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Article

Molecular docking, synthesis and *in vitro* antimalarial evaluation of certain novel curcumin analogues

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The receptor protein *Pf*ATP6 has been identified as the common target of artemisinin and curcumin. The work was initiated to assess the antimalarial activity of six curcumin derivatives based on their binding affinities and correlating the *in silico* docking outcome with *in vitro* antimalarial screening results. A ligand library of thirty two Knoevenagel condensates of curcumin were designed and docked against *Pf*ATP6 protein and six compounds with the best binding scores were synthesized and screened for their antimalarial activity against the sensitive 3D7 strain of *Plasmodium falciparum*. ADME/Tox, pharmacokinetic and pharmacodynamic profiles of the designed compounds were analyzed and reported. 4-FB was found to have similar binding energy to the standard artemisinin (-6.75 and -6.73 respectively) while 4-MB, 3-HB, 2-HB, B, 4-NB displayed better binding energy than curcumin (-5.95, -5.89, -5.68, -5.35, -5.29 and -5.25 respectively). At a dose of 50 µg/mL all the six compounds showed 100% schizont inhibition while at 5µg/ml, five showed more than 75% inhibition and better results than curcumin. 4-FB showed the best activity with 97.8% schizonticidal activity. The *in vitro* results superimpose the results obtained from the *in silico* study thereby encouraging development of promising curcumin leads in the battle against malaria.

Keywords: Curcumin/synthesis. Curcumin/antimalarial activity. Curcumin/Knoevenagel condensates. *Pf*ATP6. Malaria. Docking. *In vitro.* ADME/Tox.

INTRODUCTION

Globally malaria is beginning to show signs of abatement, yet it still ravages millions across the globe. Its worldwide figures border on two hundred million clinical cases and half a million deaths (World Malaria Report, 2014). Several people in India succumbed to this dreaded disease in the past couple of years. Counteraction to most of the standard antimalarials is increasing at a startling rate resulting in substituting combinations of sulfadoxine and pyrimethamine with artesunate-mefloquine in many states of India (NVBDCP, 2014). Information regarding the development of resistance to the most effective drugs like artemisinin, mefloquine and piperaquine in Greater Mekong areas and south East Asian countries of Thailand and Cambodia has also come to light (World Health Organization, 2014). Resistance to artemisinins has also been recently detected in the border areas of India and Myanmar (Tun *et al.*, 2015). Thereby, newer, more potent and less toxic antimalarial leads, which can effectively cripple malaria are the need of the hour. Despite the urgent requirement of a new and potent antimalarial agent, drug discovery for malaria is an uphill task (Olliaro, 2001; Gelb, 2007). The continuous evolution of the drug discovery methods and high quality lead generation process is likely to deliver potential compounds with better therapeutic activity (Ratti, Trist, 2001). Turmeric has been widely reported as a principle component of traditional remedies for treating malaria and fever in India, Nigeria and Samoa (Odugbemi *et al.*, 2007; Uhe, 1974; Shankar, Venugopal, 1999). Curcumin 1,7-*bis*-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (diferuloyl methane), a major

