Original Research Article

Influence of substituents on the antiplasmodial and cytotoxic activities of 5-chloro-3,4-dihydro-*1H*-pyrano[3,4-b]-1,10-phenanthrolinone as antimalarial agent

Abstract

Background: Malaria is one of the most common parasitic disease in tropical and subtropical regions. *Plasmodium falciparum*, one of the causative agent of malaria is resistant to many type of antimalarial drugs. This study present *in vitro* assessment of the antiplasmodial activity of substituted phénanthrolinone compounds.

Methods: A series of 7 substituents were used to substitute the chlorine on the 5 position in the 5-chloro-3,4-dihydro-*1H*-pyrano[3,4-b]-1,10-phenanthrolinone ring. *In vitro*antiplasmodial activities were evaluated on chloroquine-sensitive and resistant strains of *P. falciparum*.

Results: The results showed that compounds **7** and **8** possessing N,N-diethylamino side chain had the best antiplasmodial activities. In addition, the cytotoxic activities were evaluated on HeLa cells and compound **8** was the least cytotoxic of all studied compounds.

Conclusion: The synthesis andantimalarial activity of substituents of compound**2**were carried out.This study has shown that compounds with basic and lipophilic substituents exhibited the best antiplasmodial activity.

Keywords: malaria disease, *Plasmodium falciparum,* 1,10-phenanthrolinone, antimalarial activity

Introduction

Malaria is one of the tropical parasitic diseases with the highest global mortality. According to the World Health Organization, it was estimated 212 million cases of malaria occurs worldwideand 429 000 deaths were registered in 2015 [1]. Over 81% of case and 91% of the deaths in 2015 were in Africa, and the majority of the remaining being in India, Southeast Asia and South America. Malaria is a disease caused by parasites from the genus *Plasmodium* and is transmitted to a human host when the vector, an infected female *anopheles* mosquitos feeds on a human host. *P. falciparum* and *P. vivax* are responsible for most of human malarial infection. For many African tropical countries, this disease is one of the major public health problem [2]. In addition, malarial burden is exacerbated with the spread of drug resistance of *P. falciparum* to antimalarial drugs and of Anopheles to insecticides [3]. Fighting against *Plasmodium* drug resistance requires the development of new candidates as potential efficient antimalarial drugs. Inour previous studies, antimalarial activity of diaza analogues of phenanthrene were evaluated for their antimalarial activities against both chloroquine-sensitive and chloroquine-resistant *P. falciparum* strains. These studies revealed that 4-chloro-3-(2-chloroethyl)-2-methyl-1,10-phenanthroline **1 (figure 1)** had the most efficient activity [4-5].To obtain more information on structure-activity relationship of such compound, an efficient oxidation route involving 1.5 equivalent of SeO_2 in pyridine at 50 ºC was adopted to get the optimal yield of 5-chloro-3,4-dihydropyrano[3,4-*b*]-1,10-phenanthrolinone **2** [6]. When the reaction conditions were modified to 3 equivalent of SeO_2 at 115 ºC, two compounds were formed including compound **2**. The other compound formed, compound**3**appears in two tautomeric forms **3a** and **3b** (**figure 2**). Access to product **2** has been valued by a pharmacomodulation.The chlorine of the pyridine ring was substituted by various substituents having amino groups. Indeed, it has been shown that the introduction of amino groups having units of type morpholine, alkyldiamine, piperazine, etc., brings better

antiplasmodial activities to other family of molecules[7-9].Previous studies have shown that some fluoroquinolones such as ciprofloxacin, perfloxacin and norfloxacin with the piperazin or methylpiperazin motif in their structure have promising antiplasmodial activity *in vitro* against chloroquino-resistant *P. falciparum* strains [10-11]. Other patented series of molecules type of pyrrolidino-alkylamine with the pattern, N,N-diethylaminopropyl such as (CDRI 87/209) and (WR268954) have antiplasmodial activities against chloroquino-resistant strains [12]. From these observations, the challenge was thrown to study on one hand the reactivity of compound **2** and its analogues obtained by substitution of the chloride in position 5 with different amino group mentioned in (**figure 3**) and on the other hand to compare their antiplasmodial activity againstboth chloroquine-sensitive and resistant strains and their cytotoxicity against*HeLa* cells.

