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Synthesis and antimalarial evaluation of 1, 3, 5-trisubstituted pyrazolines

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ABSTRACT

A series of 1,3,5-trisubstituted pyrazolines were synthesized and evaluated for *in vitro* antimalarial efficacy against chloroquine sensitive (MRC-02) as well as chloroquine resistant (RKL9) strains of *Plasmodium falciparum*. The activity was at nano molar concentration. β-hematin formation inhibition activity ($BHIA_{50}$) of the pyrazolines were determined and correlated with antimalarial activity. A reasonably good correlation ($r = 0.62$) was observed between antimalarial activity (IC_{50}) and $BHIA_{50}$. This suggests that antimalarial mode of action of this class of compounds appears to be similar to that of chloroquine and involves the inhibition of hemozoin formation. Some of the compounds were showing better antimalarial activity than chloroquine against resistant strain of *P. falciparum* and were also found active in the *in vivo* experiment.

1. Introduction

Haem detoxification is a crucial biochemical process of malaria parasite [1]. The survival of the parasite is highly dependent up on proteolysis of hemoglobin of the host during its erythrocyte cycle [2, 3]. Digestion of hemoglobin releases toxic iron II ferroprotoporphyrin (Fe^{II} FPIX) moiety that subsequently oxidizes to Fe^{III} FPIX or hematin [4]. Hematin then polymerized forming inert crystals of hemozoin [5] or malaria pigment. The polymerization process is a mechanism of detoxification and can be targeted for antimalarial therapy [6]. Considerable data now support the hypothesis that antimalarial quinolines like chloroquine inhibit parasite growth by binding to hematin and preventing its aggregation to hemozoin [6–10]. But chloroquine resistance appears to arise from changes in a food vacuole membrane protein PfCRT of the parasite, rather than changes in detoxification pathway [11]. This suggests that, if a novel chemical class of drug is being discovered against the same target then that could have immense clinical value.

Pyrazolines and their derivatives have been found to possess a broad spectrum of biological activities such as antibacterial [12–14], antidepressant [15] anticonvulsant [16–18] antihypertensive [19] antioxidant [20] antitumor [21] and anticancer activities [22,23]. Recently these classes of compounds are reported to possess potential antiviral activity against flavivirus [24] and HIV [25]. Our literature survey revealed that these classes of compounds are yet to be explored for their possible antimalarial activities. As a part of the continuing effort towards antimalarial drug discovery, we identified an 1,3,5-trisubstituted pyrazoline (Fig. 1) as potential antimalarial agent targeting haem detoxification pathway of malaria parasite. This identification was done on the basis of mapping (Fig. 2) of the 1,3,5-trisubstituted pyrazoline with our earlier reported pharmacophore model [26]. The pharmacophore model contains ring aromatic (RA), positive ionisable (PI), hydrogen bond acceptor (HBA) and aliphatic hydrophobic (HY-ALI) features. Fig. 2 shows the mapping of 1,3,5-trisubstituted pyrazoline for all the pharmacophoric features except PI.

Recently Ali et al., have reported similar 1,3,5-trisubstituted pyrazolines for anti HIV activity involving one additional methyl group on ring A and isoniotinyl group at 1 position of pyrazolyl ring [25]. Molecules selected for this work contain nicotinyl group at 1 position of pyrazolyl ring without any methyl group on ring A. On the basis of pharmacophore mapping, we hypothesized that this type of 1,3,5-trisubstituted pyrazolines may show potential antimalarial activity targeting haem detoxification pathway of malaria parasite. In order to validate the hypothesis experimentally, herein we report the antimalarial activity of 1,3,5-trisubstituted pyrazolines by synthesizing a series of ten molecules (**1a**–**j**) and evaluating them against chloroquine sensitive (MRC-02) as well as chloroquine resistant (RKL9) strains of *P. falciparum*. In this study, only the HY-ALI feature associated with 'B' ring of the 1, 3, 5-trisubstituted

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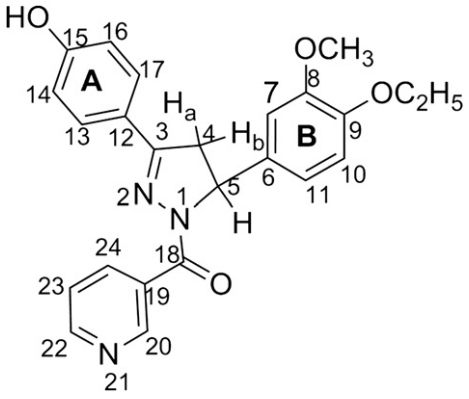


Fig. 1. Structure of 1,3,5-trisubstituted pyrazoline.

pyrazoline moiety was changed by keeping the basic skeleton intact. The compounds were also evaluated for β-hematin formation inhibition activity and correlated with their antiplasmodial activity against CQ sensitive as well as resistant strains of malaria parasite. From the correlations, the mode of action of this class of compounds was proposed to follow haem detoxification inhibition pathway. Three compounds (**1a**, **1f** and **1g**) found most active *in vitro* against CQ resistant malaria parasite were also tested *in vivo* against *P. berghei*.

2. Results and discussion

2.1. Chemistry

1,3,5-trisubstituted pyrazolines (**1a**–**j**) were synthesized according to the method shown in Scheme. In the first step, syntheses of chalcones were carried out by the well-known Claisen-Schmidt reaction and products were purified by recrystallization from methanol (60–70% yield). In the second step, chalcone and nicotinic acid hydrazide were refluxed in *n*-butanol in order to synthesize the desired product. Factors such as the structure and position of the substituents have profoundly influenced the rate of the reaction. Haloginated chalcones (**1a**, **1f** and **1i**) took 8 h for complete conversion followed by unsubstituted (**1b**, 12 h), methyl substituted (**1c** and **1d**, 16 h) and alkoxy substituted (**1e** and **1g**, 24 h) chalcones. Compound **1h** contains both halogen and alkoxy groups, took 18 h for complete conversion. The generally accepted interpretation of this reaction, involves the initial formation of an aryl hydrazone with subsequent nucleophilic attack of nitrogen upon the carbon-carbon double bond at β position. Hence the electropositive nature of β carbon may control the overall rate of the reaction. The electropositive nature of β carbon is controlled by the aromatic ring directly connected to it. Halogens being electron withdrawing in nature significantly increase the positive character of β carbon lead to faster reaction while electron donating alkyl and alkoxy groups contributed for slower reaction. However this phenomenon was not observed in case of nitro group (**1j**). The possible explanation might be the *meta*-directing nature of nitro group. In compound **1j**, nitro group is placed at meta position of the phenyl ring and by virtue of its *meta*-directing nature electron density at β carbon increases and its electropositivity decreases leading to longer time for completion of reaction.

A variety of methods have been reported for the synthesis of 1,3,5-trisubstituted pyrazolines [27–31]. Among the simplest methods, condensation of an α,β-unsaturated ketone with an acid hydrazide in glacial acetic acid (bp 117 °C) under reflux was reported by Fischer and Knövenagel [27]. Comparing similar type of molecules reported in literature [25], we found that the use of *n*-butanol (bp 115 °C) as reaction medium has significantly improved the yield. This is essentially because acetic acid protonates the pyrazoline and pyridine rings resulting improved solubility in water. Hence during processing, significant quantities of the products were washed away by water. *N*-butanol being very low acidic in nature didn't protonate the products and thus improved the yield quantitatively.

Structures of compounds **1a**–**j** were confirmed by MS and NMR techniques. All of the 1,3,5-trisubstituted pyrazolines possesses similar basic skeletal structure. Proton NMR signals were assigned by comparing the spectra of the products (**1a**–**j**) with their corresponding chalcones. Signals around δ value 3.1 and 3.9 ppm recorded as doublet of doublets (dd) were assigned to 4-H_a and 4-H_b protons. The *J* values were calculated for above signals and found to be around 17 Hz and 5 Hz for signal around 3.1 ppm and 17 Hz and 11 Hz for signal around 3.9 ppm respectively. 5-*H* proton (δ around 5.9 ppm) of pyrazoline ring showed a 'dd' pattern of *J* values around 11 Hz and 5 Hz respectively and most likely interacting with 4-H_a and 4-H_b protons. In the deuterium exchange, protons at δ value 10 ppm was exchanged which was assigned as O*H* proton.