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hydrophobic polyphenol is derived from the rhizome (turmeric) of the herb *Curcuma longa*. Chemically, it is a *bis*-α,β–unsaturated β-diketone that exhibits keto-enol tautomerism. It has, of late generated considerable amount of interest due to a plethora of therapeutical properties which includes antioxidant, anti-inflammatory, antimicrobial, and anticarcinogenic activities. It exhibits hepatoprotective and nephroprotective activities, suppresses thrombosis, protects against myocardial infarction, and has hypoglycemic and antirheumatic properties (Aggarwal, Harikumar, 2009; Kunnumakkara, Anand, Aggarwal, 2008; Ahsan *et al.*, 1999; Dubey *et al.*, 2008; Liang *et al.*, 2008). Moreover, it has been shown to be extremely safe at high doses in various animal and human models (Hatcher *et al.*, 2008). Cheng *et al.* (2001) reported administration of curcumin up to 8g/day to 12g/day for a period of 3 months without any toxic effects (Cheng *et al.*, 2001) Curcumin along with artemisinin has been noted to prevent revival of malaria parasites and death in *in vivo* studies (Reddy *et al.*, 2005; Nandakumar *et al*., 2006). In combination with *Andrographis paniculata* and *Hedyotis corymbosa* extracts, it has shown a synergistic effect *in vitro* and also in *in vivo* rodent malaria models (Mishra *et al.*, 2009). Recent studies have brought to light the efficacy of curcumin against *Plasmodium falciparum* cultures and *Plasmodium berghei*-infected mice (Manohar *et al*., 2013). Derivatives and analogues of curcumin have been proclaimed to have improved efficacy against *P. falciparum* cultures than the parent molecule (Mishra *et al.*, 2008). Replacement or removal of the phenolic group leads to a loss of activity which suggests that two unsubstituted phenolic groups are necessary for curcumin's antimalarial activity (Eckstein *et al*., 2003). *P. falciparum* Ca(2+)-ATPase (*Pf*ATP6) the parasite orthologue of mammalian Sarcoplasmic–Endoplasmic Reticulum $Ca2^{+}$–ATPase (SERCA), has been confirmed to be the molecular target of artemisinins, the most potent of all antimalarials. Through docking simulation studies, it was found that curcumin effectively inhibited *Pf*ATP6 through hydrophobic interactions and hydrogen bonds, leading to its antimalarial action (Knoevenagel, 1898). Though studies targeting the *Pf*ATP6 protein with curcumin and artemisinin have been individually proclaimed (Hong-Fang, Liang, 2009), *in silico* studies on a series of curcumin derivatives, their consequent synthesis based on binding energies and their correlation with *in vitro* antimalarial studies as not been reported earlier. Knoevenagel condensates are products obtained by the Knoevenagel condensation reaction, which is a nucleophilic addition of an active hydrogen compound to a carbonyl group followed by a dehydration reaction in which a molecule of water is eliminated. The study involved designing and synthesizing Knoevenagel condensates of curcumin as per their binding affinities to the *Pf*ATP6 protein, interpretation of the ADME/Tox, pharmacokinetic, pharmacodynamic data and in *vitro* antimalarial assessment in the hope of obtaining new drug candidates with potent schizonticidal activity.

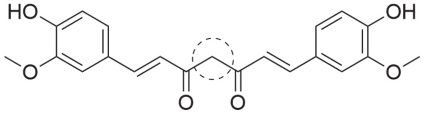
MATERIAL AND METHODS

Target identification

The protein *Pf*ATP6, also known as *Pf*SERCA is a 139 kDa protein composed of 1228 amino acids which share 51% identity with mammalian SERCA protein and has been proved to be a major molecular drug target of artemisinin antimalarials (Eckstein *et al*., 2003). Hong-Fang Ji and Liang Shen used *Pf*ATP6 as drug target for curcumin binding which indicated its interactions with *Pf*ATP6 through hydrophobic and hydrogen bonds and its subsequent inhibition (Hong-Fang, Liang, 2009). As a result, in the present study derivatives of curcumin were designed and targeted against *Pf*ATP6 to unveil their antimalarial potential. In absence of crystallographic structure of *Pf*ATP6, the modeled structure of *Pf*ATP6, (PDB ID: 1U5N) designed by Krishna and Salas-Burgos based on open conformation template 1SU4 was used (Krishna *et al*., 2009; Salas-Burgos *et al*., 2004)

Ligand dataset preparation and optimization

A ligand library of 32 curcumin derivatives was designed using ChemOffice 2010 (designing software). Selection of the ligands was based on structural similarity to the parent compound curcumin. The parent curcumin structural skeleton was retained, and modification was made via the addition of an aryl aldehyde to the active hydrogens at position 4 of the curcumin nucleus (Figure 1) in the hope of increasing its antimalarial efficacy. Table I shows the positions of different substituents on the parent curcumin molecule. The positions of the different substituents are indicated in Figure 2. The "Prepare ligand" protocol of DS3.5 was used to prepare the ligands which removes duplicate structures, standardizes the charges



(1*E*,6*E*)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione

FIGURE 1 - Curcumin and the position of its modification.

