Trop J Nat Prod Res, July 2019; 3(7):225-230 ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Design, Synthesis and Antimalarial Evaluation of New Trimethoxy Benzaldehyde Chalcones

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ARTICLE INFO

Article history:
Received 26 May 2019
Revised 02 August 2019
Accepted 07 August 2019
Published online 25 August 2019

ABSTRACT

Proteases are validated drug target for inhibition of *Plasmodium falciparum*, the most virulent malaria parasite. This study guided by previous reports, designed trimethoxy benzaldehyde chalcone derivatives as potential protease inhibitors and antimalarial agents. They were synthesized by Schmidt-Claisen condensation reaction. The structures of these compounds were established using Fourier transform infrared (FT-IR), Proton, Carbon-13, as well as two-dimensional nuclear magnetic resonance (NMR) spectroscopy, and Mass Spectrometry (MS). The synthesized compounds were screened for *in-vivo* antimalarial activity in mice infected with *Plasmodium berghei* parasite, using curative model. (E)-1-(2,4-dimethoxyphenyl)-3-(2,3,4-trimethoxy-phenyl) prop-2-en-1-one (**P2**) displayed a significant activity, with activity comparable to that of quinine (10 $mgkg^{-1}$) and chloroquine (25 mg kg^{-1}) at a dose of 100 $mgkg^{-1}$ in the curative test. However, (E)-1,3-bis(2,3,4-trimethoxyphenyl) prop-2-en-1-one (**P1**) and (E)-1-(2,4-dichlorophenyl)-3-(2,3,4-trimethoxyphenyl) prop-2-en-1-one (**P13**) did not show any significant activity ($p < 0.05$). Compound **P2** was found to be devoid of electron deficient ring A (benzaldehyde ring). This suggests that, electron density on the rings are not determinants for antimalarial activity of the chalcone as proposed earlier and, present compound **P2** as a candidate for further optimization and evaluation for prophylactic and suppressive activities.

Keywords: Malaria, Protease, Chalcones, Synthesis, Schmidt-Claisen condensation.

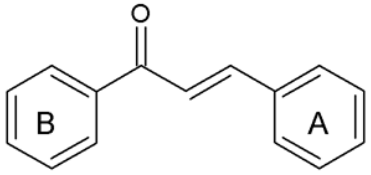
Introduction

Malaria remains a human disease of global significance and a major cause of high infant mortality in endemic nations. In Nigeria, the disease still remains a major cause of death with a morbidity rate of, around 110 million of clinically diagnosed cases.[1] It is also associated with significant socio-economic burden.[2]

Parasites of the genus *Plasmodium* cause the disease by degrading human hemoglobin as a source of amino acids for their growth and maturation.[3] To date, clinically relevant resistance has emerged, towards all classes of antimalarial drugs except for the artemisinins. However, prominent reports have recently noted delayed parasite clearance suggestive of decreased drug sensitivity in Southeast Asia.[4] Hence, new antimalarials with novel targets are urgently needed to combat the disease.

One of the most studied potential antimalarial drug targets are the proteases.[3,5, -9] Proteases have key roles on the degradation of host's hemoglobin within the food vacuole of blood-stage parasites, a process essential for the survival of the parasite.[3]

Interestingly, chalcone (Figure 1) was identified through molecular modeling studies as *Plasmodium* parasite cysteine protease inhibitor[10] and was also isolated from Chinese licorice roots as potent antimalarial compound.[11] Since then, there was substantial interest by the scientific community to develop chalcone as an effective antimalarial drug. Various derivatives have been isolated from plants or synthesized and were found to possess promising antimalarial activity.[12-17] Thereafter, several efforts were made to determine the features necessary for the antimalarial activity by optimization through quantitative structure activity relationship (QSAR) and molecular modeling studies.[9,18,19] The strong dependence of antimalarial activity on the specific substitution of rings **A** (Benzaldehyde ring) &**B** (Acetophenone ring) was noted. This led to a proposal which was put forward by Li *et al*, [10] on the importance of electron withdrawing substituents on ring **A** (decreased electron density) and electron donating substituents on ring **B** (increased electron density) for good antimalarial activity of chalcones. Similarly, Liu *et al*. [20,21] and Go *et al*. [22] synthesized a series of substituted chalcones as antimalarial agents. From structure activity relationship (SAR) analysis, size and hydrophobicity of substituents on both rings of chalcones were identified as critical parameters.