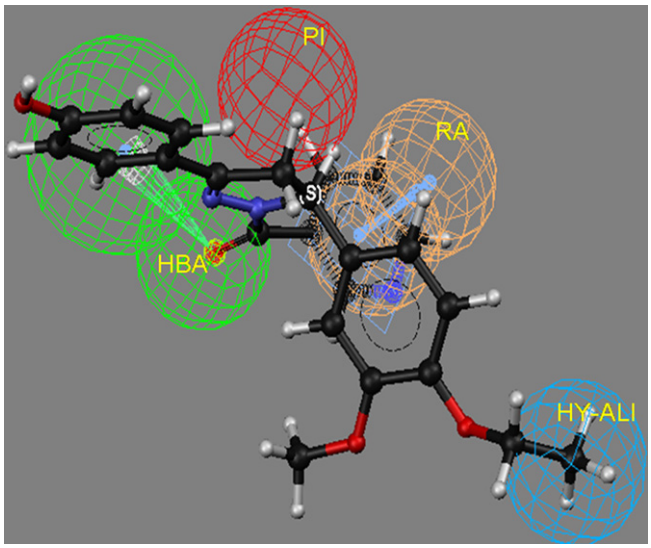
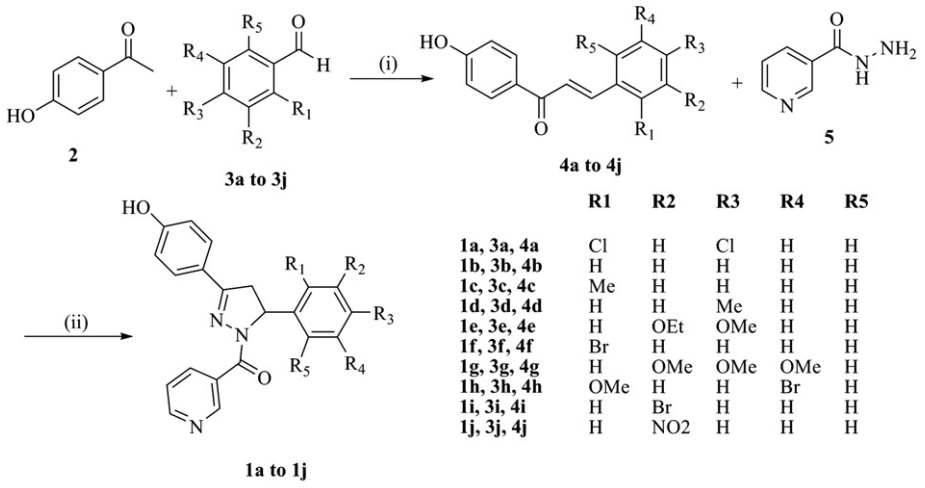


Fig. 2. Pharmacophore mapping of 1,3,5-trisubstituted pyrazoline. The blue contour represents the hydrophobic aliphatic feature (HY-ALI), the orange contour represents ring aromatic feature (AR), the green contour represents hydrogen bond acceptor feature (HBA) and the red contour represents positive ionizable feature (PI).

2.2. In vitro antiplasmodial activity

In vitro antiplasmodial activity (Table 1) of **1a**–**b** and **1f**–**h** were found comparable with chloroquine diphosphate ($IC_{50} = 0.021$ μM) against chloroquine sensitive strain (MRC-02) of *P. falciparum*. Compounds **1a**, **1c**, **1d**, **1f** and **1g** of this series have shown better activity than chloroquine diphospahte ($IC_{50} = 0.177$ μM) against chloroquine resistant strain (RKL9) of parasite. Compound **1g** was found to be most potent against resistant ($IC_{50} = 0.0425$ μM) as well as sensitive ($IC_{50} = 0.0265$ μM) strains of *P. falciparum*. For compound **1g**, R_2, R_3 and R_4 are methoxy groups. Interestingly compound **1b**, for which R_1–R_5 are all hydrogen, also showed antiplasmodial efficacy close to **1g** against chloroquine sensitive strain ($IC_{50} = 0.0304$ μM). However, against resistant strain, compound **1b** ($IC_{50} = 0.1305$ μM) was found to be 3 fold less active than **1g** ($IC_{50} = 0.0425$ μM). This indicates that HY-ALI feature plays an important role for antimalarial activity. **1a**, **1f** and **1i** are the three halogen containing compounds showed very good activity against



[a] Reagents and conditions: (i) Methanolic NaOH, r.t, 24 hours; (ii) n-butanol, reflux

Scheme. Synthesis[a] of 1, 3, 5-trisubstituted pyrazolines.

both chloroquine sensitive and resistant strains. Methyl group in place of R_1 (**1c**) and R_3 (**1d**) also delivered good activities. **1e** and **1h** are the two compounds containing two HY-ALI features each but showed moderate to poor *in vitro* antiplasmodial activity. In this series only the HY-ALI feature was changed and all other features were kept intact. **1b** and **1j** are lacking in HY-ALI feature were predicted as poorly active, which was found valid for both of the compounds.

Table 1
Cytotoxicity, i*n vitro* and *in vivo* antimalarial activity of 1,3,5-trisubstituted pyrazolines.

Compounds	Cytotox.[a] (μM)	Activity vs MRC-02 strain[b] (μM)	Cytotox./ antimal. ratio	Activity vs RKL9 strain[c] (μM)	Cytotox./ antimal. ratio
1a	1.250	0.0375 ± 0.005	33.33	0.0680 ± 0.019	18.38
1b	1.875	0.0304 ± 0.006	61.67	0.1305 ± 0.031	14.36
1c	1.812	0.0625 ± 0.022	28.99	0.1019 ± 0.002	17.78
1d	2.187	0.0555 ± 0.016	39.40	0.0887 ± 0.005	24.65
1e	3.562	0.0755 ± 0.024	47.49	0.2140 ± 0.049	16.64
1f	3.812	0.0272 ± 0.005	140.14	0.0640 ± 0.003	59.56
1g	0.828	0.0265 ± 0.005	31.24	0.0425 ± 0.005	19.48
1h	2.660	0.0495 ± 0.011	53.73	0.2420 ± 0.089	10.99
1i	3.062	0.0260 ± 0.011	117.76	0.0786 ± 0.003	38.95
1j	3.750	0.1065 ± 0.056	35.21	0.4235 ± 0.041	8.85
CQ[d]	66.233	0.0210 ± 0.003	3153.95	0.177 ± 0.004	374.19

[a] Cytotoxicity (maximum nontoxic dose, MNTD) against HeLa cells.
[b] Antiplasmodial activity against chloroquine sensitive (MRC-02) strain of *P. falciparum*, IC_{50}, μM ± SD; results of two separate determinations.
[c] Antiplasmodial activity against chloroquine resistant (RKL9) strain of *P. falciparum*.IC_{50}, μM ± SD; results of two separate determinations.
[d] Chloroquine diphosphate.

2.3. In vitro cytotoxicity

The maximum concentration of DMSO in any well was 0.1% and did not affect cell growth. All the ten molecules of this series were found to be at least 10 times more toxic than chloroquine (Table 1). Yet for all the compounds, the cytotoxic/antiplasmodial ratios were >1, indicating better selectivity against *P. falciparum* even though a long cell-drug exposure time (72 h) was given for cytotoxicity test. Selectivity ratios of two most potent antimalarial compounds **1f** and **1g** were 59.56 and 19.48 for resistant strain of *P. falciparum*. In case of sensitive strain those values were 140.14 and 31.24 respectively. The most potent 1,3,5-tritrisubstituted pyrazoline **1g** was found to be most toxic and second best active **1f** was found to be least toxic among the ten compounds under study. Selectivity ratio of **1f** was found best for both sensitive and resistant strains of malaria parasite.

2.4. In vivo antiplasmodial activity

The three most active compounds (**1a**, **1f** and **1g**) found in the *in vitro* antiplasmodial assays, were tested in mice infected with *P. berghei* by oral administration (Table 2). Compound **1a** showed 69% average suppression at 50 mg/kg/day in comparison to 100% suppression by **CQ** at 8 mg/kg/day. The HPLC retention time (Rt) of **1a**, **1f** and **1g** was 12.67, 11.64 and 11.87 respectively. This signifies that with two chlorines **1a** is most hydrophobic followed by **1f** and **1g**. Their *in vivo* antimalartial activity followed the same trend and showed a correlation. The mean survival time (MST) was found in accordance with the inhibition data.