TABLE I - Data set of compounds used for docking study

Sl No.	Ligand	R_1	R_2	R_3	R_4	R_5
1.	B	H	H	H	H	H
2.	2-HB	OH	H	H	H	H
3.	3-HB	H	OH	H	H	H
4.	2,4-DHB	OH	H	OH	H	H
5.	3,4,5-THB	H	OH	OH	OH	H
6.	3-MEOB	H	OCH_3	H	H	H
7.	4-MEOB	H	H	OCH_3	H	H
8.	2,5-DMEOB	OCH_3	H	H	OCH_3	H
9.	4-H,3-MEOB	H	OCH_3	OH	H	H
10.	4-FB	H	H	F	H	H
11.	3,4-DFB	H	F	F	H	H
12.	4-MB	H	H	CH_3	H	H
13.	3,4-DMB	H	CH_3	CH_3	H	H
14.	2,4-DMB	CH_3	H	CH_3	H	H
15.	4-CLB	H	H	Cl	H	H
16.	2,3-DCLB	Cl	Cl	H	H	H
17.	4-NB	H	H	NO_2	H	H
18.	4-DMAB	H	H	$N(CH_3)_2$	H	H
19.	3-NB	H	NO_2	H	H	H
20.	4-ISOPB	H	H	$(CH_3)_2CH$	H	H
21.	4-H,3,5-DMB	H	CH_3	OH	CH_3	H
22.	2,4,5-TMB	CH_3	H	CH_3	CH_3	H
23.	4-EOB	H	H	OC_2H_5	H	H
24.	2,4,6-TMB	CH_3	H	CH_3	H	CH_3
25.	4-EB	H	H	C_2H_5	H	H
26.	3-F,4-MB	H	F	CH_3	H	H
27.	3-F,2-MB	CH_3	F	H	H	H
28.	4-CNB	H	H	CN	H	H
29.	4-AB	H	H	NH_2	H	H
30.	2,4-DNB	NO_2	H	NO_2	H	H
31.	4-MTB	H	H	SCH_3	H	H
32.	4-HB	H	H	OH	H	H

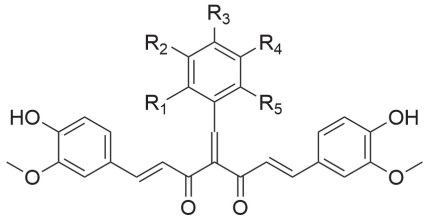


FIGURE 2 - Position of substituents on the benzaldehyde group attached to curcumin via Knoevenagel condensation.

of common groups, calculates the ions and ionization of the ligand's functional groups, generates isomers and tautomers, 2D-3D conversion, verifying and optimizing the structures, and other tasks established by user-defined parameters. Energy minimizations of all the ligands were done by applying CHARMM force field.

Docking of receptor with ligand

*Pf*ATP6 was used as receptor molecule for the docking study to probe the binding free energy between the ligand library and receptor using AutoDock 4.2 (Morris *et al*., 2009). Autodock Tools (ADT) was used to optimize the receptor and ligand molecules. For preparation of the receptor molecule, polar hydrogens, Kollman charges and AD4 type of atoms were added, while Gasteiger charges were added on the ligands and maximum numbers of active torsions were given. AutoGrid4 was used to prepare a grid map of interaction energies around LEU 263, PHE 264, GLN 267, ILE 977, ILE 981, ALA 985, ASN 1039, LEU 1040, ILE 1041 and ASN 1042 with a grid box of 90 X 90 X 90 Å3 centered on X, Y, Z = 52.27, 16.45, 11.48 with a grid spacing of 0.375Å. The residues used in the current

study have been considered and validated as the binding pocket of *Pf*ATP6 as per earlier reports (Jung *et al*., 2005; Garah *et al*., 2009; Shandilya *et al.*, 2013). Molecular docking was performed using Lamarckian Genetic Algorithm (LGA), keeping the receptor molecule rigid throughout the docking simulation and rest of the docking parameters was set to default values. Ten different poses were generated for each ligand and scored using AutoDock 4.2 scoring functions and were ranked according to their docked energy. AutoDock Tools, PyMOL (Delano, 2002; Lerner, Carlson, 2008) and LigPlot+ (Laskowski, Swindells, 2011) were used for post docking analysis.