(2*E*)-1,3-diphenylprop-2-en-1-one

Figure 1: General Structure of Chalcone

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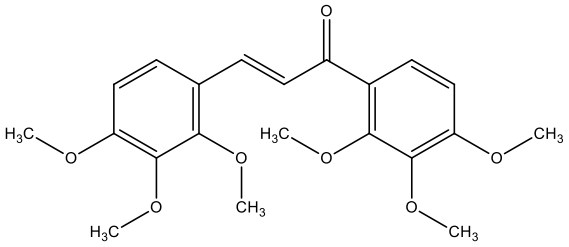
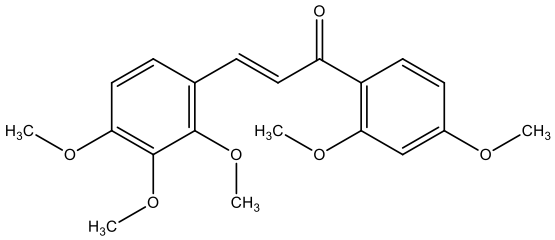
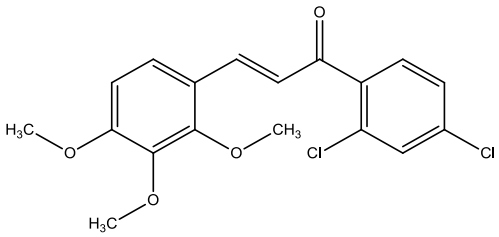
Citation: Hamza AN, Idris AY, Musa AM, Olorukooba AB. Design, Synthesis and Antimalarial Evaluation of New Trimethoxy Benzaldehyde Chalcones. Trop J Nat Prod Res. 2019; 3(7):225-230. doi.org/10.26538/tjnpr/v3i7.2

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Table 1: Chemical description of P1, P2, and, P13

Compound ID	Molecular formula	2D- Representation/IUPAC Name
P1	$C_{21}H_{24}O_7$	 (*E*)-1,3-bis(2,3,4-trimethoxyphenyl)prop-2-en-1-one
P2	$C_{20}H_{22}O_6$	 (*E*)-1-(2,4-dimethoxyphenyl)-3-(2,3,4-trimethoxyphenyl)prop-2-en-1-one
P13	$C_{18}H_{16}Cl_2O_4$	 (*E*)-1-(2,4-dichlorophenyl)-3-(2,3,4-trimethoxyphenyl)prop-2-en-1-one

However, in a study conducted by Kumar *et al*, [23] it was observed that chalcones which are methoxylated in their aryl ring **A** and electron deficient at ring **B** are better antimalarials than those in which these groups are interchanged. These observations clearly indicate that increase in the electron density on ring **A** significantly enhanced the antimalarial activity which is evidently in contrast to earlier reports linking potent antimalarial activity with electron deficient ring **A** of chalcones.[10] Thus, there is no clear conclusion of the substitution pattern on the chalcone rings that confer better antimalarial activity.
Therefore, this research studied previous works [10,21,23] to design other chalcone derivatives not previously studied with substitution contrary to Li *et al.* [10] proposal and, evaluated for *in vivo* antimalarial activity. Ring A was substituted with 2,3,4 trimethoxy group (electron dense ring A) in all the three compounds synthesized and the other aromatic ring (ring B) was substituted with groups of varying electronic character (Table 1).