Table 2
In vivo antimalarial activity against *P. berghei* ANKA strain in swiss mice.

Compounds	% Suppression on day 4[a]	Mean survival time[a] (MST in days) ± SE	% Suppression on day 4[b]	Mean survival time[b] (MST in days) ± SE	Rt (min)[e]	Purity (%)[e]
1a	68.93	13.22 ± 1.19	60.55	11.55 ± 1.02	12.67	98.72
1f	47.52	10.33 ± 1.98	34.98	9.00 ± 1.06	11.64	98.91
1g	43.70	11.00 ± 0.78	32.78	9.00 ± 1.55	11.87	98.83
CQ	100[c]	All alive	–	–	–	–
Control	0[d]	6.44 ± 0.97	–	–	–	–

[a] At 50 mg/kg/day.
[b] At 25 mg/kg/day.
[c] At 8 mg/kg/day.
[d] Without drug.
[e] HPLC.

Table 3
Relationship between pK_a, normalized antiplasmodial IC_{50} values, and inhibition of β-hematin formation for 1,3,5-trisubstituted pyrazolines and chloroquine diphosphate.

Compounds	pK_{a2}, pK_{a1}	VAR[a]	α[b]	$IC_{50} \times \alpha$[c]	$IC_{50} \times \alpha$[d]	$BHIA_{50}$[e]
1a	9.08, 6.65	1001.20	0.79	0.0295	0.0542	1.53 ± 0.025
1b	8.95, 6.78	1254.89	1.00	0.0300	0.1305	1.58 ± 0.454
1c	8.89, 7.08	2049.04	1.63	0.1020	0.1663	2.43 ± 0.010
1d	9.01, 6.80	1303.19	1.03	0.0576	0.0921	1.57 ± 0.142
1e	8.93, 6.84	1464.13	1.16	0.0879	0.1524	2.39 ± 0.023
1f	9.11, 7.20	2457.64	1.95	0.0532	0.1253	1.82 ± 0.462
1g	8.98, 6.95	1676.66	1.33	0.0354	0.0567	1.70 ± 0.002
1h	9.10, 6.90	1551.03	1.23	0.0611	0.2991	1.89 ± 0.002
1i	9.01, 6.90	1545.72	1.23	0.0320	0.0968	2.06 ± 0.034
1j	8.95, 6.38	606.30	0.48	0.0512	0.2046	2.07 ± 0.630
CQ[f]	9.61, 8.55	5889.85	4.69	0.0985	0.8307	1.89 ± 0.008

[a] Vacuolar accumulation ratio (VAR) calculated using Eq. (1) and assuming vacuolar pH of 5.5 and external pH of 7.4.
[b] Vacuolar accumulation ratio relative to **1b**.
[c] Normalized antiplasmodial activity against chloroquine sensitive (MRC-02) strain of *P. falciparum*.
[d] Normalized antiplasmodial activity against chloroquine resistant (RKL9) strain of *P. falciparum*.
[e] β-hematin inhibitory activity, IC_{50}, mM ± SD; results of two separate determinations.
[f] Chloroquine diphosphate.

2.5. Correlation of antiplasmodial activities with β-hematin formation inhibition

To establish the mode of action, vacuolar accumulation ratio (VAR) of all the compounds were theoretically calculated from experimentally determined pK_a values (Table 3) using Eq. (1) [32]. The two pK_a values of CQ diphosphate were determined experimentally and were found close to the previously reported values [32]. In Eq. (1), VAR represents the ratio of drug inside and outside of the parasite food vacuole. $pH_v = pH$ inside food vacuole (assumed to be pH 5.5) and $pH_e = pH$ externally (assumed to be pH 7.4).

$$VAR = \frac{Q_v}{Q_e} = \frac{\left\{1 + \frac{[H^+]_v}{K_{a2}} + \frac{[H^+]_v^2}{K_{a1}K_{a2}}\right\}}{\left\{1 + \frac{[H^+]_e}{K_{a2}} + \frac{[H^+]_e^2}{K_{a1}K_{a2}}\right\}} \quad (1)$$

The calculated VAR and the VAR relative to **1b** (α) are shown in Table 3. Compound **1b** was taken as reference molecule to calculate α because this molecule was not substituted at B ring. VAR values for the compounds of this series are calculated in the range of 606 (**1j**) to 2458 (**1f**). For chloroquine diphosphate the value was 5890. A significant difference between VAR values were observed between **1f** (VAR = 2458) and **1i** (VAR = 1546) in which bromine is present at ortho and meta position respectively. In case of **1h** (VAR = 1551) bromine is present at meta position and its VAR value was found closer to **1i**. The relative VAR (α) values for all the compounds were calculated in the range 0.48–1.95 and that for chloroquine diphospahte was 4.69. Due to higher pK_a values, chloroquine diphosphate achieved 2.5–9 times better accumulation in the parasite food vacuole in comparison to the 1,3,5-trisubstituted pyrazolines (**1a**–**j**). Normalized IC_{50} values ($IC_{50} \times \alpha$) were obtained by multiplying experimentally obtained antimalarial IC_{50} with α. Normalized IC_{50} values are the theoretically expected IC_{50} values, if the compounds accumulate in the vacuole to the same extent as **1b**. Having adjusted for the differential accumulation of the compounds, the normalized antiplasmodial IC_{50} values and the inhibition of β-hematin formation ($BHIA_{50}$) should show a clear correlation. Hawley et al. [9] and Kaschula et al. [32] reported direct proportionality between normalized antiplasmodial activity and inhibition of β-hematin formation of quinoline antimalarials using chloroquine sensitive strains of *P. falciparum*. In this study we have taken both chloroquine sensitive (MRC-02) and chloroquine resistant (RKL9) strains of *P. falciparum*.

The quantities of the compounds required to inhibit the formation of β-hematin by 50% ($BHIA_{50}$) were found in a narrow

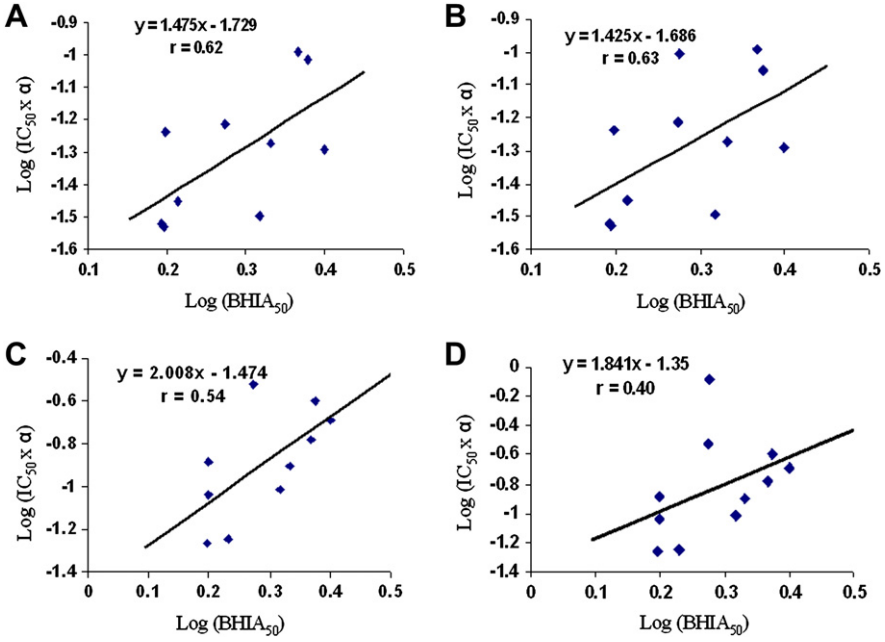


Fig. 3. Plot of the log of the normalized antiplasmodial IC_{50} ($IC_{50} \times \alpha$) versus the log of the activity against the inhibition of β-hematin formation. (A) Study involving chloroquine sensitive (MRC-02) strain without chloroquine diphosphate. (B) Study involving chloroquine sensitive (MRC-02) strain with chloroquine diphosphate. (C) Study involving chloroquine resistant (RKL9) strain without chloroquine diphosphate. (D) Study involving chloroquine sensitive (RKL9) strain with chloroquine diphosphate.