Chemicals and reagents

Curcumin (80%), artemisinin (98%), piperidine and benzaldehyde groups used for synthesis were obtained from Sigma Aldrich. Methanol used as a reaction medium was obtained from Qualigens. Sodium hydrogen phosphate, dichloromethane used in column chromatography and petroleum benzine, chloroform used in purification and recrystallization process were obtained from Spectrochem. All the chemicals used were of analytical grade. The chemicals used in the continuous malaria culture and antimalarial drug testing such as RPMI, sodium bicarbonate, D-sorbitol and DMSO were obtained from Sigma Aldrich. Plasma and O +ve blood were obtained from a voluntary donor.

Chemistry

Curcumin obtained from Sigma was further purified and separated from its demethoxy and *bis*-demethoxy analogs by column chromatography using Silica gel impregnated with sodium hydrogen phosphate as the solid phase and dichloromethane as the eluent (Almeida *et al.*, 2005). Synthesis of curcumin derivatives was based on a method to similar to the one used by Padhye and his co-workers (2009). Curcumin (0.01 mol) was dissolved in an adequate amount of methanol in small portions with continuous stirring. The aryl benzaldehyde groups (0.01 mol) were dissolved separately in a minimum amount of methanol. The benzaldehyde mixture is added drop wise to the curcumin solution with constant stirring. Using a pilot study, it was determined that the requirement of piperidine as a catalyst does not exceed 5%. The reaction mixture was stirred for 48 hours and the progress of the reaction periodically monitored using TLC (toluene:methanol) (Figure 3). After the stipulated time, the reaction mixture is kept overnight at 4 ^{o}C for product separation. The supernatant liquid is allowed to evaporate at room temperature. The product is extracted using a mixture of dichloromethane and water, washed repeatedly with petroleum benzine and recrystallized with chloroform to yield required products with good yields.

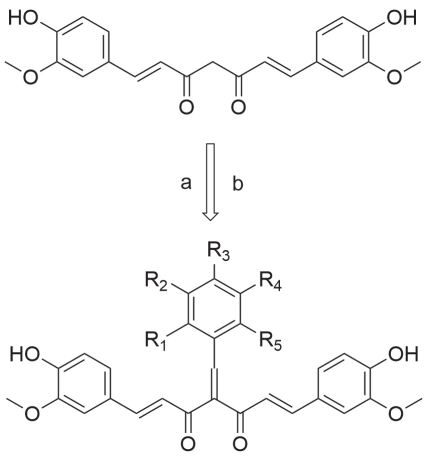


FIGURE 3 - Reaction scheme: Synthesis of Knoevenagel condensates of curcumin. Reagents and conditions: (a) Salicylaldehyde, 4-methoxybenzaldehyde, 3-hydroxy-benzaldehyde, benzaldehyde, 2,3-dichlorobenzaldehyde, 4-fluorobenzaldehyde, paranitrobenzaldehyde, 4-methyl-benzaldehyde; (b) Methanol, piperidine (<5%), 48 h.

Preparation of parasites

The sensitive 3D7 strain of *P. falciparum* was routinely maintained in stock cultures in a RPMI-1640 medium supplemented with 25 mmol HEPES, 1% D-glucose, 0.23% sodium bicarbonate and 10% heat inactivated human plasma (Trager, Jensen, 1976). The asynchronous parasites of *P. falciparum* were synchronized after 5% D-sorbitol treatment to obtain only the ring stage of the parasite. For carrying out the assay, the initial ring stage parasitaemia was maintained at 1% and 3% haematocrit.

In vitro antimalarial screening

The *in vitro* schizonticidal activity test was carried out according to the WHO Mark III protocol and the microassay methods of Reickmann and Desjardins with minor modifications (World Health Organisation, 2001; Rieckmann *et al*., 1978; Desjardins *et al*., 1979). The compounds were dissolved in 1:200 dimethyl sulfoxide (DMSO) to get a stock solution of 5mg/ml concentration. The further required dilutions of the stocks were prepared with IRPMI. To a 96-well flat bottom microtitre plate 20 microliters of the test compounds of the secondary

standard curcumin and different test dosages were charged per well in duplicates and accordingly 180 microliters of synchronized parasites in 3% hematocrit containing 1% parasitaemia was added to get the final test dose. Artemisinin as a primary standard was used for this test to validate the integrity of the assay. The negative control wells inoculated with 20 µL of CRPMI to which 180 µL of 3% hematocrit with 1% parasitaemia were added. The plates were incubated for 24 h in a water jacketed incubator at 37 °C and 5% CO_2 environment, after which thin blood smears were prepared from each well, fixed with methanol, stained with 3% Giemsa and observed under microscope. The number of schizonts (having more than 3 nuclei) was counted per 200 asexual parasites and their inhibition percentage was calculated according to the formula

