Full Paper

Synthesis and Biological Evaluation of Some Pyrazole Derivatives as Anti-Malarial Agents

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Novel series of pyrazole derivatives were synthesized and tested for their *in vivo* anti-malarial activity using mice infected with chloroquine sensitive *P. berghei* at a dose level of 50 μmol/kg. The most active compounds were further tested *in vitro* against chloroquine resistant (RKL9) strain of *P. falciparum*. The *in vivo* anti-malarial activity study indicated that compounds **2a, 2b, 8a** and **8b** had mean percent suppression of 85%, 83%, 95% and 97%, respectively at equimolar dose level of the standard drug chloroquine diphosphate. Moreover, compounds **2a, 2b, 8a** and **8b** showed *in vitro* IC_{50} values lower ($p < 0.05$) than that of the standard drug chloroquine phosphate (0.188 ± 0.003 μM) using the RKL9 strain. Compound **8b** was the most active with IC_{50} of 0.033 ± 0.014 μM. Generally, among the tested compounds, those containing a free carboxylic acid functional group on the pyrazole ring were the most active and this finding was supported by the docking results performed for the active compounds. The acute toxicity studies of the active compounds revealed that they have a good safety profile.

Keywords: Acute toxicity / Anti-malarial activity / Docking / *P. berghei* / *P. falciparum* / Pyrazoles

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Introduction

Malaria continues to be a global serious health problem. According to the WHO 2009 malaria report, half of the world population is at risk of malarial infection. An estimated 1 million deaths caused by malaria occurred in the year 2009 among which 89% were in the African region [1]. Despite the introduction of a large number of chemotherapeutic agents for the treatment of malaria there is still an unmet medical need for new effective drugs due to the emergence of resistance for current available anti-malarial drugs. This type of resistance has been reported for almost all the therapeutic agents approved for the treatment of malaria which could lead to the situation where no effective drug for treating this serious health problem becomes available [2, 3]. Therefore, the search for new and effective anti-malarial drugs remains a challenging quest for medicinal chemists.

The present work was carried out in an attempt to find new effective anti-malarial drugs among newly synthesized pyrazole derivatives series. Pyrazole derivatives were chosen because they were found to have a wide range of pharmacological activities, including anti-inflammatory [4, 5], antimicrobial [6, 7], antiviral [8] and antitumor activity [9, 10], and because they are versatile to be synthesized.

Furthermore, several reports have shown that pyrazoles have significant antimalarial activities. For instance, chloroquine-pyrazole analogues (Fig. 1) have been reported to exhibit a significant *in vitro* anti-malarial activity using an experimental model against *P. falciparum* [11, 12]. Ethyl 3-amino-5-phenylamino-4*H*-pyrazole-4-carboxylate showed a potent antimalarial activity ($IC_{50} = 1.32$ μmol/L) when tested *in vitro* against *P. falciparum* [13]. Similarly, some aryl substituted pyrazole derivatives such as methyl 5-amino-3-anisidinylpyrazole-4-carboxylic acid ($IC_{50} = 0.149$ μmol/L) [14]. There are also other reports that demonstrated the antimalarial activities of pyrazole derivatives [15, 16]. Hence, this

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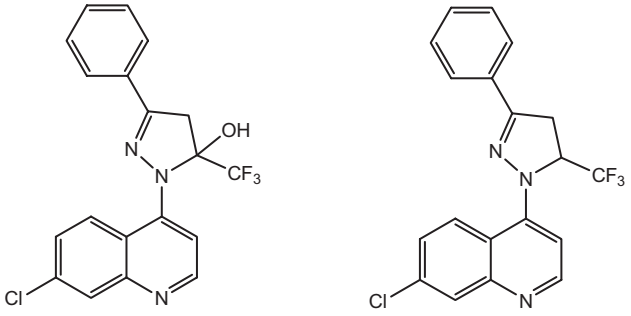


Figure 1. Reported antimalarial pyrazole derivatives.

work aims to synthesize new pyrazole derivatives and to study the effect of the molecular variation among these compounds on their anti-malarial activity.