Figure 1: Structure of compounds **1** and **2**

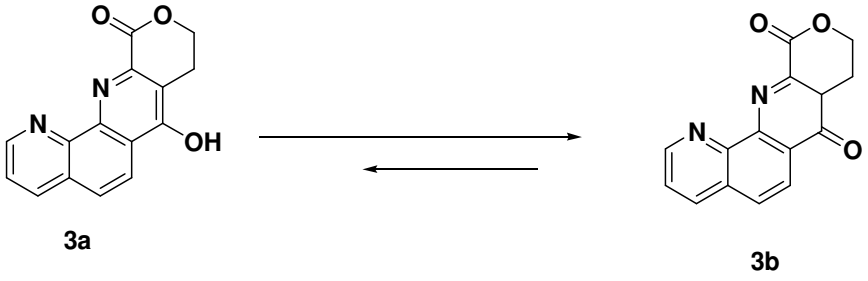


Figure 2: Structure of tautomeric forms of **3a** and **3b**

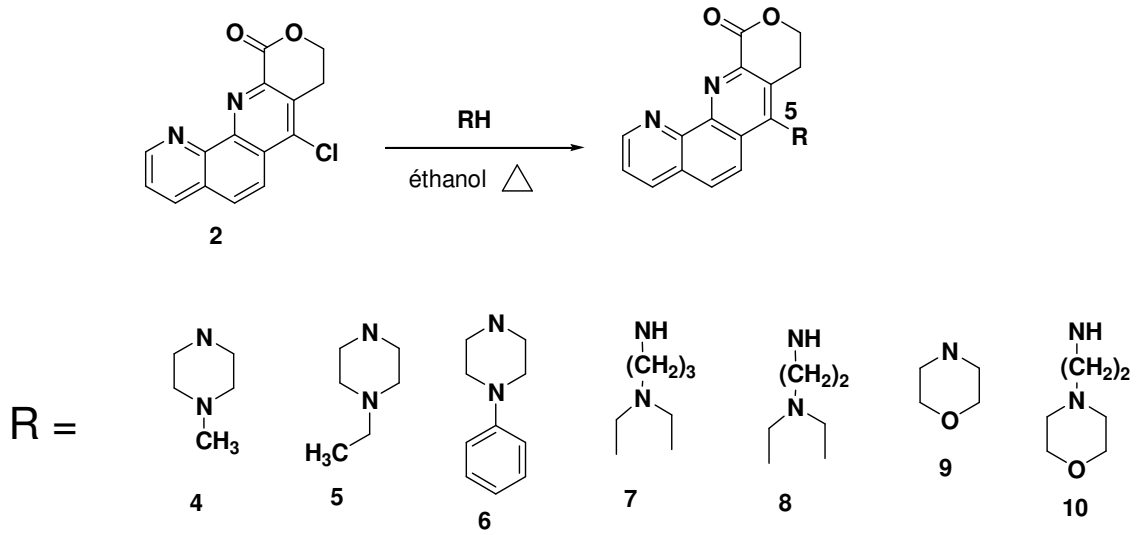


Figure 3: The amino groups used to substitute the chlorine of compound **2**

Material and methods

General Experimental.

All reagents were supplied commercially; the solvents were freshly distillated before use. ^{1}H and ^{13}C NMR spectra were measured on a Brucker 300 MHz spectrometer with $CDCl_3$, MeOD, DMSO as deuterated solvents. All chemical shifts were recorded in δ (ppm) relative to tetramethylsilane. Melting points were measured with a Stuart Scientific, melting point apparatus SMP3. The infrared spectra were taken in KBr disks using an ABB Bomen MB series spectrometer with spectra resolution of 2 cm^{-1}. The mass spectra analyses were recorded on a finnigan DSQ spectrometer operating at 70 electrons volt. Aluminium-backed silica plates with UV indicator and silica gel 37-70 µm, 200-400 mesh (SDS-13124 Peypin, France) were used in analytical thin-layer chromatography (TLC) and silica gel chromatography respectively. The spots were visualized by UV lamp (254-365 nm) or by using the iodine.