% of Schizont Inhibition = (Control- Treated/Control) × 100

Ligands drug likeness, bioavailability and ADMETox

The ligand library was passed through FAF-Drugs3 (http://fafdrugs2.mti.univ-paris-diderot.fr/) for computational screening of drug likeness, bioavailability, undesirable moieties and Pan Assay Interference Compounds (PAINS). The drug likeness was analyzed as per Lipinski's rule of 5 and bioavailability was estimated as "good" or "bad" according Egan and Veber's rule (Egan, Merz, Baldwin, 2000; Veber *et al*., 2002). FAF-Drugs3 depicts the undesirable moieties as warheads (Rishton, 1997; Rishton, 2003), frequent hitters (Roche *et al.*, 2002), promiscuous inhibitors (Mcgovern *et al*., 2002), flagged or intermediate substructure as per medicinal chemistry. According to Baell and his co-workers. (Baell *et al.,* 2010), PAINS are compounds that appear as frequent hitters (promiscuous compounds) that tend to be false positive. Furthermore, PreADMET (http://preadmet.bmdrc.org/) server was used to determine mutagenicity (Ames test) (Ames *et al.*, 1972), Blood Brain Barrier (BBB) (Ma, Chen, Yang, 2005), Plasma Protein Binding (PPB) and Human Intestinal Absorption (HIA) (Yee, 1997) properties of the selected compounds.

RESULTS

Docking studies against *Pf*ATP6 showed 4-FB to have slightly higher binding energy to standard artemisinin (-6.75 and -6.73 respectively)(Figures 4 and 5) while 4-MB, 3-HB, 4-FB, B, 4-NB displayed better binding energy against the secondary standard curcumin (-5.95, -5.89, -5.68, -5.35, -5.29 and -5.25 respectively). *In vitro* screening of the compounds at 50 µg/mL concentration showed 100% schizont inhibition in all the six compounds (Figure 6). At 5 µg/mL concentration, five showed more than 75% inhibition and better results than curcumin while 4-FB showed the best activity. The *in vitro* results validate the results obtained from *in silico* study. To affirm the *in silico* protocols, two negative controls 2, 3-DCLB and 4-MEOB with low binding energy (+21.87, +1.48 respectively), were synthesized and tested for their *in vitro* activity. The results obtained at 5 µg/mL concentration (13.43, 24.77% schizont inhibition respectively) confirms efficacy of the docking protocol. They, however, showed 100% inhibition at a dose

