the efficacious, and cheap drug Chloroquine (CQ) (**1**, Fig. 1), that was once used as a first line of defence against infection in most endemic regions.[2] This development of resistance has in part been responsible for the global rise of malaria.[3] Chloroquine induces heme accumulation in cytosol and causes membrane degradation of the parasite which finally contributes to cell death.[4] It is known that the resistance develops as a result of decreased accumulation of the drug in the parasite, due to enhanced efflux, reduced uptake and/or unavailability of drug in parasite food vacuole. Artemisinin and its semi-synthetic derivatives are renowned for their potent antimalarial activity and are only choice to treat resistant malaria parasites.[5] However, recently, first cases of clinical resistance were reported for the artemisinin class of antimalarials from the Thai–Cambodian border.[6]

To overcome the challenges of multi-drug resistance in *P. falciparum,* many approaches currently being adopted contain optimisation of treatment with available drugs including combination therapy, developing analogues of the existing drugs and evaluation of drug resistance reversers (chemo sensitizers). Among these approaches, the medicinal chemistry hybridization, which involves the rational design of new chemical entities by the fusion of two drugs, both active compounds and/or pharmacophoric units with interesting and complimentary activities is an attractive strategy. Additionally, different and/or dual mode of action may reduce undesired side effects.[7] The 7-chloroquinoline moiety present in several established antimalarials such as chloroquine and amodiaquine (**1** & **2**, Fig. 1), is thought to confer antimalarial potency to these compounds by facilitating binding to heme and consequently inhibiting hemozoin formation.[8] A number of chloroquine analogs and derivatives maintain significant activity against chloroquine resistant strains of *P. falciparum*,[9] indicating that the resistance mechanism is compound specific and not associated to modification in the structure of the drug target. On the other hand, chalcones (1,3-diaryl-2-propen-1-ones), which are the bio precursors of flavonoids, have been a prosperous source of inspiration for medicinal chemists for many years and their analogs display a broad range of biological activities including antimalarial activity.[10] They have shown antimalarial activity by inhibiting either plasmodial aspartate proteases or cysteine proteases,[11] in addition they were also shown to inhibit the parasite-induced channels[12] and cause pronounced membrane perturbations of the erythrocytes.[13] Plasmodial aspartate proteases and cysteine proteases are the attractive targets in malarial chemotherapy to overcome the drug resistance.[14] Cysteine protease falcipains are essential for degradation of hemoglobin during erythrocytic parasite

☆ Part XXI in the series, 'Advances in drug design and discovery'. CSIR-CDRI number 8281.

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http://dx.doi.org/10.1016/j.bmcl.2012.07.028