Results and discussion

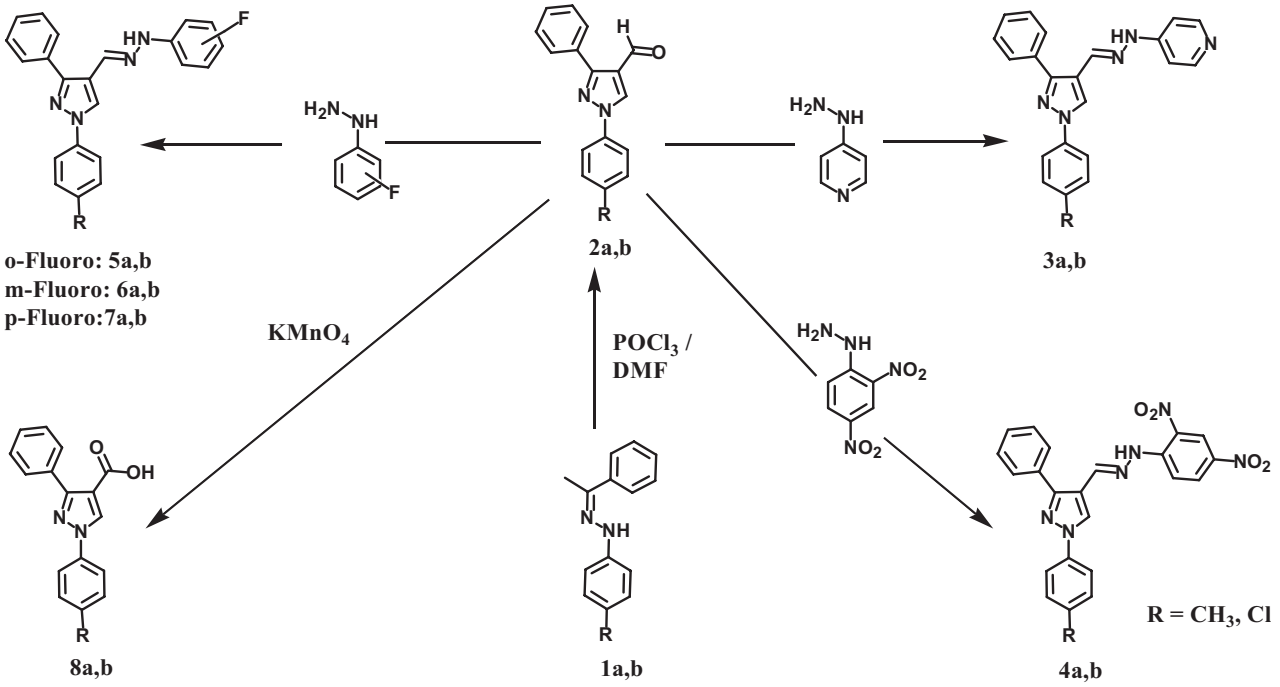
Chemistry

The target compounds were synthesized according to the steps outlined in Scheme 1. Initially the hydrazones **1a** and **1b** were subjected to Vilsmeyer Haack reaction, to yield the corresponding pyrazole aldehydes **2a**, and **2b**. The structures of the obtained aldehydes were confirmed by IR spectra that showed characteristic bands at 2726, 2669 and 1670 cm^{-1} that were attributed to the C–H and C=O stretching vibration of the aldehydic group, respectively. Moreover, ^{1}HNMR spectrum of these two compounds revealed two singlets at δ 8.55 and 10.1 that were assigned to pyrazole-C_5H and aldehydic proton. Pyrazole aldehyde **2a** and **2b** were condensed with the primary amine functional group of hydrazine derivatives, namely isonicotinic hydrazide, 2,4-dinitrophenylhydrazine and fluorophenylhydrazine derivatives in the presence of few drops of hydrochloric acid as a catalyst to give the corresponding hydrazone derivatives **3–7**. Structures of the synthesized hydrazone derivatives were proved by the absence of the aldehydic characteristic bands at 2726, 2669 and1670 cm^{-1} in their IR spectra. In addition, the absence of the singlet aldehydic peak of the starting aldehydes at δ 10.1 in their ^{1}HNMR spectrum was taken as an indication of the formation of these compounds. Oxidation of aldehydes **2a** and **2b** by $KMnO_4$ afforded the corresponding carboxylic acid derivatives **8a** and **8b**. The latter showed a characteristic IR broad band at 3170–2520 assigned for COOH stretching vibration and a sharp band at 1700–1702 attributed to C=O stretching vibration. Their ^{1}HNMR spectra showed broad D_2O exchangeable singlet at δ 12.72–12.76 characteristic for COOH.

Biological activity

In vivo anti-malarial activity

Results for the *in vivo* anti-malarial activity tests of the synthesized compounds are shown in Table 1. All the tested compounds, except **3b**, **4a**, and **6a**, showed parasitaemia suppression in mice above 50% compared to those which



Scheme 1. Sythesis of intermediate and target compounds.

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Table 1. *In vivo* antimalarial activity of the target compounds **2–8**.

Comp. No.	% Parasitaemia	% Suppression	Mean survival time (days)
2a	12 ± 1.7	85	8.6 ± 0.3
2b	13 ± 0.3	83	9.8 ± 0.6
3a	18 ± 2.3	78	13.0 ± 0.4
3b	75 ± 4.5	4	11.4 ± 0.8
4a	45 ± 4.1	43	6.6 ± 0.6
4b	18 ± 0.4	78	9.2 ± 0.5
5a	25 ± 1.6	69	6.9 ± 0.2
5b	37 ± 2.3	54	6.1 ± 0.6
6a	42 ± 2.8	46	6.8 ± 0.2
6b	39 ± 1.8	51	6.3 ± 0.7
7a	39 ± 1.8	53	7.3 ± 0.2
7b	38 ± 2.6	52	6.8 ± 0.3
8a	4 ± 0.4	95	>14
8b	2 ± 0.3	97	>14
Control	79 ± 1.6	0.0	5.8 ± 0.4
Chloroquine	0.0	100	>14

were treated with the standard drug chloroquine. Compounds **8b**, **8a**, **2a**, **2b**, **3a**, and **4b** exhibited the highest activity with percent suppression >75% (97%, 95%, 85%, 83%, 78% and 78%, respectively).

Among the initially tested compounds, those that contained free aldehydic functionality on the pyrazole nucleus **2a** and **2b** generally showed higher *in vivo* anti-malarial activity. This was probably due to stronger binding of these groups to receptor backbone compared to hydrazone analogues since such groups serve as better hydrogen donors and acceptors. This suggestion was supported by the docking results obtained for these molecules which demonstrated that while the aldehydic derivatives **2a** and **2b** exhibited strong hydrogen bonding with the backbone of dihydrofolate reductase enzyme, the hydrazone derivatives undergo fewer and weaker interactions. The activity of compound **2a** was slightly higher than that of compound **2b,** which may be due to the *p*-tolyl group which makes the aldehydic group suited to form stronger hydrogen bond by the steric effect of the methyl group.