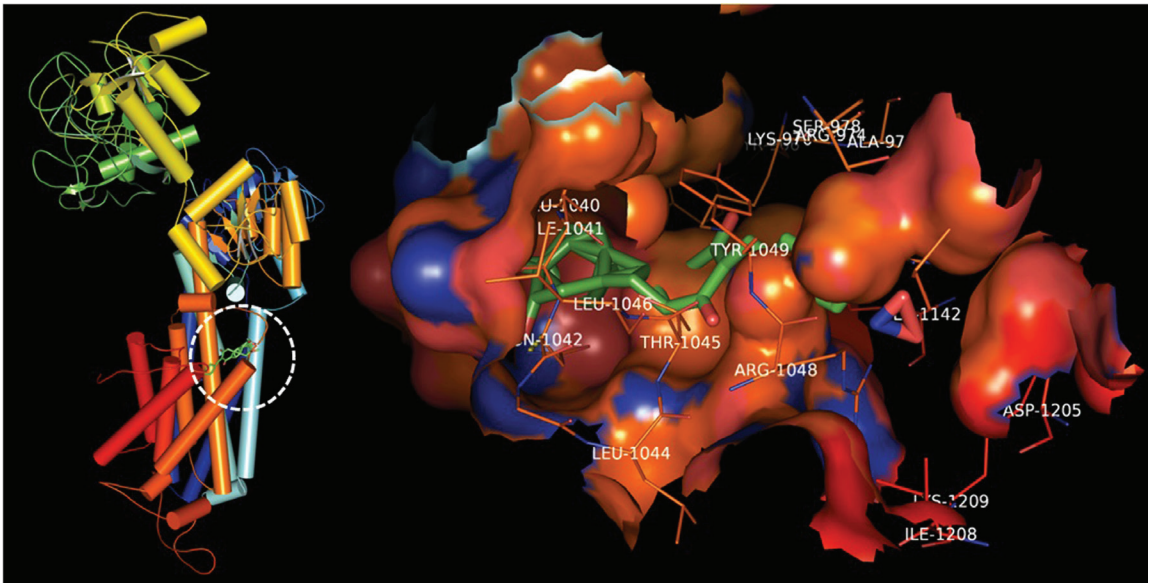


FIGURE 4 - Artemisin docked complex.

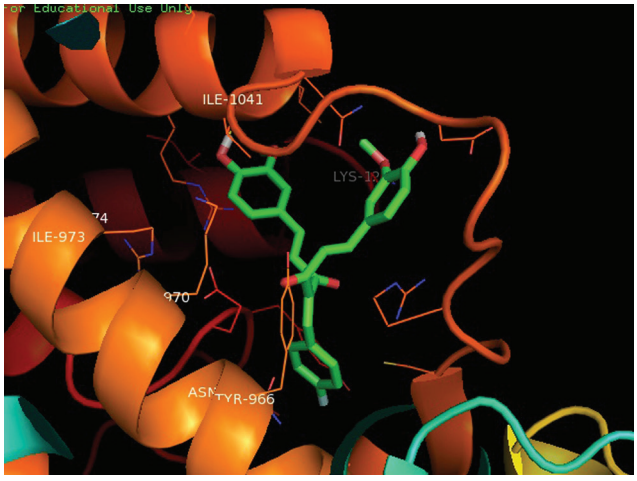


FIGURE 5 - 4-FB docked complex.

of 50 µg/mL indicating a requirement of higher dosage to produce its inhibitory activity (Table II).

Table III shows the ADME and toxicity profiles of the compounds which were analyzed using PreADMET and FAFdrugs3 online software. Of the 32 compounds two, 4-H 3,5-DMB and 3-NB showed positive for Ames mutagenicity test. The total polar surface area (tPSA) values of 29 compounds remained below 140 Å, which suggested good cell permeability capacity. Two compounds, 2,4-DNB and 3,4,5-THB had tPSA values in excess of 140 Å suggesting poor cell permeability. According to oral bioavailability rules (Egan's and Veber's rules), all the compounds fulfilled the required parameters as evident by the positive results. 4 compounds (2,3-DCLB, 2,4-DNB, 2,5-DMEOB, 4-MTB) violated more than 1 Lipinsky rules. Compounds 2,3-DCLB; 2,4,6-TMB; 2,4,5-TMB; 4-H,3,5-DMB; 4-ISOB showed slightly high Blood Brain Barrier (BBB) penetration values (2.32, 2.64, 2.61, 2.87, 2.08 respectively). Apart from 2,4-DNB the rest of the selected compounds showed significant plasma protein binding values. Compounds 4-NB and 2, 4-DNB showed 54.07% and 14.41% human intestinal absorption respectively while the rest showed above 80% absorption.

EXPERIMENTAL

General methods

Melting points were measured with a Buchi B-540 melting point apparatus and are uncorrected. IR spectra were recorded on Bruker ALPHA FT-IR spectrometer on a thin film using chloroform. ^{13}C and ^{1}H NMR spectra were recorded on Bruker Avance II 400-NMR spectrometer using tetramethylsilane (TMS) as an internal standard. Mass spectra were recorded on Waters, Q-TOF micromass (ESI-MS) spectrometer. All the commercially available reagents were used as received. All experiments were