range 1.53 mM (**1a**) to 2.43 mM (**1c**). Un-substituted **1b** showed comparable β-hematin formation inhibition activity (1.58 mM). Exceptionally, compound **1c** containing a methyl group showed better $BHIA_{50}$ than **1g** (1.70 mM), which contains three methoxy groups. In case of **1f** and **1i**, unlike VAR, the position of bromine did not make any significant difference between their $BHIA_{50}$ values. Chloroquine diphosphate showed a comparable value of 1.89 mM. Although chloroquine showed lower β-hematin inhibition activity, due to better VAR value it was found to be more potent than **1g** against chloroquine sensitive *P. falciparum*. The normalized IC_{50} ($IC_{50} \times \alpha$) values for all the compounds including chloroquine diphosphate were calculated for both sensitive and resistant strains. A plot of log ($IC_{50} \times \alpha$) against log $BHIA_{50}$ (Fig. 3) showed some correlation present between the normalized IC_{50} and the ability of the compounds to inhibit β-hematin formation. Against chloroquine sensitive and resistant strains, reasonably good correlations ($r = 0.62$ and 0.63 respectively) were observed. When chloroquine was included, the r values were found decreased to 0.54 and 0.40 for sensitive and resistant strains respectively. In case of resistant strain the value of r significantly decreased from 0.63 to 0.40 because chloroquine is less active against resistant strain. Low difference between r values was observed when chloroquine diphosphate was included against sensitive strain of parasite. This indicates, chloroquine and 1,3,5-trisubstituted pyrazolines (**1a**–**j**) have similar antiplasmodial mode of action. This finding further strengthens the hypothesis that haem polymerization inhibition may be a possible mode of action for this series of compounds (**1a**–**j**).

3. Conclusions

We reported the synthesis and evaluation of 1,3,5-trisubstituted pyrazolines as potential antimalarial agents targeting haem detoxification pathway of malaria parasite. The molecules were found active against *in vitro* culture of both chloroqine sensitive as well as chloroquine resistant strains of *P. falciparum*. Compound **1g** was found to be most active as well as most cytotoxic. The second best active compound **1f** was found to be least cytotoxic among the ten compounds. Three compounds (**1a**, **1f** and **1g**) were tested against *P. berghei* in mouse model found active. Good correlation was observed between normalized antimalarial activity ($IC_{50} \times \alpha$) and β-hematin formation inhibition ($BHIA_{50}$) against both sensitive and resistant strains. When chloroquine was included, a drastic drop in correlation was observed in case of resistant strain only. These observations indicated that the 1,3,5-trisubstituted pyrazolines are antimalarial and their mode of action may be through inhibition of haem detoxification process.

4. Experimental protocols

4.1. Chemistry

Nicotinic acid hydrazide, 2-hydroxy acetophenone and all the aromatic aldehydes were obtained from Sigma–Aldrich. Melting points were determined on Electrothermal 9100 apparatus and uncorrected. Mass spectra were acquired on a Micromass Q-ToF high resolution mass spectrometer equipped with electrospray ionization (ESI) on Masslynx 4.0 data acquisition system. ESI was used in +ve ionization mode. Vibrational spectra were recorded on a Bruker Tensor 27 FTIR spectrometer. ^{1}H NMR spectra were acquired on Bruker DPX 400 FT NMR spectrometer at 400 MHz. ^{13}C NMR spectra were acquired on Bruker AV 400 FT NMR at 100 MHz. All the NMR spectra were acquired in DMSO-d_6 at 27 °C. Purity of chalcones was checked on TLC (Merck Silica gel $60F_{254}$). Purity of the 1,3,5-trisubstituted pyrazolines were checked by HPLC. Waters HPLC system equipped with Waters 1525 binary gradient pump and 2487 dual absorbance UV detector. Separations were carried out on Waters Exterra C8 MS column (2.1 × 30 mm, 3.5 μ) with binary linear gradient of 5% methanol in water to 100% methanol in 20 min and wavelength at 254 nm.

4.2. General method for synthesis of chalcones (*4a*–*4j*)

To the solution of (8 mmole, 1.1 g) of 2-hydroxy acetophenone (**2**) in 5 mL of methanol on an ice bath, freshly prepared 2 N methanolic NaOH solution (30 ml) was added and stirred for 10 min. To this 8 mmole of appropriate aldehyde (**3a**–**j**) was added and the reaction mixture was stirred at room temperature for 24 h. The reaction mixture was cooled on an ice bath and neutralized with dilute hydrochloric acid. The precipitate appeared was separated by filtration and washed three times with 50 ml distilled water to give the crude product. The product so obtained was recrystallized from methanol. The purity of the products was checked on TLC (Merck Silica gel $60F_{254}$) by using mixture of ethylacetate and hexane as mobile phase.

4.2.1. 3-(2,4-Dichloro-phenyl)-1-(4-hydroxy-phenyl)-propenone (*4a*)

Prepared by above method from **2** (8 mmol, 1.1 g) and 2,4-dichlorobenzaldehyde (8 mmol, 1.42 g); Yield: 1.42 g, 61%; $R_f = 0.64$ in EtOAc/hexane, 3:7; Off white crystalline solid. mp:192–193 °C; ESI-MS (M + H): $C_{15}H_{10}Cl_2O_2$, *calcd.*: 293.0136, *found*: 293.0222; FTIR (cm^{-1}): 3129, 1591, 1549, 1223; ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm 6.89 (2H, d, $J = 8$ Hz), 7.53 (1H, dd, $J = 8.8$ Hz, 2 Hz), 7.74 (1H, d, $J = 2$ Hz), 7.92 (1H, d, $J = 15.6$ Hz), 7.98 (1H, d, $J = 15.2$ Hz), 8.07 (2H, d, $J = 8.8$ Hz), 10. 39 (1H, s, OH).

4.2.2. 1-(4-Hydroxy-phenyl)-3-phenyl-propenone (*4b*)

Prepared by above method from **2** (8 mmol, 1.1 g) and benzaldehyde (8 mmol, 0.89 g); Yield: 1.21 g, 67%, $R_f = 0.45$ in EtOAc/hexane, 3:7. Colourless crystalline solid. mp: 180–181 °C; ESI-MS (M + H): $C_{15}H_{12}O_2$, *calcd.*: 225.2625, *found*: 225.2032; FTIR (cm^{-1}): 3115, 1646, 1598, 1565, 1337, 1219; ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm 6.89 (2H, d, $J = 8$ Hz), 7.43 (3H, m), 7.65 (1H, d, $J = 16$), 7.85 (2H, m), 7.88 (1H, d, $J = 16$ Hz), 8.05 (2H, d, $J = 8$), 10. 46 (1H, s, OH).

4.2.3. 1-(4-Hydroxy-phenyl)-3-o-tolyl-propenone (*4c*)

Prepared by above method from **2** (8 mmol, 1.1 g) and 2-methylbenzaldehyde (8 mmol, 1.12 g); Yield: 1.20 g, 63%, $R_f = 0.53$ in EtOAc/hexane, 3:7; Paleyellow crystalline solid. mp: 171–172 °C; ESI-MS (M + H): $C_{16}H_{14}O_2$, *calcd.*: 239.1072, *found*: 239.2142; FTIR (cm^{-1}): 3127, 1647, 1600, 1560, 1386, 1218; ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm 2.37 (3H, s, CH_3), 6.83 (2H, d, $J = 8.2$ Hz), 7.22 (3H, m), 7.70 (1H, d, $J = 15.5$ Hz), 7.85 (1H, d, $J = 14.9$ Hz), 7.89 (1H, s), 7.99 (2H, d, $J = 8.6$ Hz), 10. 39 (1H, s, OH).

4.2.4. 1-(4-Hydroxy-phenyl)-3-p-tolyl-propenone (*4d*)

Prepared by above method from **2** (8 mmol, 1.1 g) and 4-methylbenzaldehyde (8 mmol, 1.15 g); Yield: 1.14 g, 60%, $R_f = 0.56$ in EtOAc/hexane, 3:7; Yellow crystalline solid. mp: 197–198 °C; ESI-MS (M + H): $C_{16}H_{14}O_2$, *calcd.*: 239.1072, *found*: 293.1841; FTIR (cm^{-1}): 3117, 1646, 1596, 1553, 1226; ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm 2.28 (3H, s, CH_3), 6.82 (2H, d, $J = 8.6$ Hz), 7.19 (2H, d, $J = 7.9$ Hz), 7.51 (1H, d, $J = 15.6$ Hz), 7.68 (2H, d, $J = 8.0$ Hz), 7.77 (1H, s), 7.99 (2H, d, $J = 8.6$ Hz), 10.37 (1H, s, OH).