By realizing the possible *in vivo* metabolism of the aldehydic group to its corresponding carboxylic acid, we synthesized and tested the biological activity of compounds **8a** and **8b** and an excellent anti-malarial activity was observed which was more pronounced than that found with the two aldehydic derivatives **2a** and **2b**. This finding was also rationalized by performing molecular docking for these compounds where compound **8a** was shown to form six hydrogen bonds and some hydrophobic interaction while compound **8b** formed four hydrogen bonds and few hydrophobic interactions with the receptor backbone. This finding is quite interesting because of the possible long term toxicity of aldehyde derivatives which have very reactive nature and may undergo many nucleophilic reactions with vital electrophiles present in the body [17, 18]. These side effects can be avoided by preparing their carboxylic acid analogous, such as in **8a** and **8b**.

In vitro antiplasmodial activity

The most active compounds **2a, 2b, 3a**, **4b, 8a** and **8b,** which showed reasonable *in vivo* activity, were further examined for their antiplasmodial activities against chloroquine resistant (RKL9) strain parasite (Table 2). All the tested compounds showed better activity than chloroquine diphosphate (IC_{50} = 0.188 μM) against chloroquine resistant (RKL9) strain parasite (Table 2). Compound **8b** was found to be the most potent against RKL9 strain (IC_{50} = 0.033 μM) and this may be attributed to the Cl group at the *para*-position which makes the compound more lipophilic and increases the tissue penetration power.

In vivo acute toxicity study

Compounds **2a, 2b, 3a, 4b, 8a** and **8b** were further evaluated for their oral acute toxicity in male mice using a previously reported method [18, 19]. The results indicated that the tested compounds were non-toxic and well tolerated by experimental animals up to 300 mg/kg. Moreover, these compounds were tested for their toxicity through the parenteral route and the results revealed that all test compounds were non-toxic up to 100 mg/kg.

Docking

Molecular docking studies further helped in understanding the various interactions between the ligands and enzyme active sites. The determination of the three dimensional co-crystal structure of dihydrofolate reductase (PDB ID: 1J3I), complexed with a selective inhibitor, WR99210 (Fig. 2) led to the development of a model for the topography of the anti-malarial drugs binding site in dihydrofolate reductase enzyme. This compound WR99210 displayed hydrogen bond interactions with Ile 14, Asp 54 and Ile 164, in addition to hydrophobic interactions with Ile 14, Cys 15, Ala 16, Asp 54, Met 55, Phe 58 and Pro 113.

Table 2. *In vitro* antiplasmodial activity against chloroquine resistant (RKL9) strain of *P. falciprum*

Compound	IC_{50}, μM ± SD*
2a	0.051 ± 0.003
2b	0.041 ± 0.004
3a	0.076 ± 0.011
4b	0.068 ± 0.130
8a	0.036 ± 0.018
8b	0.033 ± 0.014
Chloroquine	0.188 ± 0.003

* Results of two separate determinations

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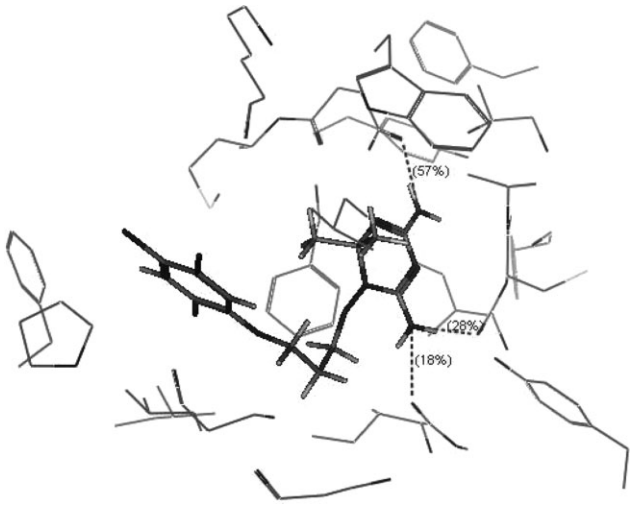

Figure 2. 3D view from a molecular modeling study, of the minimum-energy structure of the complex of WR99210 docked in DHFR (PDB ID: 1J3I). Viewed using Molecular Operating Environment (MOE) module.

The result of the docking study for compound **2a** showed that the free aldehydic group of the compound exhibited a very strong hydrogen bonding interaction with Ser111 (Fig. 3). In addition, the compound showed hydrophobic interaction with Phe 58, Phe 116, Met 55, and hydrogen bonding with Ser 108. The binding mode of compound **4b** was not different from compound **2a** (Fig. 4). Compound **4b** showed a relatively strong hydrogen bonding with Ser 111.

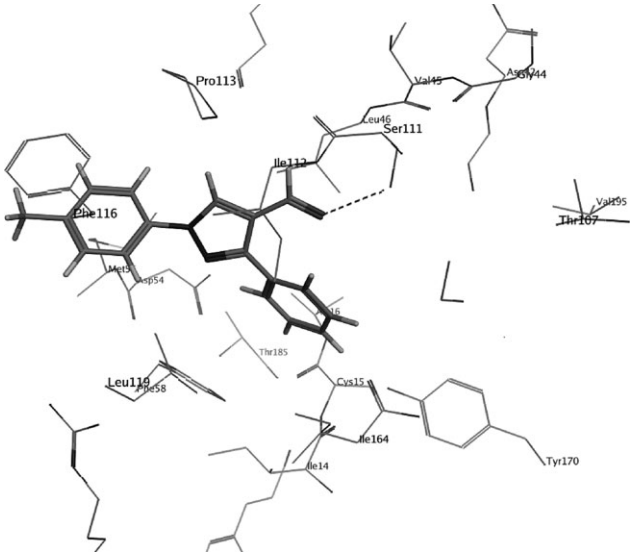

Figure 3. 3D view from a molecular modeling study of the minimum-energy structure of the complex of **2a** docked in DHFR (PDB ID: 1J3I). Viewed using Molecular Operating Environment (MOE) module.