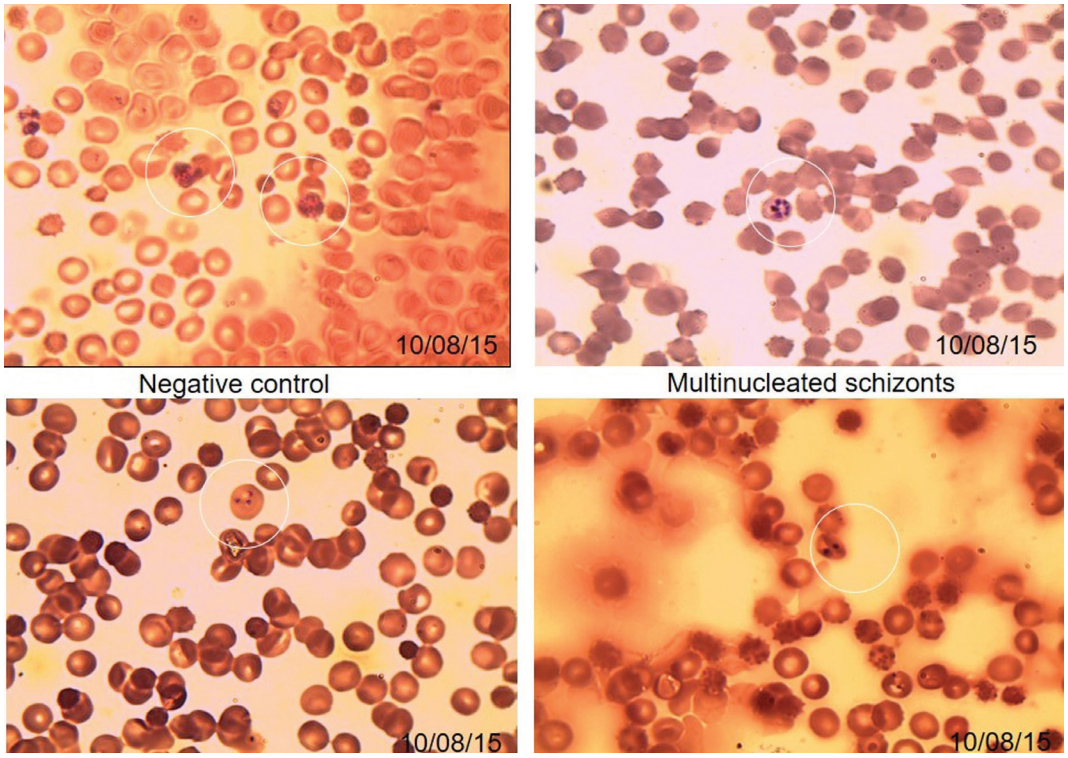


FIGURE 6 - Drug treated parasite morphology.

TABLE II - Free energy of binding and *in vitro* screening results

Sl No.	Compound na name	Free energy of binding (kcal/mol)	% Schizont Inhibition	
			5 µg/mL	50 µg/mL
1.	4-FB	-6.75	97.8	100
2.	ARTEMISININ	-6.73	100	100
3.	4-MB	-5.95	93.1	100
4.	3-HB	-5.89	89.5	100
5.	2-HB	-5.68	82.7	100
6.	B	-5.35	80.1	100
7.	4-NB	-5.29	79.9	100
8.	CURCUMIN	-5.25	79.6	100
9.	3-NB	-4.29		
10.	4-ISOPB	-4.27		
11.	3,4,5-THB	-4.22		
12.	2,4-DMB	-4.17		
13.	4-H,3,5-DMB	-4.15		
14.	4-HB	-3.87		
15.	3,4-DMB	-3.82		
16.	4-H,3-MEOB	-3.17		
17.	2,4,5-TMB	-1.82		
18.	2,5-DMEOB	-1.82		
19.	3-MEOB	-1.81		
20.	2,4-DHB	-1.79		
21.	4-EOB	-1.79		
22.	2,4,6-TMB	-1.78		
23.	4-EB	-1.75		
24.	2,3-DCLB	+21.87	13.43	100
25.	4-MEOB	+1.48	24.77	100
26.	3,4-DFB	+20.57		
27.	3-F,4-MB	+8.28		
28.	3-F,2-MB	+8.28		
29.	4-CNB	+20.72		
30.	4-AB	+25.29		
31.	4-DMAB	+16.15		
32.	4-CLB	+32.57		
33.	2,4-DNB	+2.09		
34.	4-MTB	+25.62		

monitored by thin layer chromatography (TLC). TLC was performed on prepared silica glass plates. Column chromatography was performed on silica gel (60-120 mesh, Merck Chemicals).