Materials and Methods

General Experimental Procedure
Melting points were determined in a gallenklamp melting point apparatus, and are uncorrected. Fourier Transform Infrared (FTIR) spectra were determined on Agilent model 470 spectrophotometer, and data were reported as wave numbers (cm^{-1}). ^{1}H-NMR and ^{13}C-NMR spectra were recorded using 400 MHz Agilent and 500 MHz Bruker spectrometer and are reported as chemical shift (δ) in ppm downfield from TMS as the internal standard. Mass spectra were performed in an Agilent model 5995/Gas Chromatograph ionization energy 70 eV.

Reagents, solvents, and standard drugs
The reagents were purchased from Sigma Aldrich Germany. All the starting reagents and solvents used for the experiments were of analytical grade and were used without further purification, these include; 2,3,4-trimethoxybenzaldehyde, 2,4-dimethoxyacetophenone, 2, 4-dichloroacetophenone, 2,3,4-trimethoxyacetophenone, Sodium hydroxide (50%), Ethanol, Hydrochloric acid, 10% Giemsa stain, Acacia, Chloroquine phosphate, Quinine, Artemether.

Experimental animals
Adult mice (18 – 22 g) of either sex, locally bred in the Department of Pharmacology and Therapeutics, Ahmadu Bello University (ABU), Zaria were used for the experiment. The animals were housed in clean polypropylene cages under standard laboratory conditions and allowed free access to standard rodent pellet and water *ad libitum*. All experimental protocol on animals was in accordance with Ahmadu Bello University Research Policy and guides for the use and care of laboratory animals as accepted internationally.

Malarial parasite
Chloroquine-sensitive malaria parasites (*Plasmodium berghei* NK 65) were obtained from the Department of Microbiology- Nigerian Institute of Medical Research (NIMR), Lagos. The parasites were maintained viable in new groups of mice by continuous intraperitoneal injection of 0.2 mL of infected erythrocytes every four days.[24]

Evaluation of theoretical oral bioavailability
The oral bioavailability of the designed chalcones was predicted theoretically using Lipinski's rule of five, prior to synthesis with SWISS ADME software (http://www.swissadme.ch).

Synthesis of chalcones (Claisen –Schmidt Condensation)
All the chalcones were synthesized via base catalyzed condensation of respective equimolar amounts of substituted acetophenone and substituted benzaldehyde using NaOH as a catalyst. The chemical equations for the synthesis are as shown in Figure 2. Silica gel thin layer chromatography (TLC) was used to monitor the progress of the reaction with ethyl acetate: n-hexane (7:3) as developing solvent.[23]

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

The appearance of a single new spot and disappearance of the reactants spot indicated the formation of the product, the spots were visualized under ultraviolet light at 254 nm, and by spraying with iodine vapor.

Evaluation of in vivo antiplasmodial activity
Inoculation of Plasmodium berghei parasite
Blood was collected into a heparinized capillary tube via the tail vein from donor mouse with *P. berghei*. parasitemia level of about 20 - 25% and then transferred into a sterile plain bottle. 2 mL of the blood was diluted with 10 mL normal saline such that 0.2 mL contained approximately $1x10^7$ infected red blood cells. Each mouse was then inoculated intraperitoneally with 0.2 mL of blood suspension.[25]

Curative test in mice
Evaluation of the curative potential of the compounds against established plasmodia infection was carried out as described by Ryley and Peters.[26] Briefly, the mice were inoculated with parasites as described above and left untreated until the fourth day post-inoculation. After parasitemia was established, the mice were randomly divided into seven groups of five mice each. Groups 1 - 3 were administered intraperitoneally with 25, 50 and, 100 mg/kg body weight of the test compounds, respectively. Groups 4 - 6 (positive controls) were administered intraperitoneally with 25 mg/kg of chloroquine, 10 mg/kg of quinine, and 5 mg/kg of artesunate, respectively. Group 7 was administered with 0.2 mL of 1% w/v acacia suspension (negative control) intraperitoneally. The treatment was given for four consecutive days. On the fifth day, blood was collected from the tail vein of each mouse and smeared on a slide to make a thin film[27] for parasitemia determination as describe by Iwalewa *et al*.[28]
Average percentage parasite suppression relative to the negative control was calculated for each treatment group using the formula below;[28]

$$\%\ \textbf{Parasitemia suppression} = \frac{\text{mean parasitemia}(-\text{ve Ctl}) - \text{mean parasitemia}(+\text{ve Ctl/TC})}{\text{mean parasitemia}\ (-\text{ve control})}\ \text{x}100$$