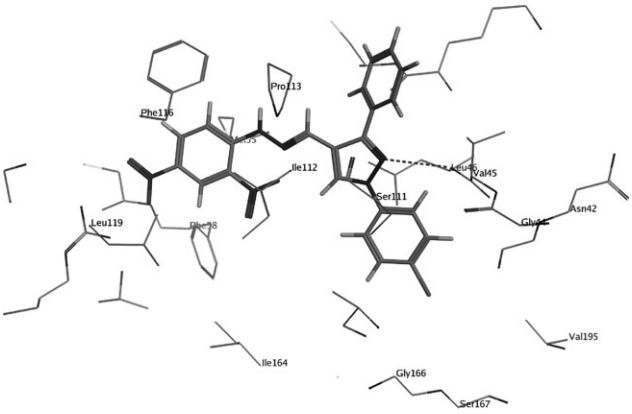

Figure 4. 3D view from a molecular modeling study of the minimum-energy structure of the complex of **4a** docked in DHFR (PDB ID: 1J3I). Viewed using Molecular Operating Environment (MOE) module.

Also it showed hydrophobic interactions with Phe 116, Met 55 and Ser 108, Tyr 170, respectively.

The molecular docking studies revealed that compounds **8a** and **8b** exhibited the strongest interaction with the target enzyme. Compound **8a** undergoes six hydrogen bonds with Ala 16, Ala 16, Leu 40, Tyr 170, Ala 16 and Tyr 170 and weak hydrophobic interactions with Cys 15, Ala 16, Leu 40, Leu 46, Met 55, Gly 166 and Ser 167 (Fig. 5). Similarly, compound **8b** binds with backbone of the receptor of the target enzyme by strong hydrogen bonding with Ile 164, Tyr 170, Ser 111 and Tyr 170 and weak hydrophobic interactions with Leu 40, Gly 44, Val 45, Leu 46, Ile 112 and Tyr 170 (Fig. 6).

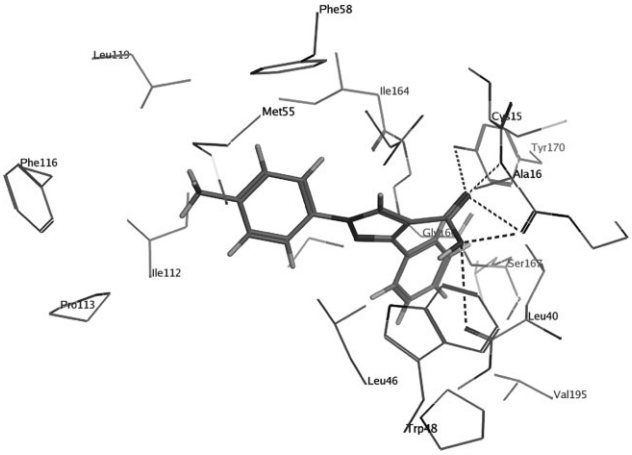

Figure 5. 3D view from a molecular modeling study of the minimum-energy structure of the complex of **8a** docked in DHFR (PDB ID: 1J3I). Viewed using Molecular Operating Environment (MOE) module.

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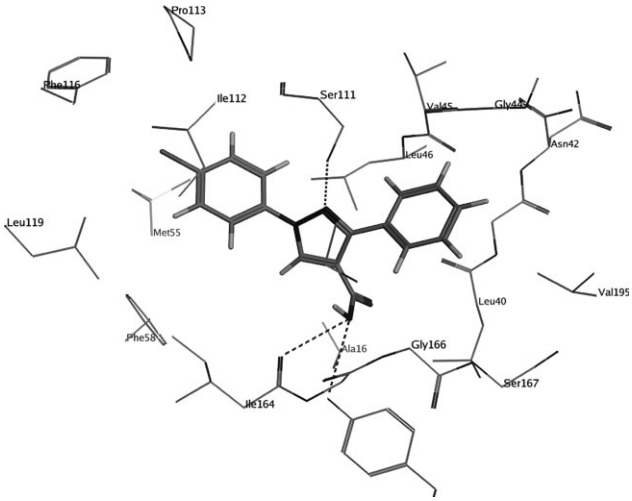

Figure 6. 3D view from a molecular modeling study of the minimum-energy structure of the complex of **8b** docked in DHFR (PDB ID: 1J3I). Viewed using Molecular Operating Environment (MOE) module.

In general the result from molecular docking for the active compounds supports the findings and the biological activities obtained from the *in vivo* and *in vitro* experimental models. Both the *in vivo* and *in vitro* anti-malarial activity study and docking results revealed that the most active compounds were those that contained free carboxylic acid group attached to the pyrazole functionality **8a** and **8b**.

Conclusion

Compounds **2a**, **2b**, **8a** and **8b** showed appreciable *in vivo* anti-malarial activity against chloroquine sensitive *P. berghei* in mice and excellent *in vitro* antiplasmodial activity against chloroquine resistant (RKL9) strain of *P. falciparum*. Among all the synthesized compounds, the two pyrazole derivatives which contained free carboxylic acid functional group **8a** and **8b** showed the highest anti-malarial activity. Docking studies for both **8a** and **8b** with DHFR (PDB ID: 1J3I) showed good binding profile. The results of acute toxicity studies for the most active compounds revealed that these compounds have a good safety margin. Therefore, compounds **8a** and **8b** would represent a fruitful matrix for the development of a new class of anti-malarial agents that would deserve further investigation and derivatization.

