Synthesis and Comparison of Antimalarial Activity of Febrifugine Derivatives Including Halofuginone

Bhupesh S. Samant[1,*] and Mugdha G. Sukhthankar[2]

[1]*Chemical Engineering Division, Institute of Chemical Technology, University of Mumbai, Matunga, Mumbai 400 019, India;* [2]*Laboratory of Environmental Carcinogenesis, Department of Pathobiology, College of Veterinary Medicine, The University of Tennessee, Knoxville, TN-37996, USA*

Abstract: Febrifugine and its derivatives including halofuginone which possess very high activity against malaria were prepared synthetically from easily available starting material, 3-hydroxy picoline, and using simple reaction conditions. Synthesis of 2-amino-5, 6-methylenedioxy benzoic acid, (which is an intermediate for the process) is described. The selectivity enhancement in nitration of 3, 4-methylenedioxybenzaldehyde towards 6-nitro isomer was done with the help of surfactant. The antimalarial activity of synthesized compounds was determined by using in vitro assays against chloroquine sensitive (D6), chloroquine resistant (W2) Plasmodium falciparum strains for susceptibility and two mammalian cell lines (neuronal cell line NG108 and macrophage cell line J774) for cytotoxicity. The IC_{50}s of halofuginone was observed to be the best among the synthesized derivatives of febrifugine.

Key Words: Halofuginone, 3-hydroxy picoline, antimalarial, antiprotozoal, coccidiostat.

1. INTRODUCTION

Malaria is the most undesirable disease all over the world. Febrifugine {(2R, 3S)-3-[3-(3-hydroxy-2-piperidinyl) acetonyl]-4(3H)quinazolinone} (Table **1**, Entry **1**) is an active component extracted from a Chinese medicinal plant, chang shan (Dichroa febrifuga Lour), that has been prescribed in traditional Chinese medicine [1]. The medicinal use of chang shan was described in an ancient Chinese pharmacopoeia for the treatment of malaria and stomach cancer and as an expectorant, emetic, and febrifuge, having side effects such as nausea and vomiting. A series of febrifugine derivatives, including halofuginone {7-bromo-6-chloro-3-[(3-hydroxy-2-piperidinyl)-2-oxopropyl]-4(3H)-quinazolinone} (Table **1**, Entry **4**), are very important heterocyclic compounds as these compounds shows activity against malaria parasite [2]. It was found that the administration of febrifugine analogs did not cause changes in body or liver weights, irritation of the gastrointestinal tract, or alteration of the levels of hepatic enzyme markers in infected mice [3]. Febrifugine caused to enhanced the production of nitric oxide and affects on the tumor necrosis factor α in mouse macrophages [4]. These studies indicate not only that febrifugine is a plausible antimalarial lead for the search of new analogs but also that it possesses unique antimalarial mechanisms.

Halofuginone inhibits collagen α1 (I) gene expression in several animal models and in patients with fibrotic disease, including scleroderma and graft versus-host disease [5]. In addition, halofuginone has been shown to inhibit angiogenesis and tumor progression [6]. It was demonstrated recently that halofuginone inhibits transforming growth factor-β (TGF-β), an important immunomodulator [7].

Halofuginone is an antiprotozoal agent of the quinazolinone derivatives group [8]. This compound has a cryptosporidiostatic effect on Cryptosporidium parvum [9]. Halofuginone has numerous applications in veterinary science as a coccidiostat [10]. Hence, febrifugine and its derivatives are very important compounds in medicinal and veterinary science. A variety of synthetic routes for febrifugine and its series of derivatives (including halofuginone) were reported in literature [1b,4,11]. However, most of the methods previously described, require the use of expensive/nonecofriendly reagents [11]. Hence, a conveniently viable method involving mild reaction conditions with enhanced yield is always appealing.

The efforts are made here to described synthesis process of febrifugine and its series of derivatives (including halofuginone) with satisfactory yields and simple reaction conditions. Synthesis of 2-amino-5,6-methylenedioxy benzoic acid, (which is an intermediate for the process) has been described.

2. RESULTS AND DISCUSSION

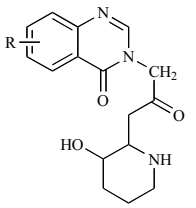
In today's research world, synthesis method which is ecofriendly and avails at simple reaction conditions with high yield is in great demand.

Purpose of this article is to describe a synthesis method for febrifugine and its series of derivatives (including halofuginone) from 2-methyl-3-hydroxy-pyridine (3-hydroxypicoline, **I**) and to study the antimalarial activity of synthesized derivatives of febrifugine by using *in vitro* assays against chloroquine sensitive (D6) and chloroquine resistant (W2) *Plasmodium falciparum* strains for susceptibility and

*Address correspondence to this author at the Max Mousseron Institute of Biomolecules, CNRS UMR 5247, Universite Montpellier II, 34095 Montpellier Cedex 5, France; E-mail: bhupesh.samant@gmail.com

1573-4064/09 $55.00+.00

Table 1. Yield, *In Vitro* Antimalarial Susceptibility and Toxicity of Febrifugine and its Derivatives



Entry	R	% Yield	M.P. (K)	IC_{50}s (ng/mL)[b]			
				P. falciparum Drug susceptibility		Mammalian cell toxicity	
				Strain W2	Strain D6	Neuronal	Macrophages
						NG108	J774
1	H	76	461-463	0.55± 0.1	0.40± 0.09	66.60± 1.95	82± 1.54
2	6,7-OCH_2O-	72	501-504	3.01± 0.5	2.50± 0.75	7563.50± 1445.6	4608.25± 688.9
3	7,8-OCH_2O-	75	508-511	4.02± 0.48	2.02± 0.9	8062.25± 1265.4	3588.90± 954.8
4	6-Cl, 7-Br	70	trans, 465-468 cis, 413-418	0.16± 0.03	0.10± 0.02	200.07± 49.2	140.30± 4.52

[a]Isolated yields; Reactions were monitored by gas chromatography and products were conformed by ^1H NMR spectroscopy.