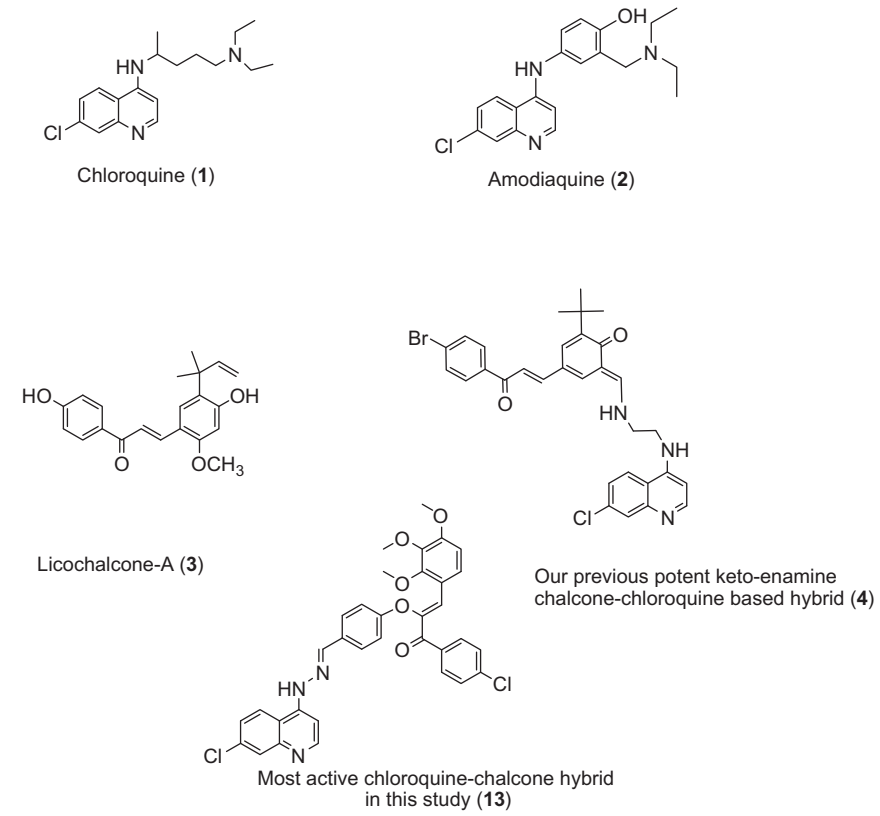
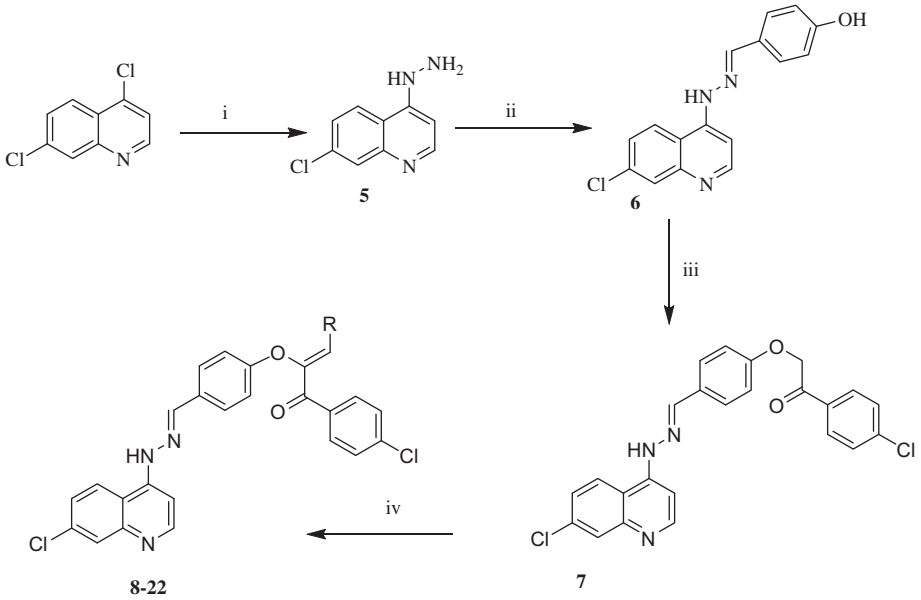


Figure 1. Chemical structures of some potent antimalarials and our synthesized potent hybrids.

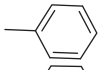
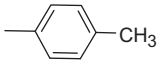
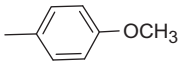
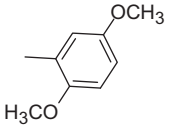
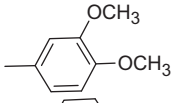
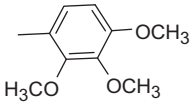
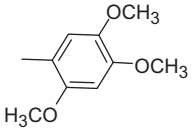
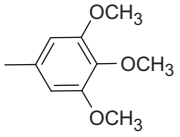
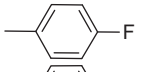
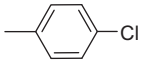
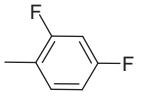
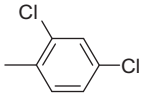
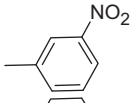
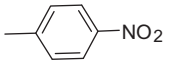


Scheme 1. Synthesis of chloroquine-chalcone hybrids. Reagents and conditions: (i) Hydrazine hydrate, ethanol, reflux, 8 h; (ii) 4-hydroxybenzaldehyde, ethanol, reflux, 2–3 h; (iii) 4-chlorophenacyl bromide, acetonitrile, K_2CO_3, rt, 2 h; (iv) Different substituted aldehydes, 10% methanolic KOH, rt.

