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RESEARCH ARTICLE

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Design, synthesis and biological evaluation of several aromatic substituted chalcones as antimalarial agents

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Abstract

Malaria is a communicable disease which is caused by protozoan's mainly Plasmodium species (*P. falciparum*, *P. ovale*, *P. vivax*, *P. malariae and P. knowlesi*). The increasing resistance of *Plasmodium* to available malarial drugs poses a great responsibility for the researchers in the field of malaria. To overcome this problem of resistance, this study aimed to design and synthesize a new class of antimalarial agent with chalcone as the main moiety. Chalcones, a member of flavanoid family, consist of two aromatic rings of 1,3-diphenyl-2-propen-1-one linked by a three carbon α,β-unsaturated carbonyl system. Five derivatives were designed and among them one was selected. The CC2 was then synthesized by esterification of Para amino acetophenone followed by treatment with hydrazide to form 2-(4 acetylphenoxy) acetohydrazide. This was then coupled with 2-Bromo substituted Diazotized esterified anilines, which was finally linked with substituted benzaldehyde to yield CC2. These were then structurally verified by Infra Red (IR) and Nuclear Magnetic Resonance (NMR) spectroscopy. The chalcone was then tested for in vitro growth inhibition assays using SYBR GREEN-1 Based assay and IC_{50} values were identified. The compound CC2 showed quite promising antimalarial activity by inhibiting cysteine protease enzyme. The acute toxicity studies of the compound were carried out as per OECD guideline 425 and the results showed no toxic signs and symptoms indicating CC2 as a safe and less toxic compound.

KEYWORDS

antimalarial drugs, chalcones, malaria, plasmodium, SYBR GREEN-1 based assay

1 | INTRODUCTION

Malaria, one among the most disastrous African nightmares often called as King of diseases (Tangpukdee, Duangdee, Wilairatana, & Krudsood, 2009), is a communicable disease caused by *Plasmodium* species. In humans, five species of plasmodium parasites have been reported to cause malaria namely *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium knowlesi*. However, *P. falciparum* infections were recorded the most across the globe. As per 2019 WHO report, African region still bears the highest of acquiring malaria with 93% cases in 2018. Also, almost 85% malarial cases were in 19 countries, which includes India and 18 African countries. Between 2010 and 2018, deaths due to malaria declined from 585,000 to 405,000 cases. Also, 67% malaria deaths (272,000 cases) were found in children aged below 5 years of age (World Health Organization, 2019). There are mainly 6 identified malarial vectors in India and they include *Anopheles culicifacies*, *Anopheles stephensi*, *Anopheles dirus*, *Anopheles fluviatilis*, *Anopheles minimus* and *Anopheles sundaicus* (Dash, Adak, Raghavendra, & Singh, 2007). India currently contributes for approximately 4% of the global malarial

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burden and adds to the 87% of the total malarial cases in South East Asia. Altogether an aggregate of 0.84 million confirmed cases of malaria and 194 malarial deaths were reported by the National Vector Borne Disease Control Programme (NVBDCP) in 2017. The states mainly Odisha, Chhattisgarh, Madhya Pradesh and Jharkhand reported 74.1% of the total malarial cases in the country. The peak value of malarial cases (40%) was reported from the Odisha state (Dhiman, Veer, & Dev, 2018).

Chalcones, a member of flavanoid family, consist of two aromatic rings of 1,3-diphenyl-2-propen-1-one linked by a three carbon α,β-unsaturated carbonyl system. Chalcones exposed their importance in the field of antimalarial drug discovery when licochalcone A, a natural product isolated from Chinese liquorice roots, was reported to exhibit potent antimalarial activity. After that a synthetic analogue 4-hydroxy-2-methoxy- 4′-butoxy chalcone was reported to have outstanding antimalarial activity. Afterward, a succession of natural and synthesized alkoxylated, hydroxylated, prenylated, oxygenated, quinolylated chalcones have been examined as antiplasmodial agents (Tomar et al., 2015). A systematic PubMed literature research survey conducted between January 2002 and December 2018 showed results of rising artemisinin resistance mutation. Also, the role of Genetic Algorithm (GA) toward the development of cure against malaria has been emerged. GAs are in silico models of Darwinian evolution which mimics the principals of genetic variations. GA consist of class of optimization routines that are mainly population based search metaheuristics. This method is employed to make in silico technique more robust as it has the character of mimicking natural selection (Hati, Bhattacharya, & Sen, 2015). Based on the mentioned situation, the need for new antimalarial agents has motivated the research to find synthetic molecules which are able to answer the problem. This resistance barricade could be tackled by the emergence of a versatile molecule having a virtuous antimalarial property and the Chalcones are apt one to resolve the same. These all information's guided us to synthesize chalcone to be a potent antimalarial agent. They mainly act by inhibiting the malarial cysteine protease and thus can be novel approach in antimalarial therapy. The chalcones are comparatively simple to synthesize requiring only normal laboratory conditions and also offering good yield.