Where; Ctl = control, TC = Test Compound.

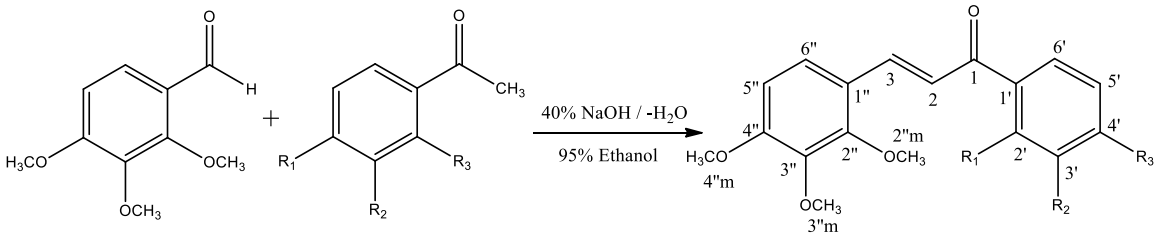
Statistical analysis
SPSS statistical software was used to determine the mean parasitemia level and standard error of the mean. The mean values of the control group were compared with the mean values of the groups treated with the test compounds using one-way analysis of variance (ANOVA) test, followed by Bonferroni post-hoc test for statistical significance and P values <0.05 were considered significant. % parasitemia suppression for each group was computed.

Results and Discussion

Synthesis and Characterization
P1 - (E)-1,3-bis(2,3,4-trimethoxyphenyl) prop-2-en-1-one
Yellow viscous liquid; Mole ratio of reactants 0.01; % Yield 57; R_f value 0.38 (n-Hexane: Ethyl acetate (7:3)); FT-IR (ν cm^{-1}) 3090 (Ar CH), 2937 (=CH), 2840 (-CH), 1647 (C=C), 1587 (CO), 1267 and 1010 (C-O-C); ^{1}H-NMR $CDCl_3$ – δ 7. 86 (d, 1H, *J* = 16.0 Hz, H3), 7.41 (d, 1H, *J* = 9.0 Hz, H6''), 7. 38 (d, 1H, *J* = 15.0 Hz, H2), 7.33 (d, 1H, *J* = 8.8 Hz, H6'), 6.70 (d, 1H, *J* = 8.8 Hz, H5''), 6.67 (d, 1H, *J* = 8.88 Hz, H5'), 3.88 (s, 3H, OMe), 3.87 (s, 3H, OMe), 3.87 (s, 3H, OMe), 3.87 (s, 3H, OMe), 3.85 (s, 3H, OMe), 3.83 (s, 3H, OMe); ^{13}C-NMR 191.35 (C1), 156.71 (C4''), 155.56 (C4'), 153.67 (C2''), 153.56 (C2'), 142.1 (C3'), 142.43 (C3''), 138.42 (C3), 127.17 (C1'), 125.70 (C2), 125.65 (C6''), 123.40 (C6'), 122.22 (C1''), 107.65 (C5'), 107.27 (C5''), 62.07 (OCH_3 2''), 61.47 (OCH_3 2'), 61.03 (OCH_3 3''), 60.88 (OCH_3 3'), 56.10 (OCH_3 4''), 56.06 (OCH_3 4'); Mass spectrum (m/z) M^+ 389.