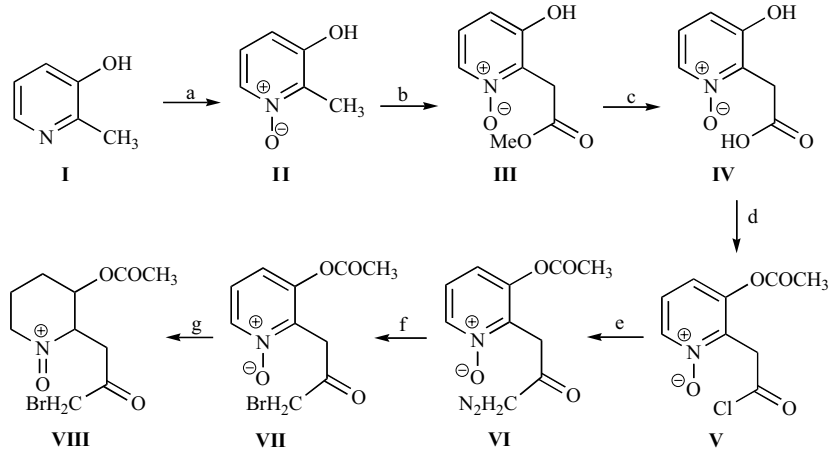
[b]Values are means ± standard deviations; three independent experiments were done with replicates of three.

two mammalian cell lines (neuronal cell line NG108 and macrophage cell line J774) for cytotoxicity .

The synthesis of halofuginone i.e. {7-bromo-6-chloro-3-[(3-hydroxy-2-piperidinyl)-2-oxopropyl]-4(3H)-quinazolinone} (**XIV**); demonstrates the general way in which these compounds were prepared.

3-hydroxy-picoline is very easily available, nonhazardous and cheap raw material. It was obtained by two different methods, hydrogenolysis of N-(3-hydroxy-2-picolyl) trimethyl ammonium iodide and the action of ammonium hydroxide on 2-acetofuran at high temperature and pressure [12]. Oxidation of 3-hydroxy-picoline to 3-hydroxy-2-methyl-pyridine-1-oxide (**II**) was done with *meta*-chloroperbenzoic acid to give 92% of N-oxide (Scheme **1**).

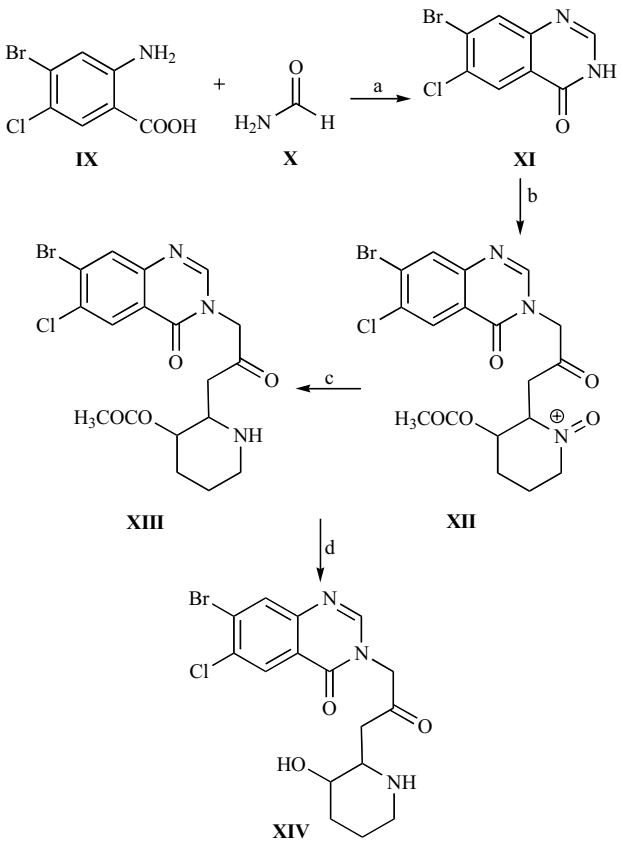
The purpose behind formation of N-oxide is to protect nitrogen of pyridine ring and to make pyridine ring more reactive. The presence of highly electronegative oxygen atom (attached to nitrogen) pulls lone pair electrons of nitrogen and pi electrons from ring towards it; hence second and sixth positions of pyridine ring become more reactive. In



Scheme 1. Preparation of 1-[2'-(3'-(acetoxy)-1'-(oxo)-piperidino)]-3-bromo-2-propanone; 3-acetyloxy-2-(3-bromo-2-propanone)-piperidine-1-oxide (**VIII**). Reagents and conditions: (a) I (1 equiv, 0.1 moles), M-CPBA (30 mL), maintain temperature below 283 K; (b) II (0.6 equiv, 0.06 moles), MeOH (25 mL), PhLi in 40 mL ether; (c) 6 N HCl (75 mL), 4 h reflux; (d) AC_2O (5 mL), PCl_5 (2 g); (e) V (0.2 equiv, 0.02 moles), CH_2N_2 in ACOH (15 mL); (f) HBr in ACOH (30% in 20 mL) maintain temperature below 283 K; (g) H_2 / Ni, 473 K, EtOH (60 mL).

subsequent step, the action of methanol and dry ice with phenyl lithium in acidic medium caused esterification of 3-hydroxy-2-methyl-pyridine-1-oxide to form 3-hydroxy-2-(1-methylaceto)-pyridine-1-oxide (**III**) in 75% yield.

1-[2'-(3'-(hydroxy)-1'-(oxo)-pyridino)]-acetic acid; 3-hydroxy-2-acetic acid-pyridine-1-oxide (**IV**) was formed when **III** was refluxed in acidic medium. Then chlorination of side chain was done with the help of phosphorus penta chloride to form 1-[2'-(3'-(acetyloxy)-1'-(oxo)-pyridino)]-acetyl chloride; 3-acetyloxy-2-acetyl chloride-pyridine-1-oxide (**V**) in 75% yield. In **V**, hydroxy group at third position was protected by acetyl group by the action of acetic anhydride. Chloride group was replaced to diazo group by the action of diazomethane to give **VI**, which was finally converted in to bromomethyl ketone (**VII**) in 80% yield by the action of hydrogenbromide. In the next step, reduction of pyridine ring of compound **VII**, in the presence of nickel catalyst gave piperidine ring (**VIII**) in 90% yield. Substituted pyridines at second position were reduced more rapidly than pyridine itself. This is because the group at second position decreased the ability of nitrogen to bind with the catalyst in reduction reaction.