The functions of Cysteine proteases in malarial parasites have been identified from studies conducted over protease inhibitors. The best function of plasmodium cysteine proteases was the characteristic hydrolysis of hemoglobin. As hemoglobin is processed, the haem portion is converted into hemozoin pigment, and the globin moiety was hydrolyzed to its constituent amino acids. Hemoglobin hydrolysis is essential to provide amino acids for parasite protein synthesis, to balance the osmotic stability of malaria parasites and to provide space for the growing intra erythrocytic parasite. Evidence regarding the cysteine proteases in the hydrolysis of hemoglobin came from studies of the effects of cysteine protease inhibitors on cultured parasites (Ettari, Bova, Zappalà, Grasso, & Micale, 2009; Rosenthal, 2004). So obstructing the Cysteine Protease can eventually destroys the *Plasmodium* species and thus Falcipain 2 (FP-2) would be a potential target for the antimalarial drug action as its inhibition leads to death of the *Plasmodium*. It can be studied by looking into consideration of FP-2 with E-64, which is an epoxide and a potential FP-2 inhibitor. The crystal structure is also available in Protein data bank as 3BPF. The E64 proceeds toward FP-2 and binds with it tightly and inhibit it (Salawu, 2018).

2 | MATERIALS AND METHODS

2.1 | Molecular docking

Five novel chalcone analogues (CC1, CC2, CC3, CC4 and CC5) were designed with pyrazolone functions (as shown in Figure 1) as cysteine protease inhibitors which could be used as a better antimalarial agent. In order to reveal the possible intermolecular interactions behind the inhibitory activities of novel chalcone derivatives, a molecular modeling study was performed using docking program Argus lab 4.0.1.version. Hence five designed compounds were docked into binding site of the enzyme cysteine protease and recorded the possible docking scores. Based on the docking score one compound was selected from the designed series with better binding affinity and binding mode.

2.1.1 | Preparation of receptor

The cysteine proteases crystal structure (PDB entry: 3BPF) was taken from RCSB Protein Data Bank (http://www.rcsb.org) which is composed of falcipain 2 and its inhibitor, E64. The ligand from 3BPF was extracted from the crystal structure. Then the protein was filtered out by removing the water molecules. The addition of hydrogen atoms was performed to correct the tautomeric and ionization states of corresponding amino acid residues. The energy minimization of the refined protein was also carried out. Then the newly modified protein structure obtained was saved in PDB format and used for future docking studies.

2.1.2 | Determination of the active site

The active site determination (PDB entry: 3BPF) was performed by using scfbio-iit.res.in: an energy-based method for the prediction of protein-ligand binding sites. The active sites determined were selected and saved as the binding site for the docking studies.

2.1.3 | Preparation of ligands

All the ligand molecules were built by using Marvin Sketch 19.17.0 version software. The structures obtained were saved in mol format which then can be imported into the work area of Argus lab 4.0.1.version. The geometry optimization was done by using Universal Force Field molecular mechanics method. Hydrogen bonds were added to