86 **Synthesis of the 5-chloro-3,4-dihydro-*1H*-pyrano[3,4-b]-1,10-phenanthrolinone (2)**

87 To a solution of 4-chloro-3-(2-chloroethyl)-2-méthyl-1,10-phenanthroline **1** (0.20 g, 0.69
88 mmole) in anhydrous pyridine (5 ml) was added SeO_2 (0.2g, 0.69 mmole). The solution was
89 refluxed for 3 hours. The hot solution was filtered and the solvent was evaporated to give a
90 residue.After addition of a little amount of water to the residue, it was extracted with
91 chloroform. The organic layer was dried over anhydrous Na_2SO_4 and evaporated under
92 vacuum. The crude material was purified by column chromatography through silica gel
93 eluted by dichloromethane/methanol (95/5) to give lactone compound (**2**).

94

95 **General methods for preparation of compounds 4 to 9.**

96 To a stirredsolution of 5-chloro-3,4-dihydropyrano[3,4-*b*]1,10-phenanthrolinone **2** (0.11g,
97 0.39 mmole) in distillated absolute ethanol (5 ml), the amine compound (substituent) was
98 added. The solution was refluxed for 3 hours. Cooled downto room temperature, the solution
99 was evaporated under vacuum pressure. The crude mixture was suspended in 50 ml of water
100 and pH of the solution was adjustedwith Na_2SO_4 solution to pH= 8. The mixture was
101 extracted with dichloromethane (3x50 ml), then the organic layer dried over Na_2SO_4, filtered
102 and evaporated. The remaining oil was treated with ether cooled and the precipitate was
103 filtered. When the compound did not precipitate, the purification was carried out by flash
104 chromatography with dichloromethane-methanol as eluent.

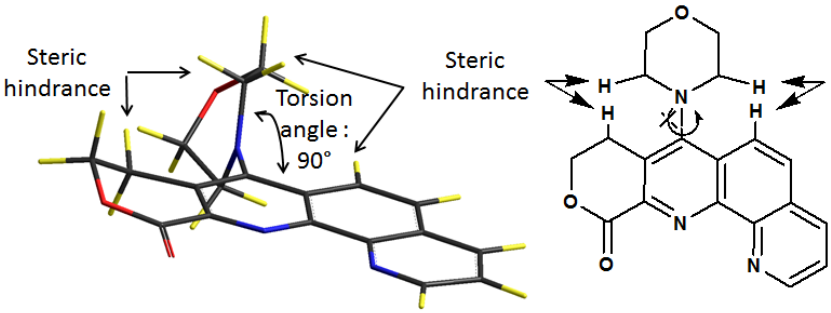
105

106 **Antiplasmodial and cytotoxic activities evaluation**

107 *In vitro* antimalarial activity was evaluated against 3 references *P. falciparum* strains. FcB_1-
108 Colombia and FcM_{29}-Cameroun are chloroquine resistant and F_{32}-Tanzania is chloroquine
109 sensitive. *P. falciparum* was cultured in schizont stage of development by using the method
110 described by Trager et al[13] with modifications[14]. Cultures were synchronized by 5% of