*4.2.5. 3-(4-Ethoxy-3-methoxy-phenyl)-1-(4-hydroxy-phenyl)-propenone (**4e**)*

Prepared by above method from **2** (8 mmol, 1.1 g) and 4-Ethoxy-3-Methoxybenzaldehyde (8 mmol, 1.45 g); Yield: 1.54 g, 65%, $R_f = 0.51$ in EtOAc/hexane, 4:6; Yellow crystalline solid. mp: 212–214 °C; ESI-MS (M + H): $C_{18}H_{18}O_4$, *calcd*.: 299.1283, *found*: 299.1613; FTIR (cm^{-1}): 3075, 2968, 1643, 1593, 1503, 1250; ^1H NMR (400 MHz, DMSO-d_6) δ/ppm 1.28 (3H, t, $J = 6.9$ Hz, CH_3), 3.81 (3H, s, CH_3), 4.02 (2H, q, $J = 6.9$ Hz, CH_2), 6.84 (2H, d, $J = 8.6$ Hz), 6.94 (1H, d, $J = 8.3$ Hz), 7.27 (1H, d, $J = 8.3$ Hz), 7.46 (1H, s), 7.57 (1H, d, $J = 15.4$ Hz), 7.72 (1H, d, $J = 15.5$ Hz), 8.01 (2H, d, $J = 8.6$ Hz), 10.35 (1H, s, OH).

*4.2.6. 3-(2-Bromo-phenyl)-1-(4-hydroxy-phenyl)-propenone (**4f**)*

Prepared by above method from **2** (8 mmol, 1.13 g) and 2-Bromobenzaldehyde (8 mmol, 1.1 g); Yield: 1.55 g, 64%; $R_f = 0.59$ in EtOAc/hexane, 3:7; Paleyellow crystalline solid. mp: 168–170 °C; ESI-MS (M + Na): $C_{15}H_{11}BrO_2$, *calcd*.: 325.1325, *found*: 325.1485; FTIR (cm^{-1}): 3201, 1641, 1601, 1560, 1342, 1220; ^1H NMR (400 MHz, DMSO-d_6) δ/ppm 6.90 (2H, d, $J = 8.7$ Hz), 7.39 (1H, m), 7.38 (1H, m), 7.48 (1H, d, $J = 7.9$ Hz), 7.94 (2H, d, $J = 2.5$ Hz), 8.08 (2H, d, $J = 8.9$ Hz), 8.16 (d, 1H, $J = 7.8$ Hz), 10.39 (1H, s, OH).

*4.2.7. 1-(4-Hydroxy-phenyl)-3-(3,4,5-trimethoxy-phenyl)-propenone (**4g**)*

Prepared by above method from **2** (8 mmol, 1.1 g) and 3,4,5-trimethoxydenzaldehyde (8 mmol, 1.58 g); Yield: 1.65 g, 66%, $R_f = 0.48$ in EtOAc/hexane, 4:6; Yellow crystalline solid. mp: 236–238 °C; ESI-MS (M + H): $C_{18}H_{18}O_5$, *calcd*.: 315.1232, *found*: 315.1901. FTIR (cm^{-1}): 3115, 1645, 1595, 1282; ^1H NMR (400 MHz, DMSO-d_6) δ/ppm 3.64 (3H, s, CH_3), 3.80 (6H, s, CH_3), 6.84 (2H, d, $J = 8.5$ Hz), 7.14 (2H, s), 7.55 (1H, d, $J = 15.5$ Hz), 7.78 (1H, d, $J = 15.5$ Hz), 8.03 (2H, d, $J = 8.5$ Hz), 10.37 (1H, s, OH).

*4.2.8. 3-(5-Bromo-2-methoxy-phenyl)-1-(4-hydroxy-phenyl)-propenone (**4h**)*

Prepared by above method from **2** (8 mmol, 1.1 g) and 5-Bromo-2-methoxybenzaldehyde (8 mmol, 1.75 g); Yield: 1.61 g, 60%, $R_f = 0.64$ in EtOAc/hexane, 4:6; Redish-yellow crystalline solid. mp: 199–200 °C; ESI-MS (M + H): $C_{16}H_{13}BrO_3$, *calcd*.: 333.0126, *found*: 332.9661; FTIR (cm^{-1}): 3166, 1648, 1603, 1255; ^1H NMR (400 MHz, DMSO-d_6) δ/ppm 3.88 (3H, s,CH_3), 6.87 (2H, d, $J = 8$ Hz), 7.20 (1H, d, $J = 7.8$ Hz), 7.56 (1H, d, $J = 16$ Hz), 7.91 (2H, m), 8.07 (2H, d, $J = 8.4$ Hz), 8.17 (1H, s), 10.35 (1H, s, OH).

*4.2.9. 3-(3-Bromo-phenyl)-1-(4-hydroxy-phenyl)-propenone (**4i**)*

Prepared by above method from **2** (8 mmol, 1.1 g) and 3-Bromobenzaldehyde (8 mmol, 1.49 g); Yield: 1.61 g, 67%, $R_f = 0.61$ in EtOAc/hexane, 3:7; Paleyellow crystalline solid. mp: 174–175 °C; ESI-MS (M + Na): $C_{15}H_{11}BrO_2Na$, *calcd*.: 325.1325, *found*: 325.4017; FTIR (cm^{-1}): 3201, 1641, 1601, 1560, 1342, 1220; ^1H NMR (400 MHz, DMSO-d_6) δ/ppm 6.83 (2H, d, $J = 8.0$ Hz), 7.68 (1H, d, $J = 16$ Hz), 7.98 (3H, m), 8.03 (2H, m), 8.07 (2H, d, $J = 8$ Hz), 10.51 (1H, s, OH).

*4.2.10. 1-(4-Hydroxy-phenyl)-3-(3-nitro-phenyl)-propenone (**4j**)*

Prepared by above method from **2** (8 mmol, 1.1 g) and enzaldehyde3-nitro (8 mmol, 1.2 g); Yield: 1.31 g, 61%, $R_f = 0.53$ in EtOAc/hexane, 4:6; Offwhite solid. mp: 222–223 °C; ESI-MS (M + H): $C_{15}H_{11}NO_4$, *calcd*.: 270.2601, *found*: 270.1700; FTIR (cm^{-1}): 3142, 1650, 1600, 1559, 1516, 1344, 1223; ^1H NMR (400 MHz, DMSO-d_6) δ/ppm 6.82 (2H, d, $J = 7.9$ Hz), 7.75 (2H, m), 8.10 (2H, d, $J = 8.1$ Hz), 8.14 (1H, m), 8.23 (1H, d, $J = 8.4$ Hz), 8.30 (1H, d, $J = 8$ Hz), 8.74 (1H, s), 10.53 (1H, s, OH).

*4.3. General method for synthesis of 1, 3, 5- trisubstituted pyrazolines (**1a**–**1j**)*

To the solution of (4 mmole) of the appropriate chalcone (**4a–j**) in 10 mL of *n*-butanol, (4 mmole, 0.55 g) of nicotinicacidhydrazide was added and the reaction mixture was refluxed for 8–24 h. Conversion was monitored in every 60 min interval on pre-coated silica TLC plates (Merck, $60F_{254}$) by using mixture of acetone and petroleum ether (40:60 V/V) as mobile phase. The excess of solvent was removed under reduced pressure and the reaction mixture was cooled on an ice bath. The products precipitated out at low temperature were washed five times with 50 ml distilled water, reconstituted in minimum amount of methanol and dried under reduced pressure. Purity of the products was checked by HPLC.