Experimental

General procedures

Melting points were determined in open glass capillaries using electro thermal BUCHI (B-540) hot storage melting-point apparatus and are uncorrected. The infrared (IR) spectra were recorded on FTIR-8400 Shimadzu infrared spectrophotometer using Nujol mules or KBr plates. ^{1}HNMR spectra was recorded on BRUKER Avance DMX400 400-MHz FT-NMR spectrometer using one or two of the following solvents DMSO-d6, $CDCl_3$ and MeOD and the chemical shifts are given in δ (ppm) downfield from tetramethylsilane (TMS) which served as an internal standard. Splitting patterns were designated as follows: s: singlet; d: doublet; m: multiplet. Elemental analyses was performed on Perkin Elmer 2400 elemental analyzer and values obtained were within ±0.4% of the theoretical values. Follow up of the reactions and checking the purity of the compounds was made by thin layer chromatography (TLC) on silica gel-precoated aluminum sheets (Type 60 GF_{254}, Merck) and the spots were detected by exposure to UV-lamp at λ = 254nm and/or iodine chamber. Parasites were counted using BIO-PLUS microscope.

Chemistry

*Synthesis of 3-phenyl-1-(p-tolyl)-1H-pyrazole-4-carboxaldehyde **(2a)** and 1-(4-chlorophenyl)-3-phenyl-1H-pyrazole-4-carboxaldehyde **(2b)***

Phosphorous oxychloride (2.42 g, 15.8 mmol) was added dropwise to an ice cold dimethylformamide (6.54 g, 6.93 mL, 89.5 mmol) with continuous stirring over a period of 45 min. 1-(1-Phenylethylidene)-2-(*p*-tolyl) hydrazine (1.5 g, 6.88 mmol) or 2-(4-chlorophenyl)-1-(1-phenylethylidene) hydrazine (1.7 g, 6.88 mmol) was then added and the reaction mixture was allowed to attain room temperature. The mixture was then heated at 70°C for 2 hours, allowed to cool and poured onto crushed ice (55 g) and water (100 ml). Finally the mixture was boiled and the copious white precipitate obtained after cooling was filtered, dried and recrystallized from methanol as white needles.

Compound **2a**, yield % = 76, m.p. = 120–121°C, microanlysis for $C_{17}H_{14}N_2O$ (262.31), calculated C% = 77.84, H% = 5.38, N% = 10.68, Found C% = 77.47, H% = 5.61, N% = 10.92. IR (cm^{-1}): 1670 (C=O), 1610 (C=N). ^{1}H-NMR ($CDCl_3$): δ 2.50 (s, 3H, CH_3), 7.31–7.63 (m, 5H, phenyl-H), 7.75 (d, J = 8.0 Hz, 2H, tolyl-$C_{3,5}$H), 7.85 (d, J = 8.0 Hz,2H, tolyl-$C_{2,6}$H), 8.55 (s, 1H, pyrazole-C_5H), 10.1 (s, 1H, CHO).

Compound **2b**, yield % = 71, m.p. = 134–136°C, microanlysis for $C_{16}H_{11}ClN_2O$ (282.72), calculated C% = **67.97**, H% = 3.92, N% = 9.91, Cl% = 12.54, Found C% = 68.22, H% = 3.75, N% = 10.21, Cl% = 12.32. IR (cm^{-1}): 1670 (C=O), 1600 (C=N). ^{1}H-NMR ($CDCl_3$): δ 7.45–7.55 (m, 5H, phenyl-H), 7.82 (d, J = 7.8 Hz, 2H, chlorophenyl-$C_{3,5}$H), 7.85 (d, J = 7.8 Hz, 2H, chlorophenyl-$C_{2,6}$H), 8.52 (s, 1H, pyrazole-C_5H), 10.11 (s, 1H, CHO).

*Synthesis of N′-[3-phenyl-1-(4-methylphenyl)-1H-pyrazol-4-yl)methylene]isonicotinohydrazide **(3a)** and N′-[1-(4-chlorophenyl)-3-phenyl-1H-pyrazol-4-yl)-methylene]isonicotinohydrazide **(3b)***

Three drops of HCl were added to a solution of the selected aldehyde **2a** or **2b** (2 mmol) and isonicotinic hydrazide (2 mmol, 0.27 g) in 25 mL of ethanol. The mixture was heated under reflux for 7 h with stirring. The reaction mixture was then cooled and the yellow precipitate formed was filtered, washed with ethanol, dried and recrystallized from methanol/water (1:1) for **2a** and from ethanol/water (1:1) for **2b**.

Compound **3a**, yield % = 72, m.p. = 212–215°C, microanlysis for $C_{23}H_{20}N_4$ (352.43), calculated C% = 78.38, H% = 5.72,

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N% = 15.90, Found C% = 78.51, H% = 4.29, N% = 16.92. IR (cm^{-1}): 3120 (NH), 1610 (C=N). ^{1}H-NMR ($CDCl_3$): δ 2.50 (s, 3H, CH_3), 7.32–7.53 (m, 5H, phenyl-H), 7.55 (d, J = 7.8 Hz, 2H, tolyl-$C_{3,5}$H), 7.82 (d, J = 7.8 Hz, 2H, tolyl-$C_{2,6}$H), 7.85 (d, J = 6.1 Hz, 2H, pyridine-$C_{2,6}$H), 8.45 (s, 1H, pyrazole-C_5H), 8.71 (d, J = 6.1 Hz, 2H, pyridine-$C_{3,5}$H) 8.85 (s, 1H, CH).