111 D-sorbitol (Sigma, France) which lysed the old stage parasites. The evaluation was performed
112 in 96 well plates (TPP, Suisse) which were filled with 100 ml of *P. falciparum* culture
113 (parasitemia =1.5%, and hematocrit =1.5%) to which was added 100 ml of culture medium
114 containing the compounds to be tested at different dilutions. After 24 h incubation at 37 °C
115 with 5% CO_2, 7400 Bq of [^{3}H]-hypoxanthine (Perkin Elmer) was added to each well. The
116 plates were further incubated for additional 24 hours. After nucleic acids collection on a
117 nylon filter (Perkin-Elmer), [^{3}H] hypoxanthine incorporation was measured with a β counter
118 (1450-Microbeta Trilux-Perkin Elmer). Results were then analysed for the determination of
119 the 50% inhibitory concentration (IC_{50}) and compared to controls by using the dose response
120 curves of excel software.
121 Cytotoxicity of the drugs was estimated on *HeLa* cells which were cultured in similar
122 conditions as for *P. falciparum* except for the 5% human serum which was replaced by 5%
123 fetal calf serum (Boehringer) as described elsewhere [15]. After addition of drugs at various
124 concentrations, cell growth was estimated by [^{3}H] hypoxanthine incorporation after 48 hours
125 incubation.
126
127 **Computational methods**
128 Structure of heme was extracted from the 5sw7.pdb file obtained from RCSB protein
129 databank (http://www.rcsb.org). Structure of compounds (**8,9**) were built in HyperChem
130 Release 8.0.5 pro for Windows (Hypercube Inc. Gainesville, Florida). The geometries were
131 optimized using the semi-empirical AM1 programme in singly-excited configuration
132 interaction. (RHF [Restricted Hartree-Fock], charge 0, spin multiplicity 1, lowest state,
133 orbital criterion, five occupied and five unoccupied orbitals.) to an rms (root mean square)
134 gradient of 0.01 invacuo (Polak-Ribière method). The binding mode of **8** and **9** to heme was
135 analyzed by use of ArgusLab 4.0 using a bounding ox of 25x25x25 Å, using grid (0.4 Å),

136 Argusdock engine, flexible ligand, high precision, and no augmented torsions. The
137 energetically most favourable binding pose of **8** (binding energy = -8.0 kcal/mol) and
138 **9**(binding energy = -7.28 kcal/mol) with heme, were then subjected to molecular dynamic
139 using Hyperchem 8.05. The system was first optimized in MM+ to an rms gradient of 0.5.
140 Then a molecular dynamics program was run for 1 ps, with 0.001 ps steps, relaxation time 0.1
141 ps, to a simulation temperature of 300 K. This was followed by MM+ geometry optimization
142 to an rms gradient of 0.2. The molecular dynamics run was repeated and a further MM+
143 protocol was carried out to a gradient of rms 0.01.
144



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146 **Figure 4: Restricted conformation of compound 8**

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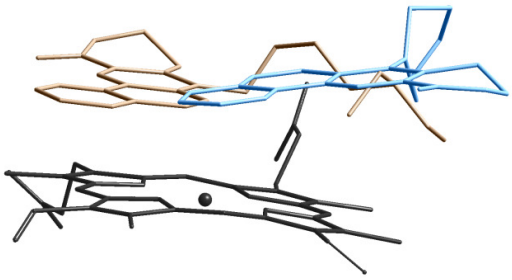

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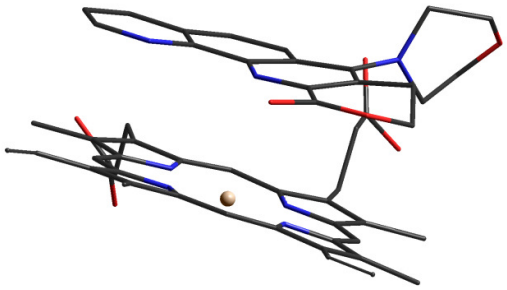
149 **Figure 5: Superimposition of the best docked poses of 8 (brown) and 9 (blue) with heme**
150 **(black)**

151



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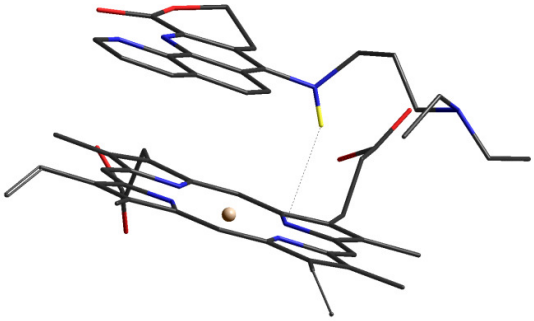
153 **Figure 6: Refined interactions for 9 with heme (molecular dynamics)**

154



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156 **Figure 7: Refined interactions of 8 with heme (molecular dynamics) showing H-bonding**

157

158 **Results and discussion**

159 All the synthesized compounds (**1** to **10**) were screened for their antiplasmodial activities

160 against the three strains of *P. falciparum* strains (chloroquine- resistant FcB_1 and FcM_{29} and

161 chloroquine-sensitive F_{32}). Five molecules were screened for their cytotoxicity toward *HeLa*

162 human cell line.