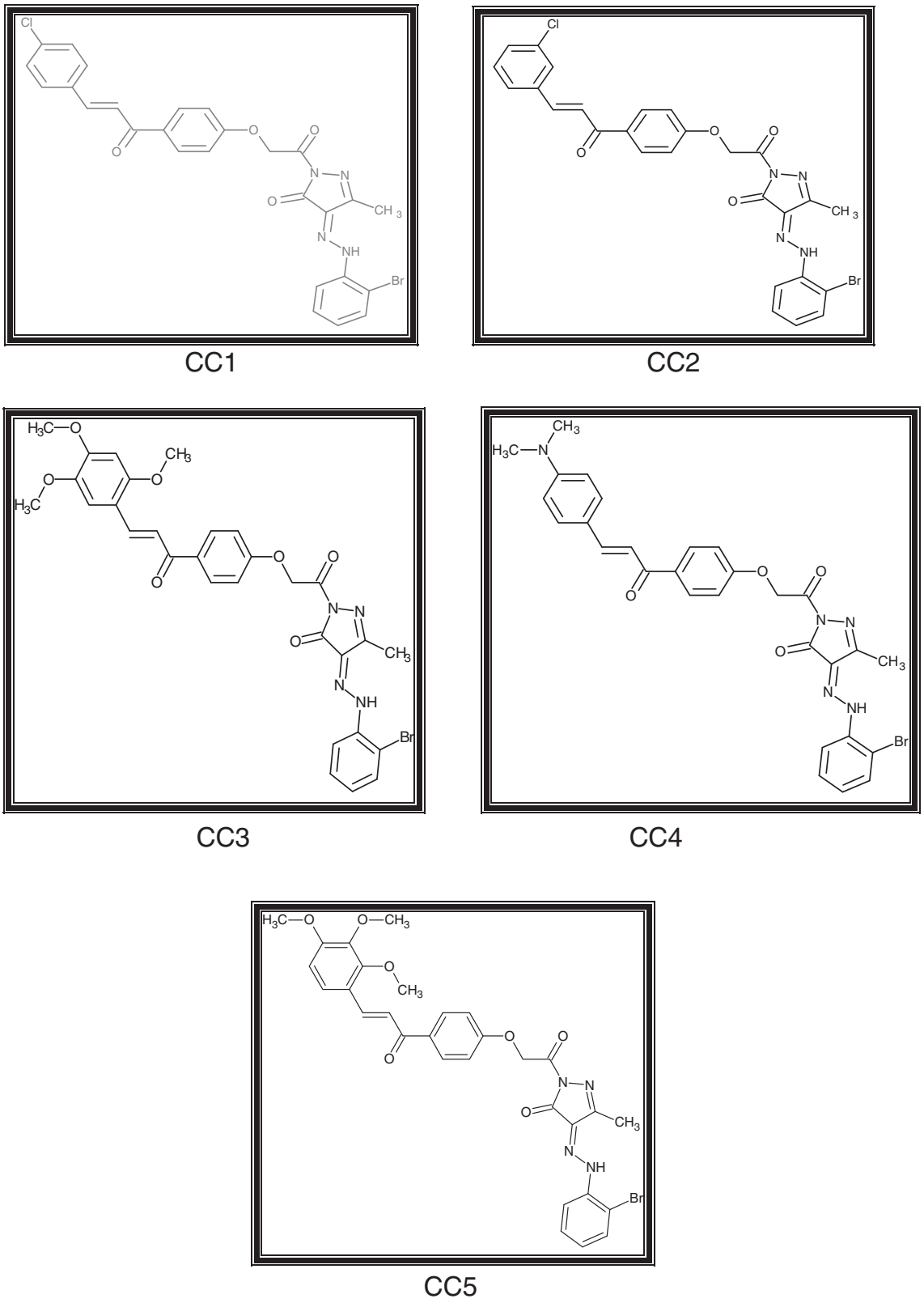


FIGURE 1 Structure of designed compounds. The pictures depict the structure of the five designed chalcone derivatives

each molecule and check the hybridization level and valency of atoms in the ligand. Clean hybridization option was done to make sure of the exact hybridization pattern of the molecule. Final geometry optimization was done by using semi empirical quantum mechanics PM3 method. The energy minimized structures were saved in PDB format for further docking studies.

2.1.4 | Docking protocol

Argus lab 4.0.1.version was used in the coupling between protein and ligand using Argus dock with a fast and simplified medium strength potential. Argus lab is a simple available molecular modeling and drug designing software, with user friendly interface and is free licensed software. The coupling of a ligand at the binding site was performed by "Argus Dock" as the coupling engine. "Regular accuracy" was selected in the coupling accuracy menu, "Dock" was selected as the type of calculation, "Flexible" for the ligand and "A score" was used for the scoring function. The junction site box was set to (26.33 × 22.66 × 17.24) angstroms to enclose the entire active binding site of the protein with a grid resolution of 0.4 Å. The coupling process is repeated until a constant coupling score value is obtained. The resulting final coupled structures were saved in PDB format and the coupling snapshots were sent into Molegro Molecular viewer 7 version 2019.7.0.0. This program can generate the amino acid residue around each segment of the synthesized final derivatives and interpret the different drug-receptor interactions, such as hydrogen bonds, hydrophobic interaction and electrostatic attraction force.

2.2 | Synthesis of 3-chloro chalcone derivative (CC2)

2.2.1 | Chemistry

All chemicals were purchased from Sigma-Aldrich. Melting Point was determined by using BUCHI-M-560 apparatus. The NMR spectra were recorded BRUCKER 500 MHz spectrometer in CDCl3/DMSO–d6/CD3OH. Chemical shifts are reported in parts per million (ppm) from Tetramethylsilane. The progress of reaction was examined by thin layer chromatography using 60F-254 precoated silica gel (Merck, India).

2.2.2 | Synthesis of ethyl (4-acetylphenoxy) acetate

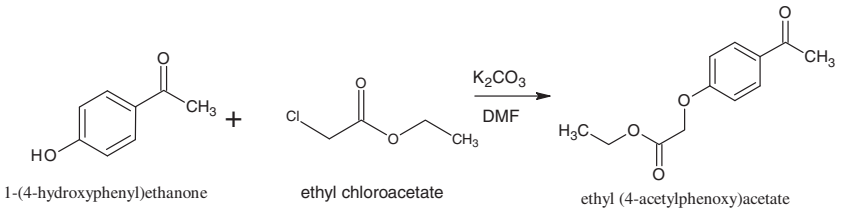
P-Hydroxy Acetophenone (5 mmol) and potassium carbonate (5.5 mmol) were taken and dissolved in Dimethyl Formamide (DMF) (5 ml) (Razavi et al., 2013). The solution was allowed to stir at room temperature for several minutes and then ethyl chloroacetate (5.2 mmol) was added drop wise to the mixture. The solution was then heated to 90 °C for 5 hr. After completion of the reaction, the mixture was allowed cool down to room temperature and diluted with cold water. The precipitate was filtered off and washed. The solid was dried and recrystallized from ethanol.