development. Licochalcone-A (**3**, Fig. 1) was the first chalcone reported with potent antimalarial activity against chloroquine resistant *P. falciparum*.[15] Subsequently, several synthetic chalcones were synthesized and reported as significant antimalarial agents.[16]

As part of our ongoing drug discovery program in developing potential antimalarial agents,[17] we recently designed and synthesized a series of potent novel keto-enamine chalcone-chloroquine based hybrids.[17a] Inspired by these encouraging results, herein, we report, design and synthesis of CQ-chalcone based hybrid molecules and their antimalarial evaluation against resistant strain of *P. falciparum*. The synthesis of target (**8**–**22**) and intermediate (**5**–**7**) compounds was performed as outlined in the Scheme 1. Synthesis of the compound **5** was achieved by the nucleophilic substitution of the 4-Chloro of 4,7-dichloroquinoline with hydrazine hydrate. The resulting compound **5** was easily condensed with 4-hydroxy benzaldehyde affording the compound **6**.

Table 1
In vitro antimalarial activity against chloroquine sensitive strain 3D7, chloroquine resistant strain K1 of *P. falciparum*, in vitro cytotoxicity of compounds using VERO cell line and Log*P* values

Compd	R	Molecular weight	In vitro antimalarial activity IC_{50} (nM)		Selectivity index (SI)	Log*P*
			3D7	K1		
7		449	36.70	125.87	800.36	5.41
8		537	78.77	110.20	2364.07	7.19
9		551	111.68	97.87	1624.96	7.67
10		567	48.09	96.91	2050.61	7.06
11		597	250.41	125.19	668.89	6.93
12		597	84.03	97.77	1442.69	6.93
13		627	123.50	82.93	837.293	6.81
14		627	90.49	89.50	727.88	6.81
15		627	95.31	122.61	970.21	6.81
16		527	57.30	114.70	962.25	5.75
17		555	29.06	102.07	6199.63	7.34
18		571	144.69	199.49	1210.36	7.74
19		573	138.81	170.22	1040.86	7.50
20		605	295.42	NA	559.50	8.30
21		582	128.12	314.86	1341.02	8.05
22		582	>500	NA	NA	8.05
CQ			7.68	463+	30612	3.73

IC_{50}: Concentration corresponding to 50% growth inhibition of the parasite.
SI: IC_{50} values of cytotoxic activity/IC_{50} values of antimalarial activity.
Log*P*: Calculated by Chem Draw Ultra software.
NA: Not active.

Furthermore, the nucleophilic substitution on compound **6** by using 4-chlorophenacyl bromide in the presence of potassium carbonate, furnished compound **7** in quantitative yields. Finally, the target compounds were synthesized by a base (KOH) catalyzed Claisen-Schmidt condensation of compound **7** with the appropriate aldehydes at room temperature to furnish chloroquine-chalcone hybrid compounds (**8–22**).[18] The detailed reaction conditions are illustrated in Scheme 1. The structures of all the new synthesized compounds were confirmed by ^{1}H NMR, ^{13}C NMR, IR spectroscopy and mass spectrometry. The purity of these compounds was ascertained by TLC analysis. (See Supplementary data).

Table 2
Inhibition of in vitro β-hematin formation

Compd	Inhibition of β-hematin formation IC_{50} (μg/mL)
7	16.4
8	5.66
9	5.58
10	17.6
11	25.0
12	3.46
13	3.52
14	10.6
15	10.8
16	3.74
17	15.6
18	8.38
19	ND
20	5.91
21	5.72
22	12.8
CQ	3.75

Data are the mean of three different experiments in triplicate.
The IC_{50} represents the concentration of compound that inhibit β-hematin formation by 50%.
ND: Not Done.

Table 3
Inhibition of hydrogen peroxide mediated hemin decomposition

Compd	Percentage of remaining hemin
Control	43
Chloroquine	65
12	59
13	63
16	53

Results were expressed as the percentage of remained hemin in the reaction mixture. Data are the mean of three different experiments in triplicate.