Scheme 2. Preparation of halofuginone {7-bromo-6-chloro-3-[(3-hydroxy-2-piperidinyl)-2-oxopropyl]-4(3H)-quinazolinone} (XIV). Reagents and conditions: (a) IX (0.5 equiv., 0.05 moles), X (55 mL), heat at 373-375 K for 1 h then at 470-475 K for 0.5 h.; (b) VIII (1 equiv., 0.01 moles), NaOMe in MeOH (45 mL, 1 N); (c) Zinc powder in ACOH (25 mL), refulx for 4 h; (d) HCl (30 mL, 6 N).

Condensation of 2-amino-4-bromo-5-chlorobenzoic acid (**IX**) and formamide (**X**) gave 6-bromo-7-chloro-4-quinazolone (**XI**) in 60% yield (Scheme **2**). This on reacting with 3-acetyloxy-2-bromomethylketo-piperidine-1-oxide (**VIII**) in acidic media gave **XII** in 75% yield. The nitrogen oxygen bond of compound **XII** was cleaved by reduction, with the help of zinc and acetic acid to form **XIII**, which on refluxing with hydrochloric acid gave 7-bromo-6-chloro-3-[(3-hydroxy-2-piperidinyl)-2-oxopropyl]-4(3H)-quinazolinone (**XIV**) in 75% yield; as a model compound. Halofuginone obtained in a form of recimic mixture, which was then isolated in *cis* and *trans* isomers by the action of sodium hydroxide and extraction process with $CHCl_3$. Spectral analysis and physical constants obtained for febrifugine and it's derivatives are similar to those reported in literature [13].

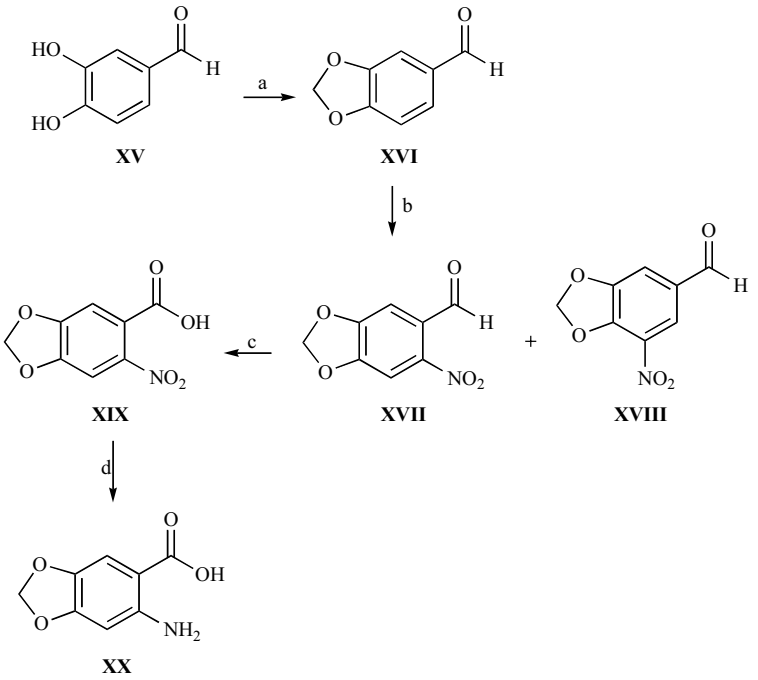
Table **1** contains yields and melting points of different derivatives of febrifugine. Analysis of febrifugine (Entry **1**) was done with the ^1H NMR technique. For 3-[2-oxo-3-cis-(3-hydroxy-2-piperidyl)propyl]-6,7-methyledioxy-4-quinazol (Entry **2**), the procedure of preparation is similar to compound **XIV**, with only difference being reactant **IX** in Scheme **2** which was replaced with 2-amino-4,5-methylenedioxy benzoic acid.

Scheme **3** describes the procedure for preparation of 2-amino-4,5-methylenedioxy benzoic acid (**XX**) from 3, 4-Dihydroxybenzaldehyde (**XV**). **XV** was converted in to 3, 4-methylenedioxybenzaldehyde (**XVI**) in 75% yield, by the action of CH_2Br_2 and K_2CO_3 in DMF.

Nitration of **XVI** gave a mixture of 3, 4-methelenedioxy-6-nitrobenzaldehyde (**XVII**) and the corresponding 5-nitro isomer (**XVIII**) in ratio 2:1 [14]. However, this reduces the yield of process. Hence, to increase the selectivity in nitration reaction towards 6-nitro isomer, surfactant (sodium dodecyl sulfate, SDS) was used in process. This surfactant formed micelles as aggregates in the reaction media. 3, 4-methylenedioxybenzaldehyde dissolved in those micelles to exhibit favorable orientation, such that polar aldehyde group resides at the interface, pointing towards water whereas relatively nonpolar methylene group resides at the nonpolar core of micelle. Hence, attacking polar nitro electrophile attacks at 6^{th} position which was in water where as 5^{th} position was inside micelle protected from nitration (Fig. **1**). In recent years, the use of surfactants for enhancing selectivity of reaction towards particular product is increasing due to easily avail reaction conditions [15].

3, 4-methelenedioxy-6-nitrobenzaldehyde (**XVII**) was then oxidized to the corresponding 3,4-methelenedioxy-6-nitro benzoic acid (**XIX**) in 91% yield, by the action of potassium permanganate. Catalytic reduction of which yield 90% of 2-amino-4,5-methylenedioxy benzoic acid (**XX**).