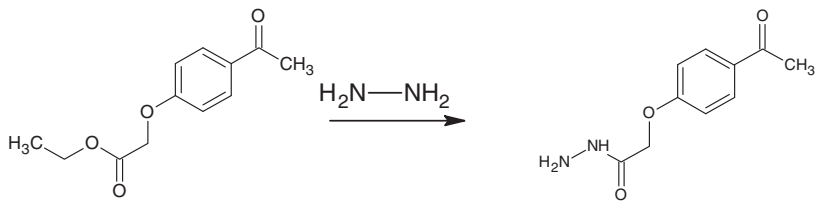
2.2.3 | Synthesis of 2-(4-acetylphenoxy) acetohydrazide

In a 500 ml round bottom flask take required quantity of ethanol (20 ml), to this add 7 g of Ethyl (4-acetylphenoxy) acetate and 1.3 ml of hydrazine hydrate (Kardile, Holam, Ankit, & Ramani, 2011). This was then refluxed for 5–6 hr. After completion of the reaction, the mixture was added into ice-coldwater, which resulted in separation of solid, which was filtered, dried and recrystallized from ethanol.

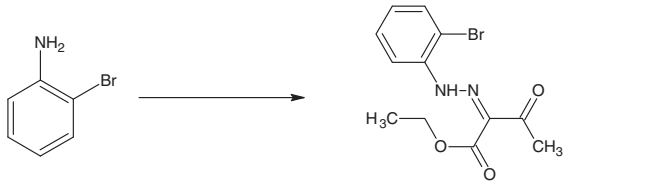
2.2.4 | Diazotization

Substituted aromatic amines (2-bromo aniline) 0.01 mol was taken in a beaker and then add 40 ml of hydrochloric acid (8 ml) and water (6 ml) (Organic Chemistry Portal, n.d.). The solution was then cooled to 0–5 °C in ice water. To this add a cold solution of sodium nitrite (0.03 mol). The diazotization salt solution was filtered directly into the cold solution of ethyl acetoacetate (0.01 mol) and sodium acetate (0.122 mol) in 50 ml of ethanol. The resultant solid was filtered off,





washed with water and then recrystallized from ethanol. The purity of the compound was established by single spot on TLC.



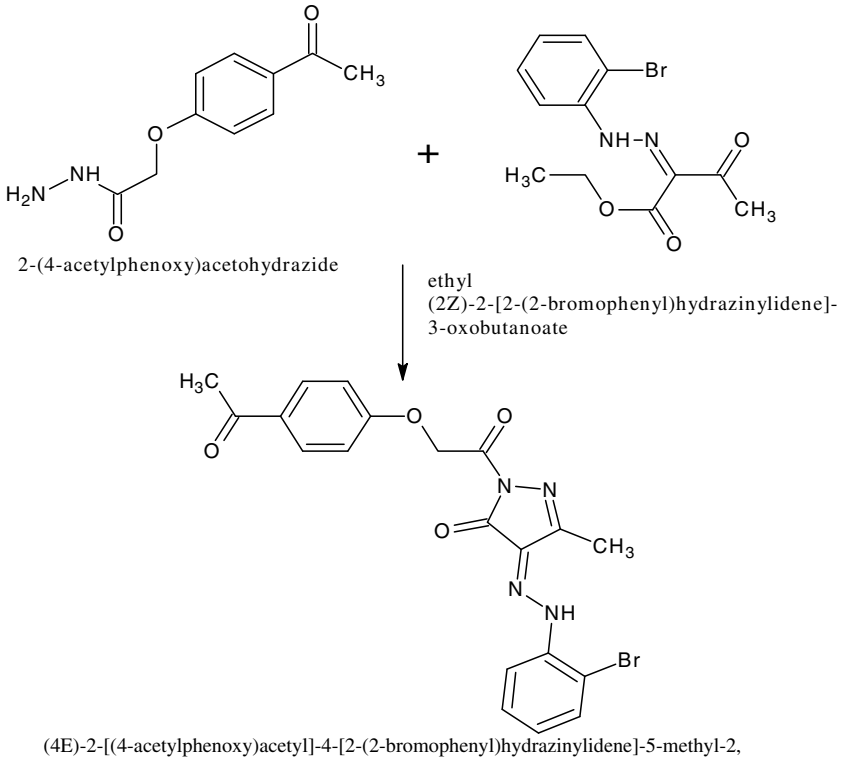
2-bromoaniline ethyl (2Z)-2-[2-(2-bromophenyl)hydrazinylidene]-3-oxobutanoate

2.2.5 | Cyclization

The two mixtures namely, diazotized and esterified aromatic substituted amines (0.002 mol) and 2-(4-acetylphenoxy) acetohydrazide(0.002 mol)in glacial acetic acid were taken in a round bottom flask and refluxed for 5 hr, cooled and then allowed to stand overnight (Delos & Detar, 1958). The product was precipitated out by addition of cold water. The resultant solid separated by filtration. It was then dried and recrystallized from ethanol. The purity of the resultant compound was determined by single spot on TLC.