All the synthesized chloroquine-chalcone based hybrids (**8**–**22**) and intermediate compound **7** were evaluated for their in vitro antimalarial efficacy against 3D7 (CQ sensitive) and K1 (CQ resistant) strains of *P. falciparum*.[19] Most of the compounds showed potent antimalarial activity as compared to CQ against K1 strain, on the other hand some of the compounds showed comparable activity to CQ against 3D7 strain. The results have been summarized in Table 1. Among 16 tested compounds, **13** was found to be the most potent compound of the series with IC_{50} value of 82.93 nM against chloroquine resistant K1 strain. It was almost sixfold more potent than chloroquine. Five compounds of the series (**9**, **10**, and **12–14)** showed IC_{50} in the range of 80–100 nM and six compounds (**7**, **8**, **11**, and **15**–**17)** showed IC_{50} ranging between 100–130 nM. The structure-activity relationship studies for these compounds suggest that the presence of either methyl or methoxy functionalities on phenyl ring improves their activity. The compounds **9** and **10** consisting methyl and methoxy groups on phenyl ring showed IC_{50} 97.87 and 96.91 nM, respectively. The replacement of electron releasing groups with those of electron withdrawing groups like Cl and NO_2 on phenyl ring resulted in reduced antimalarial activity as shown by compounds **18**, **20**, **21**, **22** (Table 1), however, in the case of compound **17** which consists of fluorine on phenyl ring the activity was found to be comparable to compounds with electron releasing groups. The substitution pattern as well as number of substituent's on the phenyl ring also altered the activity profile, wherein compound **13** consisting three methoxy groups on phenyl ring at 2nd, 3rd, and 4th positions showed IC_{50} value of 82.93 nM, whereas the compound **15** consisting three methoxy groups at 3rd, 4th, and 5th positions showed IC_{50} value 122.61 nM, suggesting that steric factor also played a crucial role in the antimalarial activity of these hybrids against CQ-resistant K1 strain. In the case of chloroquine sensitive 3D7 strain, compound **17** containing fluorine group on phenyl ring showed maximum antimalarial activity with IC_{50} value of 29.06 nM, while, the incorporation of an extra fluorine group on phenyl ring (compound **19**), resulted in the reduction of antimalarial activity (IC_{50} = 138.81 nM). In case of compound **21** with a nitro group at *meta* position of phenyl ring showed an IC_{50} value of 128.12 nM. While changing the position of nitro group from *meta* to *para* (compound **22)**, we observed a sharp decrease in antimalarial activity (IC_{50} >500 nM). These compounds were further tested for their cytotoxicity against VERO cells using MTT assay.[20] Among all the synthesized hybrid derivatives, compound **17**, which was more potent against CQ-sensitive 3D7 strain showed good selectivity index of 6199.63 in comparison with other derivatives. While the most potent compound **13** having IC_{50} 82.93 nM against CQ-resistant strain showed selectivity index of 837.293. Rest of compounds showed SI values ranging between 387 and 2364. Compounds with selectivity index (SI) value greater than 50 are generally considered safe. Although these hybrid compounds have exhibited higher IC_{50} values against 3D7 strain but unlike CQ they have exhibited lower IC_{50} values against K1 strain, these findings make these hybrid compounds worth pursuing as promising leads.

Furthermore, to unfold the mechanism of action of these synthesized hybrid molecules, the β-hematin inhibitory activity of all the molecules were carried out.[21] The results have been summarized in Table 2. The compounds **12**, **13** consisting dimethoxy and trimethoxy groups on phenyl ring and compound **16** consisting furan ring showed stronger β-hematin inhibitory activities (3.46, 3.52 and 3.75 μg/mL, respectively) as compared to chloroquine (3.75 μg/mL). These three compounds were further analyzed for hydrogen peroxide mediated hemin degradation.[22] The results have been summarized in Table 3. The compounds **12**, **13** and **16** were found to be significantly protective against hemin peroxidation in comparison to control groups. We observed the positive correlation between these two mechanisms that is the inhibition of β-hematin formation in the digestive vacuole of parasite leads to release of excessive free hemin and further its accumulation in cytoplasm,[23] where H_2O_2 mediated secondary depredation of hemin would take place. These chloroquine-chalcone hybrids were analyzed with the assumption that their mode of action matches that of chloroquine to prevent heme polymerization in the food vacuole of the parasite. As most of these compounds showed significant activities against CQ resistant strain (K1) of malarial parasite but only few of them acted through inhibition of heme polymerization in the parasite, it might be possible that these hybrids may also affect other resistance reversal mechanism.