Compound **3b**, yield % = 74, m.p. = 265–266°C, microanlysis for $C_{22}H_{17}ClN_4$ (372.85), calculated C% = 70.8, H% = 4.60, N% = 15.03, Cl% = 9.51, Found C% = 70.54, H% = 4.86, N% = 14.74, Cl% = 9.28. IR (cm^{-1}): 3150 (NH), 1612 (C=N). ^{1}H-NMR ($CDCl_3$): δ 6.72–6.94 (m, 5H, phenyl-H), 7.05 (d, J = 8.1 Hz, 2H, chlorophenyl $C_{3,5}$H), 7.15 (d, J = 6.1 Hz, 2H, pyridine-$C_{2,6}$H), 7.20 (d, J = 8.1 Hz, 2H, chlorophenyl $C_{2,6}$H), 7.80 (s, 1H, pyrazole -C_5H), 8.05 (d, J = 6.1 Hz, 2H, pyridine-$C_{3,5}$H) 8.25 (s, 1H, CH).

Synthesis of 2-(2,4-dinitrophenyl)-1-{[3-phenyl-1-(4-methylphenyl)-1H-pyrazol-4-yl]methylene}hydrazine ***(4a)*** *and 2-(2,4-dinitrophenyl)-1-{[1-(4-chlorophenyl)-3-phenyl-1H-pyrazol-4yl]methylene}-hydrazine* ***(4b)***

Three drops of HCl were added to a solution of the appropriate aldehyde **2a** or **2b** (2 mmol) and 2,4-dinitrophenylhydrazine (2 mmol, 0.40 g) in ethanol (25 mL). The reaction mixture was heated under reflux for 7 h, cooled and resultant red precipitate formed was filtered, washed with ethanol and recrystallized from ethyl acetate.

Compound **4a**, yield % = 93, m.p. = 312–314°C, microanlysis for $C_{23}H_{18}N_6O_4$ (442.43), calculated C% = 62.44, H% = 4.10, N% = 19.00, Found C% = 62.68, H% = 4.53, N% = 18.87. IR (cm^{-1}): 3225 (NH), 1615 (C=N), 1463, 1388 (NO_2). ^{1}H-NMR (DMSO-d6): δ 7.41–7.63 (m, 5H, phenyl-H), 7.70 (d, J = 8.1 Hz, 2H, tolyl-$C_{3,5}$H), 8.01 (d, J = 8.1 Hz, 2H, tolyl-$C_{2,6}$H), 8.11 (d, J= 9.3 Hz, 1H, 2,4-dinitrophenyl-C_6H), 8.32 (d, J = 9.3 Hz, 1H, 2,4-dinitrophenyl-C_5H), 8.82 (s, 1H, pyrazole-C_5H), 8.91 (s, 1H, CH), 9.12 (s, 1H, 2,4-dinitrophenyl-C_3H), 11.71 (s, 1H, NH).

Compound **4b**, yield % = 86, m.p. = 299–301°C, microanlysis for $C_{22}H_{15}ClN_6O_4$ (462.85), calculated C% = 57.09, H% = 3.27, N% = 18.16, Cl% = 7.66, Found C% = 56.87, H% = 3.52, N% = 17.78, Cl% = 7.36. IR (cm^{-1}): 3280 (NH), 1618 (C=N), 1475, 1388 (NO_2). ^{1}H-NMR (DMSO-d6): δ 7.42–7.61 (m, 5H, phenyl-H), 7.80 (d, J = 8.1 Hz, 2H, chlorophenyl –$C_{3,5}$H), 8.00 (d, J = 8.1 Hz, 2H, chlorophenyl–$C_{2,6}$H), 8.12 (d, J = 9.6 Hz, 1H, 2,4-dinitrophenyl-C_6H), 8.32 (d, J = 9.6 Hz, 1H, 2,4-dinitrophenyl-C_5H), 8.80 (s, 1H, pyrazole-C_5H), 8.92 (s, 1H, CH), 9.11–9.21 (s, 1H, 2,4-dinitrophenyl-C_3H), 11.71 (s, 1H, NH).

Synthesis of 2-(2-fluorophenyl)-1-{[3-phenyl-1-(4-methylphenyl)-1H-pyrazol-4-yl]methylene}hydrazine ***(5a)****; 1-{[1-(4-chlorophenyl)-3-phenyl-1H-pyrazol-4-yl]-methylen}-2-(2-fluorophenyl)-hydrazine* ***(5b)****; 2-(3-fluorophenyl)-1-{[3-phenyl-1-(4-methylphenyl)-1H-pyrazol-4-yl]methylene}hydrazine* ***(6a)****; 1-{[1-(4-chlorophenyl)-3-phenyl-1H-pyrazol-4-yl]methylene}-2-(3-fluorophenyl)-hydrazine* ***(6b)****; 2-(4-fluorophenyl)-1-{[3-phenyl-1-(4-methylphenyl)-1H-pyrazol-4-yl]methylene}-hydrazine* ***(7a)*** *and 1-{[1-(4-chlorophenyl)-3-phenyl-1H-pyrazol-4-yl]methylene}-2-(4-fluorophenyl)-hydrazine* ***(7b)***

Three drops of HCl were added to a solution of the selected aldehyde **2a** or **2b** (2 mmol) and the appropriate flourophenyl-hydrazine (2 mmol, 0.25 g) in 25 mL of ethanol. The mixture was heated under reflux for 5 h, then cooled and the yellow precipitate formed was filtered, washed with ethanol, dried and recrystallized from methanol/water (4:1).