2.2.6 | Chalcone synthesis

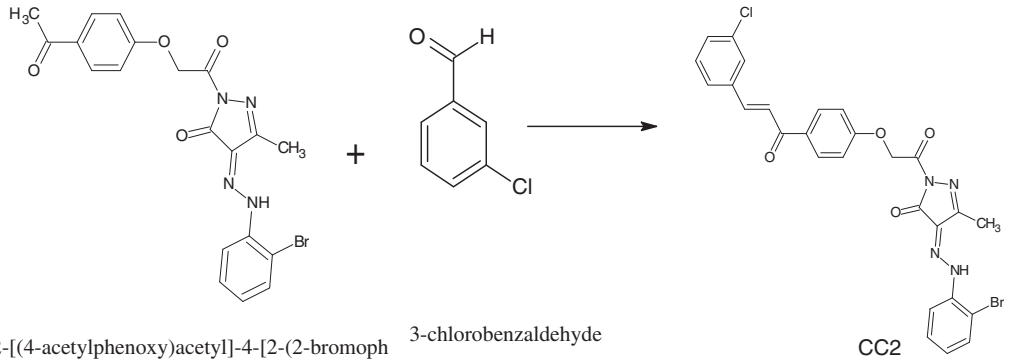
The chalcone was synthesized by reacting equimolar amount of cyclized product (10 mmol) and 3 Chloro substituted benzaldehyde (10 mmol) in ethanol (25 ml) and stirred (Syahri, Yuanita, Nurohmah, Armunanto, & Purwono, 2017). To the stirred solution 60% NaOH



2-(4-acetylphenoxy)acetohydrazide

ethyl (2Z)-2-[2-(2-bromophenyl)hydrazinylidene]-3-oxobutanoate

(4E)-2-[(4-acetylphenoxy)acetyl]-4-[2-(2-bromophenyl)hydrazinylidene]-5-methyl-2,4-dihydro-3H-pyrazol-3-one



(4E)-2-[(4-acetylphenoxy)acetyl]-4-[2-(2-bromophenyl)hydrazinylidene]-5-methyl-2,4-dihydro-3H-pyrazol-3-one

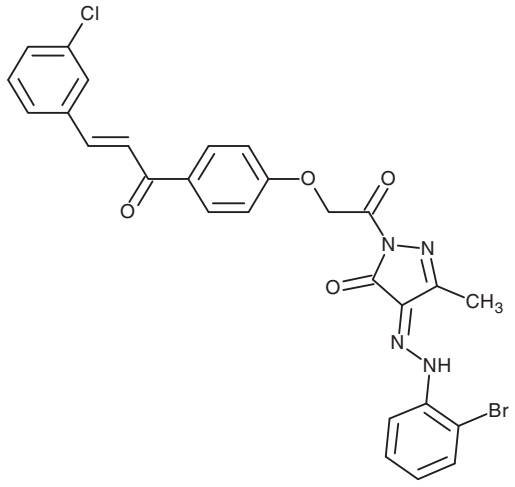
3-chlorobenzaldehyde

CC2

(15 ml) was added dropwise and stirred at room temperature overnight. After the completion of the reaction, it was poured onto crushed ice and neutralized with 2 M HCl and filtered and dried.

2.2.7 | Synthesized compound

The synthesized compound was characterized by MP, NMR and MASS spectrometric analysis, TLC was performed using 10% methanol in chloroform as mobile phase.



CC2

(2*E*)-3-(3-chlorophenyl)-1-phenylprop-2-enoyl [4-[2-(2-bromophenyl) hydrazinylidene] 5-methyl-2,4-dihydro-3*H*-pyrazol-3-one]: Amorphous powder (65%); MP- 214 to 216 °C; R_f value = 0.64 (10% Methanol:Chloroform =1:9)