In summary, a novel series of chloroquine-chalcones based hybrid antimalarials were designed and synthesized. The synthesized molecules showed significant in vitro antimalarial activity especially against CQ resistant strain (K1). Three molecules were found to be most efficacious to inhibit in vitro β-hematin formation. Activity results indicated that compounds **12**, **13** and **16** could be utilized as lead molecules for further chemical modifications to enhance their therapeutic potential as antimalarial agents in the emergence of rapid resistance against existing antimalarials. Further pharmacological and pharmacokinetic development of these hybrid molecules is currently in progress.

Acknowledgments

The authors are grateful to the Director, CDRI, Lucknow, India for constant encouragement in drug development program, S.P.

Singh for technical support, SAIF for NMR, IR, and Mass spectral data. S.R.A and G.R.P. are thankful to CSIR, New Delhi, India for financial support. This work was supported by EMPOWER grant (OLP0007) of CSIR-CDRI to KVS. This is CSIR-CDRI communication number 8281.

Supplementary data

Supplementary data (spectral data of all the compounds) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.07.028.

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18. (a) *Representative procedure for synthesis of (Z)-1-(4-chlorophenyl)-2-(4-((E)-(2-(7-chloroquinolin-4-yl)hydrazono)methyl)phenoxy)-3-(2,3,4-trimethoxyphenyl)-prop-2-en-1-one (13).*
To a solution of **7** (449 mg, 0.001 mol) in 10% methanolic KOH, 2,3,4-trimethoxy benzaldehyde (196 mg, 0.001 mol) was added and stirred at room temperature for 3 h. After completion of reaction (monitored by TLC), the reaction mixture was evaporated to dryness, the solid obtained was neutralised with dil HCl and extracted with ethyl acetate. The combined organic extracts were dried (Na_2SO_4), and concentrated in vacuo, and the residue was further purified by column chromatography (SiO_2, 1% to 2% CH_3OH in CH_2Cl_2 as eluant) afforded compound (**13**) as Yellow solid; yield: 76%; mp 218–220 °C; IR (KBr, cm^1): 3455, 3018, 2906, 1582, 1360, 1216, 766; ^1H NMR ($CDCl_3$ + CD_3OD, 300 MHz) δ: 8.44 (s, 1H), 8.10–8.05 (m, 2H), 7.85–7.83 (m, 3H), 7.68–7.64 (m, 4H), 7.45 (d, J = 8.4 Hz, 2H), 7.40–7.37 (m, 2H), 7.03 (d, J = 8.5 Hz, 2H), 6.53 (s, 1H), 3.92 (s, 3H), 3.87 (s, 3H), 3.65 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 191.1, 157.5, 156.0, 153.5, 150.3, 148.1, 146.3, 143.6, 142.2, 139.4, 135.9, 131.0, 129.5, 129.0, 128.9, 127.2, 126.1, 125.8, 125.0, 122.4, 119.3, 116.2, 115.8, 108.0, 102.0, 62.0, 62.1, 56.3; ESI-MS (m/z): 628 $(M+H)^+$; HRMS(m/z): calcd for $C_{34}H_{27}Cl_2N_3O_5$ $(M+H)^+$: 628.1406, Found: 628.1396. (b) Ducki, S.; Rennison, D.; Woo, M.; Kendall, A.; Chabert, J. F. D.; McGown, A. T.; Lawrence, N. J. *Bioorg. Med. Chem.* **2009**, *17*, 7698.
19. *In vitro antimalarial assay*: The compounds were dissolved in DMSO at 5 mg/mL. Twofold serial dilutions of test samples were made in 96 well plates and incubated with 1.0% parasitized cell suspension containing 0.8% parasitaemia (Asynchronous culture with more than 80% ring stages). The plates were incubated at 37 °C in CO_2 incubator in an atmosphere of 5% CO_2 and air mixture. 72 h later 100 μL of lysis buffer containing 2× concentration of SYBR Green-I (Invitrogen) was added to each well and incubated for one hour at 37 °C. The plates were examined at 485 ± 20 nm of excitation and 530 ± 20 nm of emission for relative fluorescence units (RFUs) per well using the fluorescence plate reader (FLX800, BIOTEK). Data was transferred into a graphic programme (EXCEL) and IC_{50} values were obtained by Logit regression analysis of dose response curves. Chloroquine was used as the standard reference drug (Singh, S.; Srivastava, R. K.; Srivastava, M.; Puri, S. K. and Srivastava, K. In vitro culture of *Plasmodium falciparum*: Utility of Modified (RPNI) Medium for drug sensitivity studies using SYBR Green I assay. *Exptl. Parasitol.* **2011**, *127*, 318.).