163

164 **Table 1**. *In vitro* antiplasmodial and cytotoxicity activities, IC_{50} in µM

	FcB_1 [b]	**FcM_{29} [b]**	**F_{32} [b]**	**HeLa**
Compounds[a]	**48 h[c]**	**48 h[c]**	**48 h[c]**	**48 h[c]**
2	98.59 ± 08	61.97 ± 07	68.66 ± 2.13	NT

3a	11.27 ± 1.70	12.03 ± 1.06	8.45 ±1.32	112 ± 3.50
4	83.33 ±070	91 ± 0.30	89.08 ± 4.06	NT
5	30.38 ±0.19	71.82 ±0.90	88.39 ±2.70	NT
6	87.80 ±100	6.58 ±1.30	46.34 ±2.44	21.95 ± 1.63
7	18.51 ±1.50	6.87 ±1.50	11.37 ±2.05	92.59 ± 3.51
8	11 ±2.13	6.86 ±1.32	6.86 ±1.13	247 ± 26.00
9	101 ± 0.01	78.35 ± 2.18	95.52 ± 4.23	NT
10	74.07 ± 090	52 ±0.02	56.87 ± 2.61	92.59 ± 9.33

a =compounds,**b** = parasites ; **c=** incubation time ; **NT** = non tested ; n = 3

Table 2: Selectivity index measured for 4 compounds

Compounds	FcB_1 [b]	FcM_{29} [b]	F32 [b]
3a	9.93	9.31	13.26
7	5.00	13.47	8.14
8	22.00	36.00	36.00
10	1.25	1.78	1.62

IC_{50} =concentration corresponding to 50% growth inhibition of the parasite

Selectivity index (**SI**) = IC_{50} values of cytotoxic activity/IC_{50} values of antimalarial activity

Antimalarial potential of compound **2** possessing a 1,10-phenanthrolinone basic framework depends essentially on the nature of the substituent used to substitute the chlorine on the position 5. Indeed, our results showed that the chlorine in the 5 position caused a loss of antiplasmodial activity. Compound **3,** where chlorine was replaced by a ketone group, had antiplasmodial activities ranging from 5 to 9 times greater than that of compound **2** against

176 the three *P. falciparum* strains. However when compared to compound **9** where the
177 substituent was morpholine, compound **2**showed a relatively higher antiplasmodial activity.
178 In compound **10**, the presence of N-ethyl chain led to a significant improvement of the
179 antiplasmodial activity on the 3 tested strains. This chain increased the lipophilicity of the
180 molecule which could facilitate its penetration and availability in the cytoplasm of the
181 parasite. Contribution of piperazine substituents (compounds **4**, **5** and **6**) on the activity of
182 compound **2** was not too significant with the exception of compound **6** where activity on
183 FcM_{29} was almost 10 times greater than compound **2**. The presence of the lipophilic aromatic
184 ring might explain this difference in antiplasmodial activity. The substituents of diaminoalkyl
185 nature (compounds **7** and **8**) were the best for their contribution in enhancing antiplasmodial
186 activity of compound **2**. These two compounds have registered higher activities than
187 compound **2** against all three of the*P. falciparum* parasite.
188 To provide amino acids, the parasite in the host erythrocyte degrade the hemoglobin which
189 releases amino acids for its maturation and toxic heme which accumulates in the food
190 vacuole. Lacking heme oxygenase activity to catalyze the porphyrin moiety, the parasite
191 polymerizes the highly free reactive heme into a metabolically inert crystalline material
192 called hemozoïn. This substance is a polymer of undefined length, in which the iron of one
193 heme is coordinated to the side chain propionate carboxylates of the next heme [16].The
194 quinoline antimalarial drugs such as chloroquine and quinidine inhibit the polymerization of
195 heme to hemozoïn, which is toxic for the *Plasmodium*.
196 Chloroquine has been demonstrated to bind to ferriprotoporphyrine with high affinity *in vitro*
197 and has been suggested that the drug can sequester heme interfering with the formation of the
198 inert pigment and the complex can lyse the parasite membrane [17]. Pashska and coworkers
199 (2004) have demonstrated that the mode of action of this compound is based on its
200 association with hemin or with different polymers of this one by noncovalent bonds [18].