Synthesized derivatives of febrifugine were tested *in vitro* against two *P. falciparum* malaria parasite clones: W2 and D6, by using the procedures described in literature [16] and their *in vitro* toxicities were tested in two mammalian cell lines; neuronal cell line, NG108 and macrophage cell line, J774 as described previously [17]. There was no substitution on aromatic ring of febrifugine (Entry **1**). On the other hand different derivatives which were synthesized, have sub-



Scheme 3. Preparation of methyl-2-amino-4,5-methylenedioxy benzoic acid (**XX**). Reagents and conditions: (a) **XV** (0.5 equiv., 0.5 moles),CH_2Br_2 (0.5 moles), K_2CO_3 (0.5 moles), CuO (4 g), DMF (450 mL), heat at 398-403 K for 2 h under N_2; (b) **XVI** (0.3 moles), SDS aqueous solution (80 mL, 40 mM), nitric acid (150 mL); (c) Separation of **XVIII** by addition of MeOH (65 mL), $KMnO_4$ (0.2 moles), H_2O (800 mL); (d) H_2/ Pd (10%), EtOH (200 mL), 473 K, 2 h.

stitution at different positions of qunazoline ring. The study of chemical structure and antimalarial activity of these synthesized derivatives of febrifugine shows that halofuginone (Entry **4)** which contains chlorine at 6th position and bromine at 7th position gave highest antimalarial efficacy among the synthesized febrifugine derivatives. Also, halofuginone showed low cytotoxicities as compared to febrifugine for mammalian cell lines. Halofuginone showed increase in selectivity indices as compared to febrifugine. IC_{50} values against parasite strains W2 and D6 by halofuginone indicated that it is the most active antimalarial derivative of febrifugine. Substitution of three membered rings at position 6, 7 and 7, 8 of qunazoline ring in compounds of entry **2** and **3** respectively in Table **1** show decreased in antimalarial efficacy in comparison with halofuginone. However, there was no substantial difference in the antimalarial activity among them self. Both these compounds showed decrease in toxicity compared to febrifugine. It was also observed that febrifugine and its derivatives were more active against *P. falciparum* malaria parasite clone D6 than W2 which indicates that there is very less resistance to these compounds in parasites. Neuronal cell line NG108 was less sensitive as compared to macrophage cell line J774 for these compounds. Halofuginone showed less specificity for these two cell lines as compared to other derivatives of febrifugine.

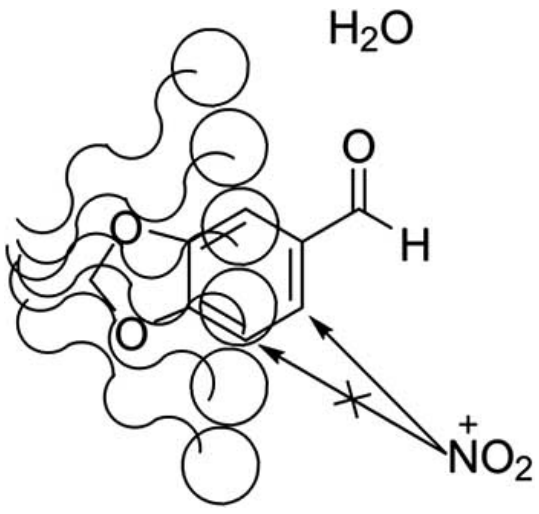


Fig. (1). Selectivity enhancement in nitration of 3,4-methylenedioxybenzaldehyde (**XVI**).

CONCLUSION

Febrifugine and its series of derivatives (including halofuginone), which are very important chemical compounds in medicinal science, can be prepared from 3-hydroxy-picoline as starting material, in which halofuginone has an enormous application in veterinary science as coccidiostat and antiprotozoal agent. The high availability of starting compound (i.e. 3-hydroxy-picoline), satisfactory yields and simple reaction conditions are the salutary features of this synthesis. Synthesis of 2-amino-5,6-methylenedioxy benzoic acid, (which is an intermediate for the process) has been described. The selectivity enhancement in nitration of 3,4-methylenedioxybenzaldehyde towards 6-nitro isomer was done with the help of surfactant. Halofuginone possess effective antimalarial activity against *P. falciparum* malaria parasite clone D6

thanW2. This study provides relationship of chemical structure and antimalarial activity.

EXPERIMENTAL SECTION

General

The reactions were monitored by GC (Chemito 8610) with flame ionization detector (FID). A 4 m long and 0.37×10^{-2} m internal diameter S.S. column packed with 10% SE-30 on chromosorb WHP was employed for the analysis. Nitrogen at the flow rate of 0.5×10^{-7} m^3 sec^{-1} was used as carrier gas. Injector and detector (FID) temperatures were maintained at 573 K. The oven temperature program was as follows: Starting at 423 K.

The reactions were monitored by GC (Chemito 8610) with flame ionization detector (FID). A 4 m long and 0.37×10^{-2} m internal diameter S.S. column packed with 10% SE-30 on chromosorb WHP was employed for the analysis. Nitrogen at the flow rate of 0.5×10^{-7} m^3 sec^{-1} was used as carrier gas. Injector and detector (FID) temperatures were maintained at 573 K. The oven temperature program was as follows: Starting at 423 K hold for 5 min rise of temperature 276 K min^{-1} up to 553 K and hold for 5 min. Samples (1 µl) were injected in split-less mode. Melting points are uncorrected. The products were purified by flash chromatography on Merck silica gel 60 (230-400 mesh ASTM). Products were confirmed using ^{1}H NMR and ^{13}C NMR spectra (Varian Gemini 300 and 200 MHz) instrument in DMSO-d_6 solutions. Peak positions are given in parts per million (δ) from tetramethylsilane as internal standard, and coupling constant values (*J*) are given in Hertz. IR spectra in Nujol mulls were recorded on a Perkin Elmer Spectrum BX FTIR spectrometer. UV spectra were determined by using Chemito 2100 UV spectrophotometer.

3-hydroxy-2-methyl-pyridine-1-oxide (II)

Dropwise addition of 30 mL meta-chloroperbenzoic acid in 11.5 g (0.1 moles) of 3-hydroxy-picoline was done in 10 min, in that period temperature was maintained below 283 K. Then solution kept to room temperature for 15 min. to form 12 g (92%) of **II**.

1-[2'-(3'-(hydroxy)-1'-(oxo)-pyridino)]-ethylacetate or 3-hydroxy-2-(1-methylaceto)-pyridine-1-oxide (III)

9 g (0.06 moles) of **II** was added in phenyl lithium in ether (40 mL). This solution was stirred for 30 min, in that period temperature was maintained below 308 K. Then add dry ice and methanol (25 mL). Solution was stirred for 2 h. Then solution kept to room temperature for 30 min. to form 8.65 g (75%) of **III**.