*4.3.1. [5-(2,4-Dichloro-phenyl)-3-(4-hydroxy-phenyl)-4,5-dihydro-pyrazol-1-yl]-pyridin-3-yl-methanone (**1a**)*

Prepared by above method from **4a** (4 mmol, 1.17 g) and **5** (4 mmol, 0.55 g) after 8 h reflux; Yield: 1.61 g, 98%; White amorphous solid. mp: 220–221 °C; HPLC Rt (min), purity: 12.67, 98.72%; ESI-MS (M + H): $C_{21}H_{16}Cl_2N_3O_2$, *calcd*.: 412.0620, *found*: 412.0801; IR (KBr, cm^{-1}): 3200, 2967, 1664, 1596, 1502, 1465, 1260, 1092, 798; ^1H NMR (DMSO-d_6, δ): 3.10 (1H, dd, $J = 17.9$ Hz, 5.2 Hz, 4-H_a), 3.91 (1H, dd, $J = 17.8$ Hz, 12.0 Hz, 4-H_b), 5.92 (1H, dd, $J = 11.7$ Hz, 6.6 Hz, 5-*H*), 6.81 (2H, d, $J = 8.3$ Hz, 14-*H* and 16-*H*), 7.28 (1H, d, $J = 8.3$ Hz, 11-*H*), 7.39 (1H, d, $J = 8.3$ Hz, 10-*H*), 7.54 (1H, m, 23-H), 7.55 (2H, d, $J = 8.1$ Hz, 13-*H* and 17-*H*), 7.70 (1H, s, 8-*H*), 8.27 (1H, d, $J = 7.8$ Hz, 24-*H*), 8.71 (1H, d, $J = 4.4$ Hz, 22-*H*), 9.07 (1H, s, 20-*H*), 10.05 (1H, s, O*H*).

*4.3.2. [3-(4-Hydroxy-phenyl)-5-phenyl-4,5-dihydro-pyrazol-1-yl-]-pyridin-3-yl-methanone (**1b**)*

Prepared by above method from **4b** (4 mmol, 0.89 g) and **5** (4 mmol, 0.55 g) after 12 h reflux; Yield: 1.34 g, 98%; Light yellow amorphous solid. mp: 202–204 °C; HPLC Rt (min), purity: 10.90, 98.68%; ESI-MS (M + H): $C_{21}H_{18}N_3O_2$, *calcd*.: 344.1399, *found*: 344.0312; IR (KBr, cm^{-1}): 3420, 1685, 1599, 1502, 1270; ^1H NMR (DMSO-d_6, δ): 3.12 (1H, dd, $J = 17.9$ Hz, 5.2 Hz, 4-H_a), 3.85 (1H, dd, $J = 17.9$ Hz, 11.9 Hz, 4-H_b), 5.73 (1H, dd, $J = 11.5$ Hz, 5.1 Hz, 5-*H*), 6.82 (2H, d, $J = 8.4$ Hz, 14-*H* and 16-*H*), 7.25 (5H, m, 7-*H*, 8-*H*, 9-*H*, 10-*H* and 11-*H*), 7.56 (2H, d, $J = 8.1$ Hz, 13-*H* and 17-*H*), 7.53 (1H, m, 23-H), 8.24 (1H, d, $J = 7.8$ Hz, 24-*H*), 8.69 (1H, d, $J = 4.4$ Hz, 22-*H*), 9.04 (1H, s, 20-*H*), 10.04 (1H, s, O*H*).

*4.3.3. [3-(4-Hydroxy-phenyl)-5-o-tolyl-4,5-dihydro-pyrazol-1-yl]-pyridin-3-yl-methanone (**1c**)*

Prepared by above method from **4c** (4 mmol, 0.95 g) and **5** (4 mmol, 0.55 g) after 16 h reflux; Yield: 1.38 g, 97%; Light yellow amorphous solid. mp: 146–148 °C; HPLC Rt (min), purity: 11.40, 98.75%; ESI-MS (M + H): $C_{22}H_{20}N_3O_2$, *calcd*.: 358.1556, *found*: 358.0750; IR (KBr, cm^{-1}): 3153, 2800, 1657, 1597, 1506, 1277, 1160; ^1H NMR (DMSO-d_6, δ): 2.44 (3H, s, CH_3), 3.00 (1H, dd, $J = 17.8$ Hz, 4.8 Hz, 4-H_a), 3.89 (1H, dd, $J = 17.8$ Hz, 11.7 Hz, 4-H_b), 5.85 (1H, dd, $J = 11.6$ Hz, 4.7 Hz, 5-*H*), 6.81 (2H, d, $J = 8.1$ Hz, 14-*H* and 16-*H*), 7.06 (1H, m, 8-*H*), 7.16 (2H, m, 9-*H* and 10-*H*), 7.22 (1H, m, 11-*H*), 7.56 (2H, d, $J = 7.6$ Hz, 13-*H* and 17-*H*), 7.54 (1H, m, 23-H), 8.27 (1H, d,

J = 7.9 Hz, 24-*H*), 8.70 (1H, d, J = 4.6 Hz, 22-*H*), 9.06 (1H, s, 20-*H*), 10.03 (1H, s, O*H*).

4.3.4. [3-(4-Hydroxy-phenyl)-5-p-tolyl-4,5-dihydro-pyrazol-1-yl]-pyridin-3-yl-methanone (**1d**)

Prepared by above method from **4d** (4 mmol, 0.95 g) and **5** (4 mmol, 0.55 g) after 16 h reflux; Yield: 1.38 g, 97%; Straw yellow crystaline solid. mp: 188–190 °C; HPLC Rt (min), purity: 11.62, 98.56%; ESI-MS (M + H): $C_{22}H_{20}N_3O_2$, *calcd*. 358.1556, *found*: 358.5118; IR (KBr, cm^{-1}): 3435, 2993, 1645, 1598, 1507, 1286, 1241, 827; ^{1}H NMR (DMSO-d_6, δ): 2.27 (3H, s, CH_3), 3.09 (1H, dd, J = 17.9 Hz, 4.8 Hz, 4-H_a), 3.82 (1H, dd, J = 17.8 Hz, 11.7 Hz, 4-H_b), 5.68 (1H, dd, J = 11.3 Hz, 4.7 Hz, 5-*H*), 6.82 (2H, d, J = 8.1 Hz, 14-*H* and 16-*H*), 7.18 (4H, m, 7-*H*, 8-*H*, 10-*H* and 11-*H*), 7.51 (1H, m, 23-H), 7.54 (2H, d, J = 7.6 Hz, 13-*H* and 17-*H*), 8.22 (1H, d, J = 7.9 Hz, 24-*H*), 8.69 (1H, d, J = 4.6 Hz, 22-*H*), 9.02 (1H, s, 20-*H*), 10.04 (1H, s, O*H*).

4.3.5. [5-(4-Ethoxy-3-methoxy-phenyl)-3-(4-hydroxy-phenyl)-4,5-dihydro-pyrazol-1-yl]-pyridin-3-yl-methanone (**1e**)

Prepared by above method from **4e** (4 mmol, 1.19 g) and **5** (4 mmol, 0.55 g) after 24 hr reflux; Yield: 1.57 g, 95%; Purified by preparative HPLC; Bright yellow crystalline solid. mp: 198–200 °C; HPLC Rt (min), purity: 11.78, 98.69%; ESI-MS (M + H): $C_{24}H_{24}N_3O_4$, *calcd*. 418.1667, *found*: 418.2295; IR (KBr, cm^{-1}): 3066, 2977, 1595 (C═O), 1515, 1442, 1258, 1138, 1029, 836; ^{1}H NMR (DMSO-d_6, δ): 1.30 (3H, t, J = 6.84, CH_3), 3.14 (1H, dd, J = 13.5 Hz, 4.3 Hz, 4-H_a), 3.75(3H, s, OCH_3), 3.85 (1H, m, 4-H_b), 3.96 (2H, q, J = 6.8 Hz, OCH_2), 5.67 (1H, dd, J = 11.2 Hz, 4.2 Hz, 5-*H*), 6.82 (2H, d, J = 8.2 Hz, 14-*H*, 16-*H*), 6.78 (1H, m, 7-*H*), 6.89 (2H, m, 10-*H*, 11-*H*), 7.51 (1H, m, 23-*H*), 7.56 (2H, d, J = 8.2 Hz, 13-*H*, 17-*H*), 8.23 (1H, d, J = 6.6 Hz, 24-*H*), 8.72 (1H, s, 22-*H*), 9.03 (1H, s, 20-*H*), 10.03 (1H, s, O*H*).