201 Egan and colleagues have shown that the complexation between chloroquine and heme, is
202 guided by H-bonding as well as π-stacking interactions between the aminoquinoline and
203 heme[19]. In the light of this information, studying the mode of interaction of the most active
204 compound **8**and the less active one (**9**) should afford fundamental information about their
205 mode of action. First, it is obvious to note that compound**9** is conformationally restrained
206 with the morpholine ring perpendicular to the quinoline framework and that the nitrogen in
207 the 5 position cannot be implicated in H-bonding as an H-donor (figure 4and figure 5). These
208 structural specificities greatly oriented the mode of interaction of compound**9** with heme
209 when compared to compound **8**. Although the binding energies of **8** and **9** to heme are quite
210 similar, we can note that the presence of a blocked morpholine ring in**9** resulted in reduced P-
211 P stacking interactions between compound**9** and heme (figure 6), and that no H-bonding
212 between the two molecules can be founded in contrast with compound **8** which can interact
213 favourably (P-P stacking and H-bond) with heme (figure 7).
214 For the cytotoxicity measurement, compounds **3a**, **7**, **8** and **10** have least toxicity on HeLa
215 cell line with IC_{50} respectively being 112, 95, 247 and 92 µM. Indeed, most of the IC_{50} are
216 higher, lower the compound is potentially toxic. Thereby, compound **8** seems to be less
217 cytotoxic on the studied cell line. However, the selectivity index of this compoundis very low
218 (36.00)which does not allow it to be tested*in vivo*. According to Long-Ze, the selectivity
219 indexmust be greater than or equal to 100 the *in vivo* test is permitted [20].
220

221 **Conclusion**

222 Drug resistance of parasites and anopheles remains a major problem in the malaria burden. At
223 present, the best chemically solution to eradicate definitely this disease is establishment of
224 new antimalarial drug which is exempt of any resistance. The 1,10-phenanthroline skeleton
225 was synthesized and studied for it potential new antimalarial activity. Its

226 pharmacomodulation led to several compounds among which compound **8** showed higher
227 activity as compared to the parent compound **2**. This compound should be used for future
228 pharmacomodulation particularly to open the lactone ring in basic medium.
229
230 **Consent and Ethical approval**, are not applicable in this study
231
232 **References**

233 1. World Health Organization. World malaria report. 2016, 20:12. Accessed 27 January 2017.
234 Available at http://www.who.int/malaria/publications/world-malaria-report-2016/report/en/
235 2. Dambuza N, Smith P, Evans A, Taylor DT. A pharmacokinetic study of antimalarial 3,5-
236 diaryl-2-aminopyridine derivatives. Malar Res Treat. 2015,
237 http://dx.doi.org/10.1155/2015/40596

238 3. Benelli G, Mehlhorn H. Declining malaria, rising of dengue and Zika virus: insights for
239 mosquito vector control. *Parasitol Res*. 2016: 115(5):1747-54.

240 4. Yapi AD, Mustofa M, Valentin A, Chavignon O et al. *Chem Pharm Bull*. 2000: 48: 1886-
241 1889.

242 5. Yapi AD, Valentin A, Chezal JM, Chavignon O et al. In vitro and in vivo antimalarial
243 activity of derivatives of 1,10-phenanthroline framework. *Arch Pharm (Weinheim)*. 2006:
244 339(4): 201-6

245 6. Sall C, Desbois N, Paquelet S, Camacho JR, et al.An efficient route to a 5,6-
246 dihydropyrano[3,4-b]pyridine 8-one core in two steps from enaminolactones. *Tetrahedron*
247 *Lett*. 2008: 49: 1301–1304.