1-[2'-(3'-(hydroxy)-1'-(oxo)-pyridino)]-acetic acid or 3-hydroxy-2-acetic acid-pyridine-1-oxide (IV) and 1-[2'-(3'-(acetyloxy)-1'-(oxo)-pyridino)]-acetyl chloride or 3-acetyloxy-2-acetyl chloride-pyridine-1-oxide (V)

A mixture of 8 g (0.04 moles) of **III** and 75 mL of 6 N hydrochloric acid was refluxed for 4 h. The solution was cooled to 278 K. Then solution was kept at room temperature for 30 min. 5 mL of acetic anhydride was added in solution and solution was heated on steam bath for 1 h. Then 10 mL of water was added and heating was continued for 10 min more. This solution was then evaporated in vacuum. Residue (**IV**) obtained from evaporation was dissolved in 15 mL of warm toluene and evaporation was repeated. 6 g of residue obtained from above procedure was added in acetyl chloride (15 mL). 2 g of phosphorus pentachloride was added. This solution was kept in orbital shaker for 15 min., which started the crystallization process. Solution was kept to room temperature for 5 h and then it was filtered to give 5.85 g (75%) of **V**. It was washed with acetyl chloride (3×5 mL).

VI and 1-[2'-(3'-(acetoxy)-1'-(oxo)-pyridino)]-3-bromo-2-propanone or 3-acetyloxy-2-(3-bromo-2-propanone)-pyridine-1-oxide (VII)

5 g (0.02 moles) of **V** was treated with diazomethane (prepared from 10 g of nitroso methyl urea and dried with potassium hydroxide) over a period of 10 min. The solution was kept in ice bath for 10 min and at room temperature for 1 h without cooling. After that, solution was treated with 15 mL of acetic acid to neutralize excess of diazomethane. Then 20 mL of 30% hydrogen bromide in acetic acid was added with cooling so that temperature of solution was maintained below 283 K. Solution was kept at room temperature for 15 min. Then solution was washed with aqueous sodium bicarbonate. This solution was evaporated in vacuum. Compound **VII** (5 g) 80% obtained in a form of oil.

1-[2'-(3'-(acetoxy)-1'-(oxo)-piperidino)]-3-bromo-2-propanone or 3-acetyloxy-2-(3-bromo-2-propanone)-piperidine-1-oxide (VIII)

A solution of 5 g (0.016 moles) of **VII** in 60 mL ethanol was subjected to autoclave under 2 bar hydrogen pressure in the presence of Ni catalyst for 2 h. The catalyst was filtered and solution was evaporated to dryness to leave a solid. Two recrystallizations from MeOH gave 4.3 g (90%) of **VIII**.

Analysis for **VIII**: ^{1}H NMR (DMSO-d_6, TMS, 200 MHz) δ (ppm): 1.3,1.4 (2H, m, CH_2-5); 1.3,1.4 (2H, m, CH_2-6); 1.66,1.38 (2H, m, CH_2-4); 2.28 (1H, m, CH); 2.02 (3H, s, CH_3–C=O), 2.27,2.57 (2H, m, CH_2CO), 4 (1H, m, H-3), 4.5 (2H, s, CH_2-Br) . ^{13}C NMR (DMSO-d_6, TMS, 50 MHz) δ (ppm): 15.5 (C-3); 21.3 (C-6); 28.3 (C-4); 29.9 (C*H_2CO); 39.9 (OC*H_2Br); 56.5 (C-2); 62.5 (OCOC*H_3); 71.5 (C-5); 170.5 (OC*OCH_3); 199.5(C*OCH_2Br).

6-Bromo-5-chloro-4-quinazolone (XI)

Mixture of 12.5 g (0.05 moles) of **IX** and 55 mL formamide (**X**) was heated at 373-375 K for 1 h. Then solution was again heated at 470-475 K for 0.5 h. Solution was kept to attain room temperature and then cooled to 278 K. Precipitate was formed in cooling process, which was collected by filtration and washed with methanol to yield 6 g of crude product. The filtrate was diluted with 300 mL water where by additional 1.7 g of solid was obtained. The combined product was recrystallized from dimethyl formamide which formed 7.7 g (60%) of **XI**.

3-[2-Oxo-3-cis-(3-methoxy-2-piperidyl) propyl]-6-bromo-7-chloro-methylenedioxy-4-quinazolone (XIV)

To solution of 2.6 g of **XI** (0.01 moles) in 15 mL of 1 N methanolic sodium methoxide was added in a solution of 3 g (0.01 moles) of **VIII** in 45 mL of methanol. After 1 h that

reaction mixture was diluted with 200 mL of iced-water and 80 mL of 10% sodium hydroxide. This mixture was extracted twice with chloroform (2 × 10 mL). Evaporation of the combined extracts gave a gum; it was crystallized as a hydrochloride by treatment with saturated absolute alcoholic hydrochloric acid to give **XII** in 3.4 g (75%) yield. 3.4 g of **XII** was added in a (25 mL) mixture of zinc powder and acetic acid. This solution was refluxed for 4 h. Then solution was kept to attain room temperature. 30 mL of 6N hydrochloric acid was added in to solution and refluxed for 7 h. It was cooled, aqueous solution of Na_2CO_3 was slowly added with stirring so that pH of solution became 9. Solution was filtered. Filtrate evaporated to dryness in vacuum to give **XIV** as a product 2.5 g 75% yield.