P2 - (E)-1-(2,4-dimethoxyphenyl)-3-(2,3,4-trimethoxyphenyl) prop-2-en-1-one
Orange crystals; Mole ratio of reactants 0.01; % Yield 78.08; R_f value 0.64 (n-Hexane: Ethyl acetate (7:3)); mp 120 – 123°C. FT-IR (ν cm^{-1}) 2999 (Ar CH), 2944 (=CH), 2838 (-CH), 1642 (C=C), 1585 (CO), 1257 and 1093 (C-O-C); ^{1}H-NMR $CDCl_3$ – δ 7. 86 (d, 1H, *J* = 16.0 Hz, H3), 7.48 (d, 1H, *J* = 9.0 Hz, H2), 7. 34 (d, 1H, *J* = 9.0 Hz, H6''), 7.33 (d, 1H, *J* = 8.5 Hz, H6'), 6.70 (d, 1H, *J* = 9.0 Hz, H5''), 6.56 (dd, 1H, *J* = 2.59 Hz, 9.0 Hz, H5'), 6.49 (d, 1H, *J* = 2.0 Hz, H3'), 3.92 (s, 3H, OMe), 3.89 (s, 3H, OMe), 3.89 (s, 3H, OMe), 3.88 (s, 3H, OMe), 3.86 (s, 3H, OMe); ^{13}C-NMR 191.17 (C1), 164.04 (C4'), 160.38 (C2'), 155.48 (C4''), 153.79 (C2''), 142.62 (C3''), 137.62 (C3), 132.92 (C6'), 126.60 (C2), 123.68 (C6''), 122.78 (C1'), 122.71 (C1''), 107.75 (C5'), 105.21 (C5''), 98.85 (C3'), 61.58 (OCH_3 2''), 61.08 (OCH_3 3''), 56.22 (OCH_3 2'), 55.90 (OCH_3 4'), 55.69 (OCH_3 4'');

P13 - (E)-1-(2,4-dichlorophenyl)-3-(2,3,4-trimethoxyphenyl) prop-2-en-1-one
Yellow gummy solid; Mole ratio of reactants 0.01; % Yield 65.40; Rf value 0.67 (n-Hexane: Ethyl acetate (7:3)); mp 92 – 94°C. FT-IR (ν cm-1) 3093 (Ar CH), 3000 (=CH), 2847 (-CH), 1621 (C=C), 1580 (CO), 1274 & 1095 (C-O-C); ^{1}H-NMR $CDCl_3$ – δ 8.00 (d, 1H, *J* = 16.0 Hz, H3), 7.74 (d, 1H, *J* = 9.6 Hz, H6'), 7. 73 (d,1H, *J* = 4.0 Hz, H3'), 7.64 (d, 1H, *J* = 7.6 Hz, H5'), 7.64 (d, 1H, *J* = 7.6 Hz, H6''), 7.42 (d, 1H, *J* = 16.0 Hz, H2), 7.02 (d, 1H, *J* = 7.6 Hz, H5''), 4.25 (s, 3H, OMe 2''), 4.24 (s, 3H, OMe 3''), 4.24 (s, 3H, OMe 4''); ^{13}C-NMR 192.76 (C1), 156.27 (C4''), 153.68 (C2''), 142.20 (C2'), 141.89 (C3), 137.74 (C3''), 136.45 (C1'), 132.11 (C1''), 130.35 (C6'), 129.94 (C3'), 127.15 (C5'), 124.65 (C6''), 123.80 (C2), 121.06 (C4'), 107.67 (C5''), 61.51 (OCH_3 2''), 60.80 (OCH_3 3''), 56.01 (OCH_3 4''); Mass spectrum (m/z) M^+ 367.