^{1}H NMR- (500 MHz,DMSO-d6,δ,ppm) 2.35 (s,3H,CH_3), 4.24 (s,2H,CH_2), 6.871–6.958 (d,J = 5.5 Hz,2H,ArH),7.133(s,1H,ArH),7.365 (d,J = 8.0 Hz,1H,ArH),7.491(dd,J = 12.5 Hz,9.0 Hz,1H,ArH), 7.551(d, J = 9.0 Hz,1H,ArH), 7.569 (dd,J = 9.0 Hz,15.5 Hz,1H,ArH), 7.663 (d,J = 15.5 Hz,1H, =CH), 7.694 (dd,J = 7.0 Hz,15.0 Hz,1H,ArH), 7.78 (d, J = 7 Hz,1H,=CH), 7.918 (d,J = 8 Hz,1H,ArH), 7.947 (d,J = 15.5 Hz,1H, =CH), 7.978–8.11 (d,J = 7.0 Hz,2H,ArH), 11.777 (s,1H,NH). ^{13}C NMR-(500 MHz,DMSO-d6,δ,ppm) 189.7,170.7,163.9,162.1,148.2,145.1,140.1, 137.6,136.6,134.2,132.8,130.9,130.9,130.2,130.0,128.8,128.4,127.4, 126.5,126.1,125.1,121.3,115.3,114.8,114.8,66.4,11.5; ESI-MS: $[M + H]^+$ calculated 579.5721 found 578.0511.

2.3 | In vitro pharmacological studies

A number of antiplasmodial screening techniques have been identified, which include the [3H] hypoxanthine uptake test and the WHO micro-test (Dery et al., 2015; Rajesh Prasad, 2013). However, some of these mentioned techniques have limitations, such as radioactive waste disposal, expensive equipment, as well as a requirement for a high level of expertise and skill. So, the screening is performed by SYBR Green-1 based assay.

2.3.1 | SYBR GREEN-1 based assay

Principle

SYBR Green is an asymmetrical cyanine dye which binds directly to double strand DNA, preferring G and C base pairs. When incorporated into DNA, it is highly fluorescent, absorbs light at a wavelength between 390 and 505 nm, with a peak at 497 nm and a secondary peak near 254 nm. It emits light at 505–615 nm, with a peak at 520 nm.

Method

P. falciparum 3D7 was obtained from the Malaria Research and Reference Reagent Resource Centre (MR4), cell culture reagents and SYBR Green 1 were from Thermo Fisher Scientific, Dimethyl Sulfoxide (DMSO) was from Sigma, Albumax II was from Thermo Fisher Scientific. The RPMI 1640 medium (supplemented with 2 g/L sodium bicarbonate, 2 g/L glucose, 25 μg/ml gentamicin, 300 mg/L glutamine, 0.4% Albumax and human erythrocytes at 2% hematocrit) was used for parasite culture. Parasites were synchronized by treatment with 5% D-sorbitol at ring stage. The compound (CC2) was assessed for the growth inhibition of *P. falciparum*. The compounds CC2 (100 μg/ml) was dissolved in DMSO, and serially diluted twofold in 75 μl culture medium across rows of a 96 well tissue culture plate. DMSO (0.05%) or chloroquine (500 nM) was added to control wells. 75 μl parasite suspension (2% ring-infected erythrocytes at 4% hematocrit) was added to each well. The plate was incubated at 37 °C for 50 hr as described above. At the end of incubation, 75 μl of the culture was used for SYBR Green assay. 75 μl lysis buffer (20 mM Tris-Cl, 5 mM EDTA, 0.008% saponin, 0.08% Triton X-100, pH 7.5) with SYBR Green 1 (at 2X dilution) was added to each well, the plate was incubated at 37 °C for 30 min, and fluorescence was measured (Ex: 485 nm, Em: 530 nm, gain setting: 50) using a multimode microplate reader. Background fluorescence was corrected by subtracting the fluorescence of standard cultures (chloroquine-treated) from the fluorescence values of test compounds and DMSO. Fluorescence values of cultures treated with the compound were normalized as percentage of the fluorescence of DMSO-treated cultures (positive control). The relative fluorescence unit (RFU) for each concentration was plotted against logarithmic concentrations and analyzed using nonlinear regression analysis to determine IC_{50} concentrations.

2.4 | Toxicity studies

The toxicity studies of the synthesized compound CC2 was carried according to OECD Guidelines 425 (Up and Down Procedure) (OECD Guidelines for Testing, 2008).

TABLE 1 Docking score and molecular properties of designed compounds

Sl. No.	Compounds	Binding Energy (kcal/Mol)	Log *p*	Molecular Weight	HA	HD	Number of Violations
1	CC1	−10.9819	6.32	579.84	8	1	2
2	CC2	−10.9852	6.29	579.84	8	1	2
3	CC3	−9.4993	5.09	635.47	11	1	3
4	CC4	−9.3228	5.74	588.46	9	1	2
5	CC5	−9.5204	5.29	635.47	11	1	3

Abbreviations: HA, hydrogen bond acceptor; HD, hydrogen bond donor.

2.4.1 | Procedure

Food should be withheld overnight prior to dosing. The test substance (1 ml/100 g) is administered a single dose by gavage using stomach tube or suitable intubation cannula. One animal was given the test dose. If the animal dies, conduct the main test to determine the Lethal Dose at 50% (LD_{50}). If the animal survives, dose four additional animals sequentially so that a total of five animals are tested. However, if three animals die, the limit test is terminated and the main test is performed. The LD_{50} is greater than 2000 mg/kg if three or more animals survive.