3,4-Methylenedioxybenzaldehyde (XVI) and 3,4-Methylenedioxy-6-nitrobenzaldehyde, 3,4-methylenedioxy-5-nitrobenzaldehyde(XVII, XVIII)

Mixture of 69 g (0.5 moles) of 3,4-dihydroxy benzaldehyde (**XV**), 87 g (0.5 moles) of CH_2Br_2, 69 g (0.5 moles) anhydrous K_2CO_3 and 4 g of CuO was heated in 450 mL of DMF at 398-403 K under nitrogen for 2 h. The solides were removed by filtration and were washed with methanol. The combined filtrate was diluted with 1.3 lit of water and extracted with a total 3 lit of $CHCl_3$. The extract was wash with water dried and evaporated on distillation in vacuum 52.5 g (70%) of **XVI** was collected as yellow liquid. 45 g (0.3 moles) of **XVI** was added in to the mixture of 80 mL, 40 mM SDS aqueous solution and 150 mL nitric acid, with stirring and cooling (ice bath), in period of 10 min. Stirring was continued for another 10 min and the reaction mixture was poured into 700 mL of water. The precipitated crude product was collected by filtration. It was recrystallized three times from Me_2CO to give 50 g (90%) of 6-nitro isomer (**XVII**) from mother liquor and the other isomer **XVIII** (5 g) was isolated with the help of methanol. It was purified by recrystalization.

Analysis for **XVII**: mp 92 °C. ^{1}H NMR (DMSO-d6, TMS, 200 MHz) δ (ppm): 5.9 (2H, s, CH_2-5); 7.5 (2H, s, CH_2-7); 7.8 (2H, s, CH_2-3); 10.30 (1H, s, –CHO). ^{13}C NMR (DMSO-d_6, TMS, 50 MHz) δ (ppm): 101.3 (C-5); 101.9 (C-3); 116.1 (C-7); 119 (C=O); 125.5 (C-1); 142.7 (C-2); 155.5 (C-8); 155.7 (C-9). IR (KBr) cm^{-1}: 1682, 1518, 1368, 1336, 1126, 1119, 1032, 1021.

Analysis for **XVIII**: ^{1}H NMR (DMSO-d_6, TMS, 200 MHz) δ (ppm): 5.9 (2H, s, CH_2-5); 7.6 (2H, s, CH_2-7); 8.3 (2H, s, CH_2-2); 9.90 (1H, s, –CHO). ^{13}C NMR (DMSO-d_6, TMS, 50 MHz) δ (ppm): 31.2 (C-1); 100.5 (C-5); 119.1 (C-2); 121.5 (C-7); 135.7 (C-3); 148.2 (C-9); 150.7 (C-8); 191.5 (C=O).

Methyl-2-amino-4,5-methylenedioxy benzoic acid (XX)

To hot solution of 32 g (0.2 moles) of potassium permanganate in 800 mL of water, 40.8 g (0.2 moles) of **XVII** in 500 mL Me_2CO was added in 20 min. Me_2CO was distilled on water bath. The residual aqueous suspension was heated on steam bath for 20 min. and filtered. Filter cake was washed with water. The combined filtrate was acidified with hydrochloric acid to give 40 g (91%) of 6-nitro benzoic acid (**XIX**). It was purified by recrystalization from aqueous MeOH on Me_2CO.

Analysis for **XIX**: ^{1}H NMR (DMSO-d_6, TMS, 200 MHz) δ (ppm): 5.9 (2H, s, CH_2-5); 7.8 (2H, s, CH_2-7); 7.9 (2H, s, CH_2-3); 11.1 (1H, s, OH). ^{13}C NMR (DMSO-d_6, TMS, 50 MHz) δ (ppm): 101.5 (C-5); 110.3 (C-3); 116.6 (C-7); 119.3 (C-1); 143.1 (C-2); 155.1 (C-8); 155.2 (C-9); 169.8 (C=O).

A solution of 22 g (0.02) of **XIX** in 200 mL ethanol was subjected to autoclave under hydrogen pressure in the presence of 10% Pd-C for 2 h. The catalyst was filtered and solution evaporated to dryness to leave a yellow solid. Two recrystalizations from MeOH gave 17 g (90%) of **XX**. Analysis for **XX**: ^{1}H NMR (DMSO-d_6, TMS, 200 MHz) δ (ppm): 4 (1H, s, NH_2); 5.9 (2H, s, CH_2-5); 6.03 (2H, s, CH_2-3); 7.4 (2H, s, CH_2-7); 11.00 (1H, s, OH). ^{13}C NMR (DMSO-d_6, TMS, 50 MHz) δ (ppm): 70 (C=0); 100.5 (C-3); 101.1 (C-5); 103.7 (C-1); 116.5 (C-7); 138.8 (C-8); 144.6 (C-2); 154.7 (C-9).

Isolation of halofuginone in to cis and trans isomer

Halofuginone (recemate mixture) (2 g) was washed with water (100 mL), those washings and filtrates were saturated with sodium chloride salt. Then pH of that solution was adjusted to 9 by addition of 20% sodium hydroxide, extracted with $CHCl_3$ (3 × 10 mL). Extracts were dried on K_2CO_3, and concentrated in vacuum. Repeated extraction from ethanol gave trans- halofuginone (1.4 g) as colorless needles.

Analysis for Trans-Halofuginone : Colorless Solid

^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 8.25 (1H, s), 8.23 (1H, s), 8.17 (1H, s), 5.00 (2H, s), 3.94 (1H, q, J = 6.8 Hz), 3.08 (1H, ddd, J_1 = 9.9 Hz, J_2 = J_3 = 4.4 Hz,), 3.02 (1H, dd, J_1 = 15.9 Hz, J_2 = 4.0 Hz,), 2.86 (1H, d = J = 12.4 Hz), 2.76 (1H, ddd, J_1 = 8.4 Hz, J_2 = J_3 = 3.8 Hz,), 2.54 (1H, dd, J_1 = 15.8 Hz, J_2 = 8.3 Hz,), 2.45 (1H, dd, J_1 = 11.7 Hz, J_2 = 2.6 Hz), 1.90 (1H, m), 1.61 (1H, m), 1.42–1.23 (2H, m), 1.20 (3H, d, J = 6.9 Hz); ^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm): 20.7, 24.5, 33.4, 42.6, 44.9, 54.7, 59.1, 66.1, 69.8, 121.8 126. 9, 128.3, 131.7, 132.4, 147.3, 149.7, 158.7, 176.8, 202.8.

Anal. calcd. for $C_{16}H_{17}BrClNO_3$: C, 46.34; H, 4.13; Br, 19.30; Cl, 8.55; N, 10.13; O, 11.60 Found C, 46.20; H, 4.01; Br, 19.60; Cl, 8.50; N, 9.90; O, 11.55.