	R1	R2	R3
P1	OCH_3	OCH_3	OCH_3
P2	OCH_3	H	OCH_3
P13	Cl	H	Cl

Figure 2: Scheme for the synthesis of 2,3,4-trimethoxy benzaldehyde chalcones

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Three chalcone derivatives; P1, P2, and P13 that differed from each other by the substitution pattern on ring A were synthesized in a good yield. The choice of electron dense A ring chalcones as a focus for investigation is based on contradictory findings by Kumar *et al*.[23] Therefore, to verify these claims substituents on some of the active compounds from Liu *et al*.[20] findings having electron dense B ring were reversed between the two aromatic rings leading to the design of the present compounds. P1 and P2 are new chalcone derivatives not reported in any literature or chemical database as ascertained from a search on the SciFinder (www.scifinder.com). The three compounds have passed the criteria put forward by Lipinski,[29] for a chemical compound to be orally bioavailable. Prediction of theoretical oral bioavailability is an important step in the drug design process. This is to reduce the chances of drug failure at a later stage of the process due to poor pharmacokinetics. The relatively small molecular weight of Chalcones, and the various ways the core structure can be diversified depending on the substitution pattern on the two aromatic rings bestowed on them drug-like properties.[30]

P1, P2, and P13 appeared as a yellow viscous oil, orange crystals, and yellow gummy solid, respectively. This is in agreement with the appearance of natural chalcones and other chalcone derivatives reported in the literature.[10,31]

The FTIR data indicates the appearance of prominent bands at 1640 cm^{-1} (C=O stretching vibrations), 1260 and 1095 cm^{-1} (C-O-C stretching vibrations), 3040 cm^{-1} (aromatic Csp^2-H stretching vibrations), 3008 cm^{-1} (olefinic Csp^2-H stretching vibrations), 2990 cm^{-1} (aliphatic Csp^3-H stretching vibrations), 1580 cm^{-1} (C=C stretching vibrations) for both compounds. The olefinic peak observed in the region 3008 cm^{-1} is an evidence that the chalcones have been formed. Also, the characteristic α, β-unsaturated carbonyl group of the chalcones was confirmed by the prominent band between 1580 - 1587 cm^{-1}. Additionally, the methoxy function is evidenced by coupled strong vibration at 1260 and 1095 cm^{-1} for C-O-C stretching vibrations.[23]

The ^{1}H-NMR, ^{13}C-NMR, and 2D-NMR spectra were used to assign the structures of P1, P2, and P13 (Supplementary information). A noticeable change from the starting materials is the disappearance of the aldehyde and methyl ketone chemical shifts, which appear as singlet around 9.89 - 10.30 ppm and 2.50 ppm, respectively. These protons are replaced by the α, β-unsaturated ketone linker protons observed as doublets in the region of 7.38 – 7.42 ppm (H- *α*) and 7.86 - 8.00 ppm (H- *β*) with coupling constants of 15.0 - 16.0 Hz. It is evident from these coupling constants that the products formed are predominantly the trans-isomers (E-form). The configuration of the Z isomer is unstable due to the strong steric effects between the carbonyl group and the A-ring.[32] The other noticeable observations in the ^{1}H-NMR are the methoxy proton as a singlet at the region of 3.83 – 4.25 ppm. The rest of the protons appear in their expected regions with their usual coupling constants.

Additional support for the structures of P1, P2, and P13 comes from the ^{13}C-NMR spectra, where the carbonyl carbon (C=O) of the *α, β*-unsaturated ketone linker is observed in the region 191 - 192 ppm, compared to the aldehyde carbonyl group (C=O) at 195 ppm and methyl ketone carbonyl group (C=O) which appears in the region 179 ppm.[33] The α and *β*-carbon atoms with respect to the carbonyl group give rise to characteristic signals between δ 123.8 - 126.6 ppm and δ 137.6 - 141.9 ppm, respectively. The olefinic carbons (*α*- and *β*-carbon atoms) are evidence that the chalcone has been formed. The nature of carbon atoms (CH_3- or –CH) was established by means of the Distortionless Enhancement by Polarization Transfer (DEPT) experiment. The two-dimensional (COSY, HSQC, and HMBC) NMR spectroscopic data confirm the tentative structural assignment that was made using ^{1}H- and ^{13}C-NMR for P1 and P2 (new derivatives). COSY correlates two identical ^{1}H-NMR spectra; whereas, HSQC and HMBC correlate ^{1}H-NMR spectrum and ^{13}C-NMR spectrum of the same molecule at short range and long-range distances, respectively.[33] The connection between each carbon atom and the attached hydrogen atom (s) was unambiguously established using HSQC and a long-range correlation between protons and carbon was established using HMBC, which led to linking of sub-structural fragments (Supplementary information).