Compound **5a**, yield % = 82, m.p. = 252–253°C, microanlysis for $C_{23}H_{19}FN_4$ (370.42), calculated C% = 74.58, H% = 5.17, N% = 15.13, Found C% = 74.86, H% = 5.28, N% = 14.97. IR (cm^{-1}): 3155 (NH), 1612 (C=N). ^{1}H-NMR (MeOD): δ 2.50 (s, 3H, CH_3), 6.57-7.53 (m, 9H, fluorophenyl-H & phenyl-H), 7.52 (d, J = 7.8 Hz, 2H, tolyl-$C_{3,5}$H), 7.76 (d, J = 7.8 Hz, 2H, tolyl-$C_{2,6}$H), 8.41 (s, 1H, pyrazole-C_5H), 8.78 (s, 1H, CH).

Compound **5b**, yield % = 79, m.p. = 244–345°C, microanlysis for $C_{22}H_{16}ClFN_4$ (390.84), calculated C% = 67.61, H% = 4.13, N% = 14.33, Cl% = 9.07, Found C% = 67.92, H% = 3.79, N% = 14.11, Cl% = 9.28. IR (cm^{-1}): 3280 (NH), 3146 (NH), 1607 (C=N). ^{1}H-NMR (MeOD): δ 6.51–7.58 (m, 13H, fluorophenyl-H, chlorophenyl-H & phenyl-H), 7.95 (s, 1H, pyrazole -C_5H), 8.28 (s, 1H, CH).

Compound **6a**, yield % = 86, m.p. = 251–252°C, microanlysis for $C_{23}H_{19}FN_4$ (370.42), calculated C% = 74.58, H% = 5.17, N% = 15.13, Found C% = 74.71, H% = 5.44, N% = 15.42. IR (cm^{-1}): 3164 (NH), 1614 (C=N). ^{1}H-NMR (CD_3Cl): δ 2.53 (s, 3H, CH_3), 6.26–7.49 (m, 9H, fluorophenyl-H & phenyl-H), 7.53 (d, J = 7.8 Hz, 2H, tolyl-$C_{3,5}$H), 7.69 (d, J = 7.8 Hz, 2H, tolyl-$C_{2,6}$H), 8.42 (s, 1H, pyrazole-C_5H), 8.74 (s, 1H, CH).

Compound **6b**, yield % = 86, m.p. = 258–259°C, microanlysis for $C_{22}H_{16}ClFN_4$ (390.84), calculated C% = 67.61, H% = 4.13, N% = 14.33, Cl% = 9.07, Found C% = 67.88, H% = 4.41, N% = 14.58, Cl% = 8.78. IR (cm^{-1}): 3157 (NH), 1615 (C=N). ^{1}H-NMR (CD_3Cl): δ 6.24–7.56 (m, 13H, fluorophenyl-H, chlorophenyl-H & phenyl-H), 7.98 (s, 1H, pyrazole -C_5H), 8.26 (s, 1H, CH).

Compound **7a**, yield % = 85, m.p. = 248–250°C, microanlysis for $C_{23}H_{19}FN_4$ (370.42), calculated C% = 74.58, H% = 5.17, N% = 15.13, Found C% = 74.28, H% = 5.36, N% = 15.46. IR (cm^{-1}): 3170 (NH),1612 (C=N). ^{1}H-NMR (CD_3Cl): δ 2.50 (s, 3H, CH_3), 6.52 (d, J = 8.8 Hz, 2H, fluorophenyl-$C_{2,6}$H), 6.78 (d, J = 8.8 Hz, 2H, fluoophenyl-$C_{3,5}$H), 7.31–7.52 (m, 5H, phenyl-H), 7.54 (d, J = 7.8 Hz 2H, *p*-tolyl-$C_{3,5}$H), 7.58 (d, J = 7.8 Hz, 2H, tolyl-$C_{2,6}$H), 8.44 (s, 1H, pyrazole-C_5H), 8.72 (s, 1H, CH).

Compound **7b**, yield % = 81, m.p. = 264–265°C, microanlysis for $C_{22}H_{16}ClFN_4$ (390.84), calculated C% = 67.61, H% = 4.13, N% = 14.33, Cl% = 9.07, Found C% = 67.76, H% = 4.36, N% = 14.60, Cl% = 9.32. IR (cm^{-1}): 3168 (NH), 1616 (C=N). ^{1}H-NMR (CD_3Cl): δ 6.49–7.58 (m, 13H, fluorophenyl-H, chlorophenyl-H & phenyl-H), 78.01 (s, 1H, pyrazole -C_5H), 8.24 (s, 1H, CH).

Synthesis of 3-phenyl-1-(4-methylphenyl)-1H-pyrazole-4-carboxylic acid ***(8a)*** *and 1-(4-chlorophenyl)-3-phenyl-1H-pyrazole-4-carboxylic acid* ***(8b)***

Potassium permanganate (2 mmol, 316 mg) was added portionwise to a cold solution of the appropriate aldehyde **2a** or **2b** (2 mmol) in aqueous acetone (20 mL, 3:1 v/v) under continuous stirring and temperature below 20°C. The mixture was stirred until the violet color disappeared, then an aqueous NaOH solution (30 mL, 5% w/v) was added and reaction mixture was stirred for one hour. The reaction mixture was filtered and the filtrate was acidified with cold dilute hydrochloric acid with vigorous stirring. The precipitate formed was filtered, washed with excess of cold water, dried and re-crystallized from ethanol.