UV max (EtOH) nm (logλ): 242(4.62), 276(3.92), 285(3.90), 314(3.46), 327(3.42).

IR (KBr) cm^{-1}: 3300(OH, NH); 3075, 2940, 2820, 1705 (CO), 1685(CO), 1630, 1445, 1260, 1225, 1105, 1085, 745, 685.

The mother liquor from the recrystallization yielded a residue, which was subjected to TLC ($CHCl_3$-MeOH-H_2O, 75: 25: 2). The main band obtained was scraped off and suspended in 0.1 N hydrochloric acid so that pH of the solution became 2. Addition was done with ice cooling so that temperature should not rise above 281 K for 20 min. Then that suspension saturated with sodium chloride, pH was adjusted to 9 with the help of 20% sodium hydroxide and extracted with $CHCl_3$ (3 × 10 mL). Extracts were subjected to vacuum to give (0.5 g) cis-halofuginone.

Analysis for cis-Halofuginone: Colorless Solid

^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 8.23 (1H, s), 8.22 (1H, s), 8.15 (1H, s), 4.98 (2H, s), 4.75 (1H, d, J = 5.8 Hz), 2.96 (2H, m), 2.78 (1H, d, J = 11.5 Hz), 2.63, (1H, ddd, J_1 = 12.3 Hz, J_2 = 8.9 Hz, J_3 = 3.6 Hz), 2.43 (1H, dd, J_1 = 15.5 Hz, J_2 = 8.9 Hz), 2.33 (1H, dd, J_1 = 11.8 Hz, J_2 = 2.3 Hz), 2.09 (1H, s, br), 1.88 (1H, d, J = 11.5 Hz), 1.56 (1H, d, J = 12.5 Hz), 1.33 (1H, m), 1.19 (1H, m); ^{13}C NMR (DMSO-d_6 75 MHz) δ (ppm): 203.5, 158.6, 149.7, 147.3, 132.4, 131.7, 128.3, 126.9, 121.8, 70.7, 60.1, 54.8, 45.6, 43.7, 34.2, 25.8.

Anal. calcd. for $C_{16}H_{17}BrClN_3O$: C, 46.34; H, 4.13; Br, 19.30; Cl, 8.55; N, 10.13; O, 11.60. Found C, 46.30; H,

4.20; Br, 19.20; Cl, 8.44; N, 9.99; O, 11.50.

UV max (EtOH) nm (logλ): 243(4.60), 277(3.92), 285(3.91), 315(3.47), 328(3.40).

IR (KBr) cm^{-1}: 3290(OH, NH); 3060, 2930, 2845, 1689 (CO), 1620, 1450, 1258, 1200, 1100, 1090, 1020, 665.

Spectral Analysis of Febrifugine Entry 1 Table 1

Colorless needles,

IR $CHCl_3$: ν = 3320, 3030, 1730, 1670 cm^{-1}.

$C_{16}H_{19}N_3O_3$: C, 63.77; H, 6.35; N, 13.94 found: C, 63.60; H, 6.55; N, 14.00.

^{1}H NMR: $CDCl_3$ 500MHz δ = 1.30-1.38(m,1H), 1.48-1.57 (m,1H), 1.72-1.74(m,1H), 2.07-2.10(m,1H), 2.58(dt,1H, J=4.80,15.8 Hz), 3.29(m,1H), 4.83(d,1H, J=17.40 Hz), 4.89(d,1H, J=17.40 Hz), 7.51(dt,1H, J=1.2,8.1 Hz), 7.73 (d,1H, J=7.6 Hz), 7.78(dt,1H, J=1.2,8.1 Hz), 7.90(d,1H), 8.28(dd,1H, J=0.9,7.9 Hz).

Spectral Analysis of Derivatives of Febrifugine, Entry 2 Table 1

^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 1.55,1.45 (2H, m), 1.77,1.52 (2H, m), 2 (1H, s, OH), 2 (1H, d, -NH), 2.65,2.45 (2H, m), 2.79,2.69 (2H, m), 3.04 (1H, m), 3.22 (1H, q), 4.36 (2H, s), 5.9 (2H, d), 6.8 (1H, s), 7.3 (1H, d), 7.5 (1H, d); ^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm): 22.2, 30.6, 39.5, 46.2, 52.4, 60.2, 71.3, 101.2, 109.4, 114.0, 114.5, 140.4, 147.5, 147.8, 153.5, 161.8, 204.1.

Entry 3 Table 1

^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 1.55,1.45 (2H, m), 1.77,1.52 (2H, m), 2 (1H, s, OH), 2 (1H, d, -NH), 2.65,2.45 (2H, m), 2.79,2.69 (2H, m), 3.04 (1H, m), 3.22 (1H, q), 4.36 (2H, s), 5.9 (2H, d), 6.8 (1H, d), 7.3 (1H, d), 7.5 (1H, d); ^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm): 22.2, 30.6, 39.5, 46.2, 52.4, 60.2, 71.3, 101.2, 113.9, 114.5, 122.1, 134.4, 141.4, 147.8, 153.5, 161.8, 204.1.

Parasite Culture

The chloroquine-sensitive D6 strain and the chloroquine resistant W2 strain of P. falciparum were cultivated in RPMI 1640 medium with 6% human erythrocytes supplemented with 10% human serum [16]. The parasites were cultured in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 310 K.

In Vitro Drug Susceptibility Assay

Febrifugine and its analogs were tested in a cell-based *in vitro* drug susceptibility assay to determine if they were capable of interrupting Plasmodium cell metabolism and growth. A semi automated microdilution technique [17] was used to assess sensitivities of the parasites to the compounds selected. The incorporation of [3H] hypoxanthine into the parasites was measured as a function of the compound concentration to determine 50% inhibitory concentrations (IC50s).

In Vitro Toxicity Assay

The compounds selected were tested for their *in vitro* toxicities in two mammalian cell lines as described in literature [17].

REFERENCES

[1] (a) Koepfli, J.B.; Mead, J.F. Jr.; Brockman, J.A. An alkaloid with high antimalarial activity from dichroa-febrifuga. *J. Am. Chem. Soc.,* **1947**, *69*, 1837; (b) Zhu, S.; Meng, L.; Zhang, Q.; Wei, L. Synthesis and evaluation of febrifugine analogues as potential antimalarial agents. *Bioorg. Med. Chem. Lett.,* **2006**, *16*, 1854-8.