20. *Cytotoxicity assay:* Cytotoxicity of the compounds was carried out using Vero cell line (C1008; Monkey kidney fibroblast) following the method of Mosmann (1983) with certain modifications. The cells were incubated with different dilutions of test agents for 72 h and MTT was used as reagent for the detection of cytotoxicity. 50% cytotoxic concentration (CC_{50}) was determined using non-linear regression analysis of dose response curves. Selectivity Index (SI) was calculated as: SI = CC_{50}/IC_{50} (Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods.* **1983**, *65*, 55).
21. *Inhibition of in vitro β-hematin formation:* Male swiss mice, weighing 15–20 g were inoculated with 1×10^5 *P. yoelii* infected RBCs. Blood of infected animal at 50% parasitemia was collected by cardiac puncture in 2.0% citrate buffer and centrifuged at 5000 rpm for 10 min at 4 °C. The plasma was used in assay of β-hematin formation. The assay mixture contained 100 mM sodium acetate buffer pH (5.1), 50 μL plasma, 100 μM hemin as the substrate and 1–20 μg compound/drug in a total reaction volume of 1.0 mL. The control tube contained all reagents except compound. The reaction mixture in triplicate was incubated at 37 °C for 16 h in a rotary shaker. The reaction was stopped by centrifugation at 10,000 rpm for 10 min at 30 °C. The pellet was suspended in 100 mM Tris–HCl buffer pH (7.4) containing 2.5% SDS. The pellet obtained after centrifugation was washed thrice with distilled water (TDW) to remove free hemin attached to β-hematin. The pellet was solubilized in 50 μL of 2 N NaOH and volume was made up to 1.0 mL with TDW. Absorbance was measured at 400 nm. The 50% inhibitory concentration (IC_{50}) was determined using non-linear regression analysis dose response curves (Pandey, A.V.; Singh, N.; Tekwani, B.L.; Puri, S.K.; Chauhan, V.S. Assay of beta-hematin formation by malaria parasite. *J. Pharm. Biomed. Anal.* **1999**, *20*, 203).
22. *Measurement of hydrogen peroxide-mediated hemin degradation:* Hydrogen peroxide-mediated hemin degradation was also evaluated in the presence/absence of compounds by using the method of Loria et al. (**1999**) with some modifications. The inhibition was monitored in 96 well ELISA plate with a total reaction volume of 200 μL for each well consisting 25 μM Hemin (in 0.1 N NaOH), 180 μg Bovine serum albumin (in 0.2 M Sodium acetate buffer pH 5.1), 20 mM of H_2O_2 and 20 μM of Chloroquine/Compounds. The plates were incubated to equilibrate for 10 min. at room temperature after addition of hemin and bovine serum albumin. The peroxidative reaction was initiated by the addition of H_2O_2 and followed by measuring the decrease in absorption at the Soret band (405 nm) after 30 min. incubation at room temperature. Control groups (TDW instead of H_2O_2) were also included in each experiment. Results were expressed as the percentage of remained hemin in the reaction mixture. Data are the mean of three different experiments in triplicate (Loria. P.; Miller, S.; Foley, M.; Tilley, L. *Biochem. J.* **1999**, *339*, 363).
23. Ginsburg, H.; Ward, S. A.; Bray, P. G. *Parasitol. Today* **1999**, *15*, 357.