Compound **8a**, yield % = 78, m.p. = 178–180°C, microanlysis for $C_{17}H_{14}N_2O_2$ (278.31), calculated C% = 73.37, H% = 5.07,

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N% = 10.07, Found C% = 73.50, H% = 5.32, N% = 10.28. IR (cm^{-1}): 3170–2510 (br, OH), 1702 (C=O), 1605 (C=N). ^{1}H-NMR (CD_3Cl): δ 2.51 (s, 3H, CH_3), 7.32-7.54 (m, 5H, phenyl-H), 7.53 (d, J = 7.8 Hz, 2H, tolyl-$C_{3,5}$H), 7.56 (d, J = 7.8 Hz, 2H, tolyl-$C_{2,6}$H), 8.42 (s, 1H, pyrazole-C_5H), 8.71 (s, 1H, CH), 12.76 (br s, D2O exchangeable, 1H, COOH).

Compound **8b**, yield % = 74, m.p. = 191–192°C, microanlysis for $C_{16}H_{11}ClN_2O_2$(298.72), calculated C% = 64.33, H% = 3.71, N% = 9.38, Cl% = 11.87, Found C% = 64.50, H% = 3.32, N% = 9.28, Cl% = 12.11. IR (cm^{-1}): 3168–2520 (br, OH), 1700 (C=O), 1602 (C=N). ^{1}H-NMR (CD_3Cl): δ 6.49–7.58 (m, 13H, fluorophenyl-H, chlorophenyl-H & phenyl-H), 78.01 (s, 1H, pyrazole-C_5H), 8.24 (s, 1H, CH), 12.72 (br s, D2O exchangeable, 1H, COOH).

In vivo anti-malarial activity

The *in vivo* anti-malarial activity of the synthesized compounds was evaluated using the standard 4 day suppressive assay [19]. Male Swiss albino mice (weight 20–25 g, obtained from the Ethiopian Health and Nutrition Institute) were used in this study. The animals were acclimatized for a period of 7 days to room temperature (23–25°C) with relative humidity of 60–65% before starting the assay. The animals were housed in standard cages and maintained on standard pelleted diet and water [20]. Accordingly, the test mice were infected with 0.2 mL of 2×10^7 parasitized erythrocytes, (*P. berghei* ANKA strain) intravenously on day 0. Parasitized erythrocytes were obtained from the blood of a donor mouse with approximately 20–30% parasitemia which was diluted with normal saline (1:4).

After 2 h, the infected mice were weighed and randomly divided into sixteen groups of five mice per cage for each test compound. Groups 1–14 received the synthesized compounds orally which was suspended in a vehicle containing 7% Tween80 and 3% ethanol in water, at 50 μmol/kg dose level and served as treatment group. Group fifteen received the vehicle and served as a negative control. Group sixteen received the standard drug chloroquine (50 μmol/Kg) and served as a positive control [19, 21].

On days 1 to 3, animals in the treated groups were administrated the same dose of the synthesized compounds as in day 0. On day 4 (24 h after the last dose or 96 h post-infection) the weight of the animals was determined and blood smear from all mice was prepared using Giemsa stain. Level of parasitemia was determined microscopically by counting 4 fields of approximately 100 erythrocytes per field. The difference between the mean parasitemia level of the negative control group (taken as 100%) and that of the experimental group was calculated and expressed as percent suppression. Finally the survival time for each mouse was recorded except for chloroquine treated ones which were completely cured of the parasite.

In vitro anti-malarial assay

P. falciparum strain RKL9 (CQ resistant) was maintained in a continuous culture using the standard method described by Trager and Jensen [22]. Parasites were cultured in human B(+) erythrocytes in RPMI-1640 media (GIBCOBRL, Paisley, Scotland) supplemented with 25 mM HEPES buffer, 10% human AB(+) serum and 0.2% sodium bicarbonate (Sigma) and maintained under 5% CO_2 atmosphere. Cultures containing predominantly early ring stages were synchronized by addition of 5% D-sorbitol (Sigma) [23] and used for testing. Assay was carried out 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. 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 assay culture volume was 100 μL per well. Number of parasites for the assay was adjusted at 1–1.5% by dilution with fresh human B(+) RBC. 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 used. 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 using the formula: $(1\text{-}N_t/N_c) \times 100$ where, N_t and N_c represent the number of schizonts in the test and control well respectively.

Acute toxicity

The oral acute toxicity of the most active compounds **2a**, **2b**, **3a**, **4b**, **8a** and **8b** was investigated using male mice (average weight = 20 g obtained from the Medical Research Institute, Alexandria University) following previously reported method [24]. The animals were divided into groups of six mice each. The compounds were given orally, suspended in 1% gum acacia, in doses of 1, 10, 100, 200, 250, 300 mg/kg. The mortality percentage in each group was recorded after 24 h. The test compounds were also investigated for their parenteral acute toxicity in groups of mice of six animals each. The compounds or their vehicle, propylene glycol (control), were given by intraperitoneal injection in doses of 10, 25, 50, 75, 100 mg/kg. The percentage survival was followed up to 7 days [25].

Modeling study

Docking

The coordinate from the X-ray crystal structure of dehydrofolate reductase enzyme (DHFR) used in this simulation was obtained from the Protein Data Bank (PDB ID: 1J3I), where the selective DHFR inhibitor WR99210 is bound to the active site. The ligand molecules were constructed using the builder module and were energy minimized. The active site of DHFR was generated using the MOE-Alpha Site Finder, Molecular Operating Environment's (MOE Dock 2005) module to rationalize the observed biological results in the animal experiment [26]. Then ligands were docked within this active site using the MOE-Dock. The lowest energy conformation was selected and the ligand interactions (hydrogen bonding and hydrophobic interaction) with DHFR were determined.

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