2.4.2 | Animal procurement and observation

Female albino mice weighing between 24 and 38 g were obtained from Kerala Veterinary and Animal Science University, Mannuthy, Thrissur and housed in polycarbonate cages and was maintained at Animal House, College of Pharmaceutical Sciences, Govt. Medical College, Kannur. Institutional Animal Ethics Committee (IAEC) constituted as per CPCSEA guidelines with the clearance number CPCSEA/IAEC-18/19-01.

Animals are observed individually at least once during the first 30 min after dosing, followed by observing during the first 24 hr (with special attention given during the first 4 hr), and daily thereafter, for 14 days, except where they need to be removed from the study and humanely killed for animal welfare reasons or are found dead. Observations should include changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern. Attention should be directed to observations of tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma.

3 | RESULTS AND DISCUSSION

3.1 | Docking results

The docking studies were carried out on Argus lab 4.0.1 version. The synthesized derivative with good binding energy and showing more unique configurations were considered for further interaction studies. The binding energy and molecular parameters of all the designed structures are shown in Table 1.

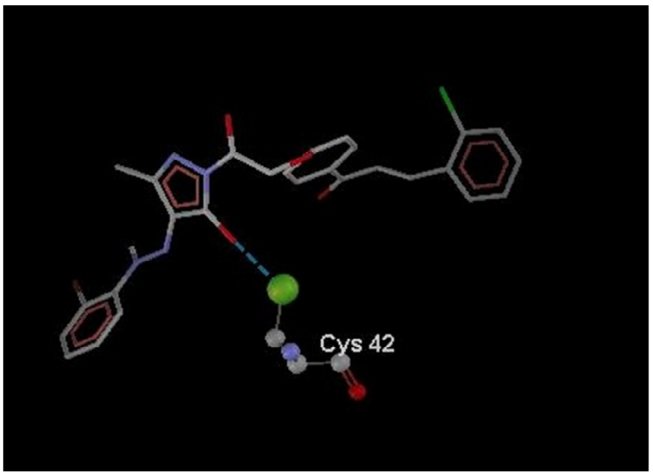


FIGURE 2 Interaction of CC2 with Cys 42 with H bonding. The figure depicts the H-bonding interaction of CC2 with the Cysteine 42 amino acid

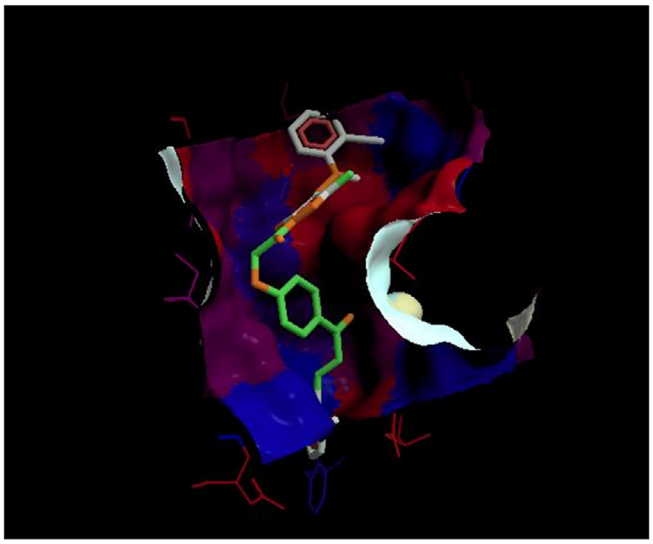


FIGURE 3 Hydrophobic interaction zone around CC2. The figure depicts the hydrophobic interaction zone around CC2 molecule

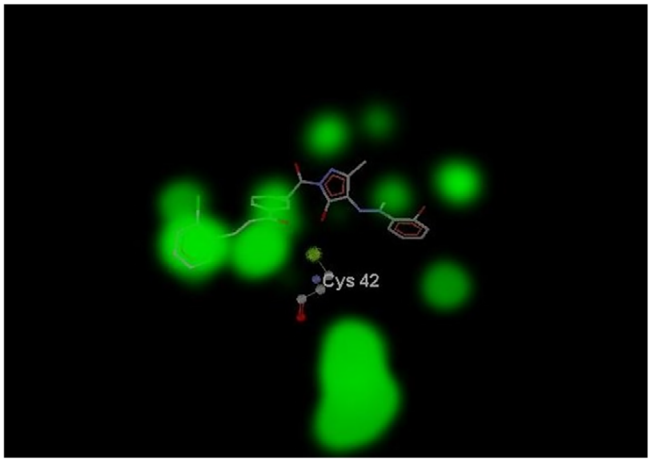


FIGURE 4 Steric hindrance zone around CC2. The figure depicts the Steric hindrance across the CC2 molecule