Mass spectral (MS) data further confirmed the structural assignment using NMR. The MS data of the compounds indicated the molecular ion peak (m/z) corresponding to their molecular weight. The molecular ion peaks $(M+1)^+$ are 389 and 368 for P1 and P13, respectively (Supplementary information).

Rane's test evaluates the curative capability of test compounds on established infection.[26] In the curative study P1, P2, and P3 did not eradicate *P. falciparum* parasite completely on four days' treatment but showed significant parasite suppressive effects. P2 showed most significant suppression (90%, $p < 0.05$) comparable to the most potent standard drug used in this study; quinine (88.5% suppression) at a dose of 100 mg/kg (Table 3). P1 with more electron density on both rings (2,3, 4-trimethoxy) exhibited low antiplasmodial activity. This is in contrast to reported good *in-vitro* activity (2.1 μM) of bis-2,4-dimethoxy chalcones.[20] From the above observation, it seems that electronic factors are not the determinant factor for the antiplasmodial activity, conformational and steric factors might be playing more role, as proposed by Liu *et al*.[21] which this study further evaluated using in-silico methods (Un-published result).

Conclusion

The chalcone derivative; P2 demonstrated the most potent antimalarial activity in vivo in mice infected with *Plasmodium berghei*, comparable to quinine. Contrary to previous findings, this work suggest that steric features are more important in deciding the antimalarial activity of chalcone derivatives than electronic feature.

Table 2: Analysis of theoretical oral bioavailability of the substituted chalcones based on Lipinski's rule of five

Compound ID	Lipinski's rule of five[b]					
	Mol.Wt[a]	HbA	HbD	nRB	MLogP	Inference
P1	388.41	7	0	9	1.36	Pass
P2	358.39	6	0	8	1.67	Pass
P13	367.22	4	0	6	3.31	Pass

(a) Molecular weight in g/mol, (b) Lipinski *et al*, 2001 (Mwt ≤ 500, MLogP ≤ 4.15, N or O ≤ 10, NH or OH ≤ 5 and number of rotatable bonds ≤ 10).[29]

Table 3: Effect of compound P1, P2 and, P13, on the parasitemia level of *P. berghei* infected mice.

Treatment groups	Dose (mg/kg)	Average parasitemia	Percentage suppression (%)
Acacia	5ml/kg	22.00 ± 1.50	0
CQ	25	3.20 ± 1.01[c]	85.45
Quinine	10	2.52 ± 0.41[c]	88.54
ART	5	5.06 ± 1.17[c]	77.00
P1	25	13.05 ± 4.40	40.68
P1	50	14.92 ± 2.29	32.18
P1	100	12.34 ± 1.15[a]	43.90
P2	25	8.28 ± 1.88[c]	62.36
P2	50	4.60 ± 0.68[c]	79.09
P2	100	2.00 ± 0.39[c]	90.90
P13	25	16.40 ± 0.40[a]	42.75
P13	50	17.40 ± 0.40	39.26
P13	100	17.74 ± 1.13	38.08

Values are presented as Mean ± SEM; Data analyzed by one-way ANOVA followed by Bonferroni 's post-hoc test; n = 5 a = $p < 0.05$, c = $p < 0.001$ versus control; CQ = chloroquine ART = artesunate.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgements

The authors wish to acknowledge the Department of Medicinal and Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria-Nigeria, for providing some of the reagents and equipment.

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