4.3.6. [5-(2-Bromo-phenyl)-3-(4-hydroxy-phenyl)-4,5-dihydro-pyrazol-1-yl]-pyridin-3-yl-methanone (**1f**)

Prepared by above method from **4f** (4 mmol, 1.20 g) and **5** (4 mmol, 0.55 g) after 8 h reflux; Yield: 1.66 g, 98%; Offwhite solid. mp: 138–140 °C. HPLC Rt (min), purity: 11.64, 98.91%; ESI-MS (M + H): $C_{21}H_{17}BrN_3O_2$, *calcd.*: 422.0504, *found*: 422.2609; IR (KBr): 3185 (br), 1603, 1517, 1440, 1339, 1277; ^{1}H NMR (DMSO-d_6, δ): 3.02 (1H, dd, J = 17.8 Hz, 5.0 Hz, 4-H_a), 3.92 (1H, dd, J = 17.8 Hz, 1.8 Hz, 4-H_b), 5.92 (1H, dd, J = 11.8 Hz, 4.9 Hz, 5-*H*), 6.81 (2H, d, J = 8.6 Hz, 14-*H* and 16-*H*), 7.22 (2H, m, 9-*H* and 10-*H*), 7.34 (1H, m, 23-H), 7.53 (1H, m, 8-*H*), 7.55 (2H, d, J = 8.6 Hz, 13-*H* and 17-*H*), 7.67 (1H, d, J = 7.9 Hz, 11-*H*), 8.28 (1H, d, J = 7.9 Hz, 22-*H*), 8.71 (1H, d, J = 3.8 Hz, 24-*H*), 9.09 (1H, s, 20-*H*), 10.05 (1H, s, O*H*). ^{13}C NMR (DMSO-d_6, δ): 40.7 (2-*C*), 60.3 (3-*C*), 115.5 (12-*C*, 14-*C*), 115.6 (18-*C*), 121.1 (5-*C*), 121.5 (10-*C*), 123.1 (8-*C*), 128.4 (11-*C*, 15-*C*), 128.8 (17-*C*), 129.4 (9-*C*), 130.3 (7-*C*), 133.0 (6-*C*), 137.2 (4-*C*), 140.3 (19-*C*), 150.1(20-*C*), 151.3(22-*C*), 156.1 (1-*C*), 159.9 (13-*C*), 163.1 (16-*C*).

4.3.7. [3-(4-Hydroxy-phenyl)-5-(3,4,5-trimethoxy-phenyl)-4,5-dihydro-pyrazol-1-yl]-pyridin-3-yl-methanone (**1g**)

Prepared by above method from **4g** (4 mmol, 1.25 g) and **5** (4 mmol, 0.54 g) after 24 h reflux; Yield: 1.68 g, 97%; Dark brown solid. mp: 184–186 °C; HPLC Rt (min), purity: 11.87, 98.83%. ESI-MS (M + H): $C_{24}H_{24}N_3O_5$, *calcd*. 434.1716, *found*: 434.2260; IR (KBr): 3193, 1596, 1509, 1454, 1329, 1281, 1238; ^{1}H NMR (DMSO-d_6, δ): 3.15 (1H, dd, J = 17.9 Hz, 5.0 Hz, 4-H_a), 3.63 (3H, s, *p*-OCH_3), 3.72 (6H, s, *m*-OCH_3), 3.83 (1H, dd, J = 11.7 Hz, 14.6 Hz, 4-H_b), 5.65 (1H, dd, J = 11.4 Hz, 4.9 Hz, 5-*H*), 6.57 (2H, s, 7-*H* and 11-*H*), 6.81 (2H, d, J = 8.6 Hz, 14-*H* and 16-*H*), 7.54 (2H, d, J = 8.5 Hz, 13-*H* and 17-*H*), 7.64 (1H, m, 23-H), 8.35 (1H, d, J = 7.7 Hz, 24-*H*), 8.75 (1H, s, 22-*H*), 9.08 (1H, s, 20-*H*), 10.09 (1H, s, O*H*). ^{13}C NMR (DMSO-d_6, δ): 42.1(2-*C*), 55.9 (m-OCH_3), 60.0 (p-OCH_3), 60.7 (3-*C*), 102.6 (5-*C*,9-*C*), 115.7 (12-*C*, 14-*C*), 121.6 (10-*C*), 124.0 (18-*C*), 128.9 (17-*C*), 131.3 (11-*C*, 15-*C*), 136.5 (7-*C*), 137.8 (4-*C*), 138.7 (19-*C*), 148.7 (20-*C*), 149.9 (6-*C*, 8-*C*), 153.2 (22-*C*), 156.6, 159.9 (13-*C*) and 162.9 (16-*C*).

4.3.8. [5-(5-Bromo-2-methoxy-phenyl)-3-(4-hydroxy-phenyl)-4,5-dihydro-pyrazol-1-yl]-pyridin-3-yl-methanone (**1h**)

Prepared by above method from **4h** (4 mmol, 1.32 g) and **5** (4 mmol, 0.54 g) after 18 h reflux. Yield; 1.73 g, 96%; Brickred-yellow solid. mp: 140–142 °C; HPLC Rt (min), purity: 12.24, 98.12%; ESI-MS (M + H): $C_{22}H_{19}BrN_3O_3$, *calcd.*: 452.0610, *found*: 452.4197; IR (KBr, cm^{-1}): 3183, 2946, 1651, 1600, 1482, 1286; ^{1}H NMR (DMSO-d_6, δ): 3.03 (1H, dd, J = 17.8 Hz, 5.0 Hz, 4-H_a), 3.83 (3H, s, OCH_3), 3.86 (1H, m, 4-H_b), 5.79 (1H, dd, J = 11.6 Hz, 4.9 Hz, 5-*H*), 6.81 (2H, d, J = 8.6 Hz, 14-*H* and 16-*H*), 7.04 (1H, d, J = 8.7 Hz, 8-*H*), 7.17 (1H, d, J = 2.0 Hz, 11-*H*), 7.34 (1H, m, 23-H), 7.43 (1H, dd, J = 8.7 Hz, 2.3 Hz, 9-*H*), 7.55 (2H, d, J = 8.4 Hz, 13-*H* and 17-*H*), 8.25 (1H, d, J = 7.8 Hz, 24-*H*), 8.70 (1H, d, J = 3.7 Hz, 22-*H*), 9.04 (1H, s, 20-*H*), 10.05 (1H, s, O*H*).

4.3.9. [5-(3-Bromo-phenyl)-3-(4-hydroxy-phenyl)-4,5-dihydro-pyrazol-1-yl]-pyridin-3-yl-methanone (**1i**)

Prepared by above method from **4i** (4 mmol, 1.21 g) and **5** (4 mmol, 0.55 g) after 8 h reflux; Yield: 1.65 g, 98%; Off white amorphous solid. mp: 144–146 °C; HPLC Rt (min), purity: 12.09, 98.43%. ESI-MS (M + H): $C_{21}H_{17}BrN_3O_2$, *calcd.*: 422.0504, *found*: 422.0550; IR (KBr, cm^{-1}): 3202, 1608, 1519, 1476, 1281, 1198;. ^{1}H NMR (DMSO-d_6, δ): 3.24 (1H, dd, J = 18.1 Hz, 5.1 Hz, 4-H_a), 3.90 (1H, dd, J = 18.0 Hz, 11.7 Hz, 4-H_b), 5.91 (1H, dd, J = 11.6 Hz, 5.1 Hz, 5-*H*), 6.83 (2H, d, J = 8.7 Hz, 14-*H* and 16-*H*), 7.53 (1H, m, 23-H), 7.58 (2H, d, J = 8.5 Hz, 13-*H* and 17-*H*), 7.69 (1H, m, 10-*H*), 7.79 (1H, d, J = 7.7 Hz, 11-*H*), 8.15 (1H, d, J = 8.1 Hz, 9-*H*), 8.19 (1H, s, 7-*H*), 8.26 (1H, d, J = 7.8 Hz, 24-*H*), 8.70 (1H, d, J = 4.5 Hz, 22-*H*), 9.05 (1H, s, 20-*H*), 10.08 (1H, s, O*H*).