[2] a) Jang, C.S.; Fu, F.Y.; Wang, C.Y.; Huang, K.C.; Lu, G.; Chou, T.C. Chang Shan, a Chinese antimalarial herb. *Science,* **1946**, *103,* 59; b) Kobayashi, S.; Ueno, M.; Suzuki, R.; Ishitani, H. Catalytic asymmetric synthesis of febrifugine and isofebrifugine. *Tetrahedron Lett.,* **1999**, *40*, 2175-8; c) Kobayashi, S.; Ueno, M.; Suzuki, R.; Ishitani, H., Kim, H.S.; Wataya, Y. Catalytic asymmetric synthesis of antimalarial alkaloids febrifugine and isofebrifugine and their biological activity *J.Org. Chem.,* **1999**, *64*, 6833-41.

[3] Mital, A. Recent advances in antimalarial compounds and their patents. *Curr. Med. Chem.,* **2007**, *14*, 759-73.

[4] Murata, K.; Takano, F.; Fushiya, S.; Oshima, Y. Enhancement of NO production in activated macrophages *in vivo* by an antimalarial crude drug, Dichroa febrifuga. *J. Nat. Prod.,* **1998**, *61*, 729-33.

[5] Pines, M.; Vlodavsky, I.; Nagler, A. Halofuginone: From veterinary use to human therapy. *Drug Dev. Res*., **2000**, *50*, 371-8.

[6] Abramovitch, R.; Itzik, A.; Harel, H.; Nagler, A.; Vlodavsky, I.; Siegal, T. Halofuginone inhibits angiogenesis and growth in implanted metastatic rat brain tumor model - an MRI study. *Neoplasia,* **2004**, *6,* 480-9.

[7] Gnainsky, Y.; Kushnirsky, Z.; Bilu, G.; Hagai, Y.; Genina, O.; Volpin, H.; Bruck, R.; Spira, G.; Nagler, A.; Kawada, N.; Yoshizato, K.; Reinhardt, D. P.; Libermann, T. A.; Pines, M. Gene expression during chemically induced liver fibrosis: effect of halofuginone on TGF-beta signaling. *Cell Tissue Res.,* **2007**, *328*, 153-66.

[8] McDougald, L.R.; Seibert, B.P. Residual activity of anticoccidial drugs in chickens after withdrawal of medicated feeds. *Vet. Parasitol.,* **1998**, *74*, 91-9.

[9] Heckeroth, A.R.; Wackwitz, C.; Gawlowska, S.; Mackenstedt, U.; Kliemt, D.; Daugschies, A. Development and validation of a cell culture based assay for *in vitro* assessment of anticryptosporidial compounds. *Parasitol. Res.,* **2007**, *101*, 161-7; Pouliquen, H. Only halofuginone is authorised against Cryptosporidiosis. *Point Vet.,* **2007**, *38*, 77.

[10] Ernst, R.A.; Vohra, P.; Kratzer, F.H.; Jr. Kuhl, H. J. Effect of halofuginone (Stenorol) on chukar partridge (Alectoris chukar). *Poult. Sci.,* **1996**, *75*, 1493-5.

[11] Takeuchi, Y.; Koike, M.; Azuma, K.; Nishioka, H.; Abe, H.; Kim, H.S.; Wataya, Y.; Harayama, T. Synthesis and antimalarial activity of febrifugine derivatives. *Chem. Pharm. Bull.,* **2001**, *49*, 721-5 ; Ashoorzadeh, A.; Caprio, V. A convergent enantioselective synthesis of the anti-malarial agent (+)-febrifugine. *Synlett,* **2005**, *2*, 346-8 reference number 4 there in.

[12] Baker, B.R.; McEvoy, F.J.; Schaub, R.E.; Joseph, J.P.; Williams, J.H. An antimalarial alkaloid from hydrangea .21. Synthesis and structure of febrifugine and isofebrifugine, *J. Org. Chem.,* **1953**, *18*, 178-83.

[13] Chien, P.L.; Cheng, C.C. Structural modification of febrifugine-some methylenedioxy analogs. *J. Med. Chem.,* **1970**, *13*, 867-70.

[14] Koepfli, J.B.; Mead, J.F.; Jr. Brockman, J.A. Alkaloids of dichroa-febrifuga .1. Isolation and degradative studies. *J. Am. Chem. Soc.,* **1949**, *71*, 1048-54; Uesato, S., Kuroda, Y.; Kato, M.; Fujiwara, Y.; Hase, Y.; Fujita, T. Conformational analysis of febrifugines and halofuginones in organic solvents. *Chem. Pharm. Bull.,* **1998**, *46*, 1-5; Takeuchi, Y.; Hattori, M.; Abe, H.; Harayama, T. Synthesis of D/L-febrifugine and D/L-isofebrifugine. *Synthesis,* **1999**, *10*, 1814-8.

[15] Chhatre, A.S.; Joshi, R.A.; Kulkarni, B.D. Microemulsions as media for organic-synthesis-selective nitration of phenol to ortho-nitrophenol using dilute nitric acid. *J. Colloid interface Sci.,* **1993**, *158*, 183-7; Samant, B.S.; Saraf, Y.P.; Bhagwat, S.S. Chlorination of aromatic compounds in micellar media: Regioselectivity. *J. Colloid Interface Sci.,* **2006**, *302*, 207-13.

[16] Trager, W.; Jensen, J.B. Human malaria parasites in continuous culture. *Science,* **1976**, *193*, 673-5.

[17] Jiang, S.P; Prigge, S.T.; Wei, L.; Gao, Y-E.; Hudson, T.H.; Gerena, L.; Dame, J.B.; Kyle, D.E. New class of small nonpeptidyl compounds blocks Plasmodium falciparum development *in vitro* by inhibiting plasmepsins. *Antimicrob. Agents Chemother*., **2001**, *193*, 2577-84.

Received: 10 November, 2008 Revised: 06 February, 2009 Accepted: 06 February, 2009