248

249 7. Weissig V; Vetro W, Tamara SR, Thomas CR. Topoisomerase II inhibitors induce
250 cleavage of nuclear and 35-kb plastid DNAs in the malarial parasite Plasmodium falciparum.
251 *DNA and Cell Biology*. March 2009: 16(12): 1483-1492. doi:10.1089/dna.1997.16.1483.
252 8. BatraS, Srivastava P, Roy K, Pandey VC, Bhaduri AP. A New Class of Potential
253 Chloroquine-Resistance Reversal Agents for Plasmodia: Syntheses and Biological Evaluation
254 of 1-[3-(Diethylamino)propyl]-3-(substituted phenylmethylene)pyrrolidines. *Journal of*
255 *Medicinal Chemistry*. 2000: 43(18): 3428-3433.
256 9. De D, Bhaduri AP, Milhous WK. A novel pyrrolidonoalkaneamine (WR268954) that
257 modulates chloroquine resistance of Plasmodium falciparum in vitro. *The American journal*
258 *of tropical medicine and hygiene*. 1993: 49(1): 113-20.
259 10. Divo Alan A, Sartorelli AC, Patton CL, Bia FJ.Activity offluoroquinolone antibiotics
260 against Plasmodium falciparum in vitro.*Antimicrob Agents Chemother*. 1988: 32(8): 1182-
261 1186.
262 11. Hamzah J, Skinner-Adams T, Davis TME. In vitro antimalarial activity of trovafloxacin,
263 a fourth-generation fluoroquinolone.*ActaTropica*. 2000: 74(1): 39-42.
264 12. Abraham JD, editor.Burger's Medicinal *Chemistry Drug Discovery*, 6th edition.
265 Department of medicinal chemistry school of pharmacy, Virginia Commonwealth University.
266 Richmond, Virginia. 2004.
267 13. Trager W and Jensen JB. Human malaria parasites in continuous culture. *Science*, 1976 :
268 193(4254): 673-675.
269 14. Benoit F, Valentin A, Pélissier Y, Diafouka F, Marion C et al. Antimalarial activity *in*
270 *vitro* of *Cochlospermumtinctorium*tubercle extracts. *Trans R Soc Trop Med Hg*. 1995: 89(2):
271 217-218.

272 15. Jullian V, Bonduelle C, Valentin A, Acebey L et al. New clerodanediterpenoids from
273 Laetiaprocera (Poepp.) Eichler (Flacourtiaceae), with antiplasmodial and antileishmanial
274 activities. *Bioorg Med Chem Lett*. 2005: 15(22):5065-70.

275 16. David J, Sullivan Jr, Matile H, Ridley RG¶[, and Goldbergi DE. A Common Mechanism
276 for Blockade of Heme Polymerization by Antimalarial Quinolines. *J Biol Chem.* 1998:
277 273(47): 31103–31107.

278 17. Surolia N, and Padmanaban G. Chloroquine inhibits heme-dependent protein synthesis in
279 Plasmodium falciparum. *Biochemistry.* 1991: 88: 4786-4790.

280 18. Pashynska VA, Van den HH, Claeys M, Kosevich MV. Characterization of noncovalent
281 complexes of antimalarial agents of the artemisinin-type and FE(III)-Heme by electrospray
282 mass spectrometry and collisional activation tandem mass spectrometry. *J Am Soc Mass*
283 *Spectrom.* 2004: 15(8): 1181-1190.

284 19. Egan TJ, Mavuso WW, Ross DC, Marques H M.Thermodynamic factors controlling the
285 interaction of quinoline antimalarial drugs with ferriprotoporphyrin IX.*J InorgBiochem.*
286 1997: 68(2): 137-145.

287 20. Lin LZ, Shieh HL, Angerhofer CK, Pezzuto JM et al. Cytotoxic and antimalarial
288 bisbenzylisoquinoline alkaloids from Cycleabarbata. *J Nat Prod.* 1993: 56(1): 22-29.