The docking results showed that all derivatives have almost similar binding energy modes with different docking scores. The docking study suggested that the compound CC2 showed a better binding energy than the other derivatives with a binding energy of −10.9852 kcal/mol. In the compound CC2, the pyrazole ring contributed to the H-bonding interaction with the bond length of Cys 42 (2.68 Å) with the active site of cysteine protease (Figure 2) and other interactions like hydrophobic and Steric hindrance were obtained. (Figures 3 and 4). This shows that the CC2 has comparatively less binding toward 3BPF compared to E64, as it blocks more catalytic residues like N-38, N-81, H-174, C-42, Q-171, C-39, G-40 and G-82 (Salawu, 2018). The results also show the CC2 has got significant binding toward FP-2 through Cys 42 binding.

3.2 | Pharmacological studies

3.2.1 | In vitro antimalarial activity by SYBR GREEN-1 based assay

The compound inhibited parasite growth at low microgram concentrations. The designed compound acts by inhibiting the plasmodium cysteine protease which is required for the degradation of hemoglobin into fragments. The compound was amorphous powder in nature and sparingly soluble in DMSO and the concentration was calculated as mass/volume.

The compound CC2 possess significant activity with IC_{50} concentration of 21.67 μg/ml. (see Figure 5 and Table 2). Also, the effect of the test compounds on the morphology of the parasite would add more value to the inhibition property of the compounds. The toxicity of compounds on a panel of mammalian cells needs to be determined to determine if they are specific for *Plasmodium*. The further question would be to ask the molecular mechanisms of inhibition of the parasite growth by these compounds.

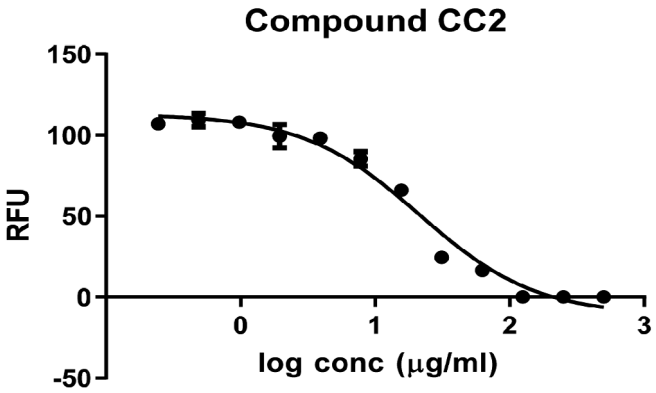


FIGURE 5 IC_{50} of CC2. The figure depicts a plot between relative fluorescence units (RFU) versus log concentration

TABLE 2 Antimalarial activity indicated by IC_{50} value

Sl. No.	Concentration	Relative Fluorescence Units (RFU)
1	2.699	0
2	2.398	0
3	2.097	0
4	1.796	16.35 ± 0.4575
5	1.495	24.46 ± 0.6357
6	1.194	65.88 ± 2.770
7	0.893	85.18 ± 6.380
8	0.592	98.11 ± 4.372
9	0.291	99.38 ± 10.24
10	−0.010	107.9 ± 0.3309
11	−0.311	109.3 ± 5.999
12	−0.612	106.9 ± 2.237
13	IC_{50} = 21.67 μg/ml	
Chloroquine (IC50 range, μg/ml) = (0.005–0.006 μg/ml)		

Note: IC_{50} value is the average of the duplicate readings with *SD*.

3.2.2 | In vivo toxicity studies

The toxicity study for the designed compound CC2 was done as per OECD guideline 425 in one swiss albino female mouse weighing 28–34 g by administering an oral dose of 2,000 mg/kg. The animal was observed for mortality as well as for behavioral changes initially during the first 30 min, followed by 24 hr, with special care given during the first 4 hr and observation continued daily for a period of 14 days. All observations were systematically recorded. After the end of 14th day, the dose was well tolerated and no morbidity features was found. So CC2 was found to be safe up to a dose 2,000 mg/kg in mouse.

4 | CONCLUSION

A group of five novel aromatic chalcone derivatives were designed and out of which a single entity (CC2) was selected on the basis of

molecular docking and Lipinski's rule of five and the compound was synthesized thereafter.

In the present study, the result obtained confirmed that the synthesized derivative was found to possess promising antimalarial property, which was proved by the in vitro SYBR GREEN-1 based assay. The designed compound acts by inhibiting the plasmodium cysteine protease which is required for the degradation of hemoglobin into fragments.

However, the effect of the test compound on the morphology of the parasite would add more value to the inhibition property of the compound. The toxicity of compound on a panel of mammalian cells needs to be determined to determine if they are specific for *Plasmodium*. Further, the molecule has to be screened for in vivo antiplasmodial studies to explore the mechanism of action and to develop the lead compound.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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