4.3.10. [3-(4-Hydroxy-phenyl)-5-(3-nitro-phenyl)-4,5-dihydro-pyrazol-1-yl]-pyridin-3-yl-methanone (**1j**)

Prepared by above method from **4j** (4 mmol, 1.07 g) and **5** (4 mmol, 0.54 g) after 16 h reflux; Yield: 1.47 g, 95%; Brown amorphous solid. mp: 230–232 °C; HPLC Rt (min), purity: 10.59, 98.37%; ESI-MS (M + H): $C_{21}H_{17}N_4O_4$, *calcd.*: 389.1250, *found*: 389.1574. IR (KBr, cm^{-1}): 3202, 1604, 1525, 1442, 1344, 1277, 1094; ^{1}H NMR (DMSO-d_6, δ): 3.29 (1H, dd, J = 18.0 Hz, 5.1 Hz, 4-H_a), 3.95 (1H, dd, J = 18.0 Hz, 11.7 Hz, 4-H_b), 5.96 (1H, dd, J = 11.5 Hz, 5.0 Hz, 5-*H*), 6.89 (2H, d, J = 8.5 Hz, 14-*H* and 16-*H*), 7.58 (1H, m, 23-H), 7.63 (2H, d, J = 8.5 Hz, 13-*H* and 17-*H*), 7.74 (1H, m, 10-*H*), 7.84 (1H, d, J = 7.6 Hz, 11-*H*), 8.19 (1H, d, J = 8.1 Hz, 9-*H*), 8.24 (1H, s, 7-*H*), 8.31 (1H, d, J = 7.8 Hz, 24-*H*), 8.75 (1H, d, J = 3.7 Hz, 22-*H*), 9.11 (1H, s, 20-*H*), 10.15 (1H, s, O*H*).

4.4. In vitro antimalarial assay

P. falciparum strains MRC-02 (CQ sensitive) and RKL9 (CQ resistant) were obtained from National Institute of Malaria Research, New Delhi, India and maintained in a continuous culture using the standard method described by Trager and Jensen [33]. Parasites were cultured in human B (+) erythrocytes in RPMI-1640 media (GIBCO-BRL, Paisely, Scotland) supplemented with 25 mM HEPES buffer, 10% human AB (+) serum and 0.2% sodium bicarbonate (Sigma) and maintained at 5% CO_2. Cultures containing predominantly early ring stages were synchronized by addition of 5% D-sorbitol (Sigma) lysis

[34], used for testing. Initial culture was maintained in small vials with 10% haematocrit, i.e. 10 μl erythrocytes containing 1.0% ring stage parasite in 100 μl complete media. The culture volume per well for the assay was 100 μl. Number of parasites for the assay was adjusted at 1–1.5% by diluting with fresh human B (+) RBC. Assay was done in 96 well microtitre flat-bottomed tissue culture plates incubated at 37 °C for 24 h in presence of two fold serial dilutions of compounds and chloroquine diphosphate for their effect on schizont maturation. Compounds were dissolved in ethanol and further diluted with RPMI 1640 medium (the final ethanol concentration did not exceed 0.5% which did not affect parasite growth). Chloroquine diphosphate was dissolved in aqueous medium. Test was done in duplicate wells for each dose of the drugs. Solvent control culture containing the same concentrations of the solvent as present in the test wells was done with RPMI-1640 containing 10% AB (+) serum. Parasite growth was found unaffected at the solvent concentrations. Growth of the parasites from duplicate wells of each concentration was monitored in Giemsa stained blood smears by counting number of schizont per 100 asexual parasites. Percent schizont maturation inhibition was calculated by the formula: (1-Nt/Nc) × 100 where, Nt and Nc represent the number of schizont in the test and control well respectively.

4.5. In vivo antimalarial assay

The in vivo efficacy was determined by Peter's 4-Day suppressive test [35] by using *P. berghei* ANKA strain and male white swiss albino mice (18–20 gm of body weight). In brief, groups of five mice each were inoculated intraperitonially with approximately 30% of *P. berghei* infected erythrocytes from a donor mouse. Four hours latter, selected test compounds were administered at a dose of 50 mg/kg/day and 25 mg/kg/day by oral route. Chloroquine (8 mg/kg/day) was taken as standard for comparison. A total of four doses were given on day 0, day 1, day 2 and day 3. Control groups received the same amount of solvent used to suspend the compounds. The tail blood smears were made on day 4 and day 7 stained with Giemsa and examined microscopically. The % parasitemia was determined and average %suppression of parasitemia in comparison to control groups. Mean survival time (MST) was calculated for the mice died during the 28 day observation period.

4.6. In vitro cytotoxicity assay

HeLa (Human cervical epithelial) cells obtained from National Centre for Cell Sciences (NCCS), Pune, India were maintained in our laboratory. Cells were routinely maintained at 37 °C in Eagles Minimum Essential Medium (EMEM, Sigma, USA) supplemented with foetal bovine serum (10%), L-glutamine (2 mM), and gentamicine (80 mg L^{-1}). Cytotoxicity was assessed using the neutral red (NR) dye uptake assay [36] using 96 well microtiter tissue (MT) culture plates (Greiner, Germany). Briefly, 2×10^3 cells were seeded in to each well of a 96 well MT plate and incubated overnight at 37 °C in a humidified atmosphere containing 5% CO_2. All the compounds were dissolved in DMSO and subsequently serial two fold dilution were prepared in EMEM to give a broad range of concentration. Prior to assessment of cytotoxicity, the preformed cell monolayer were washed with EMEM and replaced with drug solutions in duplicate keeping cell control including corresponding dilution of DMSO. The plates were incubated at 37 °C and observed microscopically daily for appearance of any morphological changes. Following 72 h incubation, the cells were fixed with 5% glutaraldehyde solution prepared in PBS, kept at room temperature for 45 min. The fixed cells were washed with PBS, stained with 0.2% NR dye aqueous solution and incubated for 1 h at room temperature. After washing the stained cells were subjected to acid alcohol lysis by adding 100 μL of 0.5% acetic acid (V/V) in 50% ethanol to each well followed by 10 min incubation. Absorbances of the resulting solutions were read at 550 nm and cell survival was calculated as the absorbance of the treated cells divided by the control (EMEM plus DMSO). Results were expressed in terms of the MNTD (maximum non toxic dose) values i.e the maximum concentration of drug where 100% cell survival observed.

4.7. Determination of pK_a values

Acid dissociation constants (pK_as) were determined spectrophotometrically as previously described [37] by using a Unicam UV 300 spectrometer of Thermo Spectronic. Absorbances of each compound (0.025 mg mL^{-1})dissolved in buffers of different pH values ranging from 5.8 to 11.2 as well as in 0.1 M NaOH and 0.1 M HCL were determined. Buffers from pH 5.8–8.0 were prepared from 0.1 M solutions of monobasic potassium phosphate (KH_2PO_4) and dibasic potassium phosphate (K_2HPO_4) and from pH 8.4–11.2 were prepared from 0.1 M glycine and 0.1 M NaOH. Analytical wavelengths (212 nm, 240 nm, 270 nm and 340 nm) were chosen at which significant difference between the absorbances of molecules in 0.1 M HCl and 0.1 M NaOH were observed. To reduce error, pK_a was determined in duplication for each compound.

4.8. Determination of inhibition of β-hematin formation

The quantitative BHIA (β-hematin inhibitory activity) assay is based on the differential solubility of hematin and β-hematin in DMSO and NaOH solution, respectively [38,39]. The method determines a 50% inhibitory concentration for β-hematin formation inhibition ($BHIA_{50}$) of the compound. In a micro centrifuge tube, 100 μL of 6.4 mM solution of hematin freshly dissolved in 0.2 N NaOH solution; 50 μL of compound dissolved in ethanol (chloroquine diphosphate was dissolved in water); 200 μL of 3 M solution sodium acetate trihydrate ($C_2H_3NaO_2.3H_2O$); 50 μL of glacial acetic acid were incubated at 37 °C for 24 h. Concentration of compounds were varied from 6 mM to 0.3 mM range. Water and ethanol were taken as controls for water soluble and ethanol soluble compounds respectively. After incubation, the tubes were centrifuged for 15 min at 3300*g*. The supernatant was discarded and the pellet was reconstituted in DMSO and again centrifuged for 15 min at 3300*g*. The supernatant was discarded and β-hematin was obtained as a pellet. Following centrifugation to isolate DMSO insoluble β-hematin, the pellet was dissolved in 0.1 N NaOH and absorbance was recorded at 405 nM to calculate $BHIA_{50}$ as previously described [39].

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Appendix. Supplementary data

Supplementary information related to this article can be found at doi:10.1016/j.ejmech.2009.10.023.

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