(1E,6E)-4-(4-fluorobenzylidene)-1,7-*bis*(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione (4-FB): TLC: toluene/methanol (4:1). Rf=0.57, yield: 76%, mp: 96–98 °C. **IR (cm^{-1}):** 3636, 1684, 1656, 1260. **^{1}H NMR ($CDCl_3$, 400 MHz):** δ 9.81 (s, 1H), 7.40 (m, 2H), 7.11 (d, 2H), 6.99 (d, 2H), 7.72 (m, 2H), 6.79 (m, 2H), 3.83 (s, 6H), 8.48 (d, 2H), 7.82 (d, 2H), 7.03 (d, 2H). **^{13}C NMR ($CDCl_3$, 400 MHz):** 162.1, 149.7, 144.9, 127.6, 128.54,

TABLE III - ADME/Tox profiles of the ligand library

Sl No.	Compds	Mol.Wt.	logP	BBB	logSw	tPSA	HIA	PPB	HB Donors	HB Acceptors	Lipinski Violation	Solubility(mg/l)	Oral Bioavailability (VEBER)	Oral Bioavailability (VEBER)	Status	Ames mutagenicity
1.	2,3-DCLB	525.38	6.58	2.32	-6.88	93.06	96.70	100	2	6	2	541.03	Good	Good	Intermediate	non-mutagen
2.	2,4-DHB	488.49	4.62	0.48	-5.41	133.52	90.35	93.85	4	8	0	2190.44	Good	Good	Intermediate	non-mutagen
3.	2,4-DMB	484.54	6.06	1.66	-6.28	93.06	95.80	90.77	2	6	1	911.31	Good	Good	Intermediate	non-mutagen
4.	2,5-DMEOB	516.54	5.27	0.08	-5.84	111.52	95.46	89.61	2	8	2	1504.85	Good	Good	Intermediate	non-mutagen
5.	3,4,5-THB	504.48	4.26	0.34	-5.28	153.75	85.02	96.95	5	9	1	2579.44	Good	Good	Intermediate	non-mutagen
6.	3,4-DFB	492.47	5.53	0.81	-6.01	93.06	95.55	95.87	2	6	1	1205.11	Good	Good	Intermediate	non-mutagen
7.	3,4-DMB	484.54	6.06	1.63	-6.28	93.06	95.80	90.65	2	6	1	911.31	Good	Good	Intermediate	non-mutagen
8.	4-AB	471.5	4.64	0.19	-5.31	119.08	93.92	91.04	4	7	0	2319.65	Good	Good	Intermediate	non-mutagen
9.	4-MEOB	486.51	5.3	0.19	-5.75	102.29	95.45	90.46	2	7	1	1548.13	Good	Good	Intermediate	non-mutagen
10.	4-CLB	490.93	5.95	1.14	-6.27	93.06	96.16	99.80	2	6	1	930.88	Good	Good	Intermediate	non-mutagen
11.	4-CNB	481.5	5.04	0.05	-5.63	116.85	95.56	91.06	2	7	1	1724.02	Good	Good	Intermediate	non-mutagen
12.	4-DMAB	499.55	5.45	0.44	-5.91	96.3	95.83	89.89	2	7	1	1351.43	Good	Good	Intermediate	non-mutagen
13.	4-FB	474.48	5.43	0.61	-5.84	93.06	95.54	94.18	2	6	1	1382.51	Good	Good	Intermediate	non-mutagen
14.	4-HB	472.49	4.97	0.72	-5.53	113.29	93.54	93.00	3	7	0	1869.61	Good	Good	Intermediate	non-mutagen
15.	4-MB	470.51	5.69	0.92	-5.97	93.06	95.67	91.07	2	6	1	1206.33	Good	Good	Intermediate	non-mutagen
16.	B	456.49	5.33	0.46	-5.66	93.06	95.53	92.18	2	6	1	1583.74	Good	Good	Intermediate	non-mutagen
17.	CURCUMIN	368.38	3.2	0.091	-3.8	93.06	99.98	88.03	2	6	0	8233.6	Good	Good	Intermediate	non-mutagen
18.	3-HB	472.49	4.97	0.75	-5.53	113.29	93.54	92.82	3	7	0	1869.61	Good	Good	Intermediate	non-mutagen
19.	2-HB	472.49	4.97	0.66	-5.53	113.29	93.54	92.16	3	7	0	1869.61	Good	Good	Intermediate	non-mutagen
20.	4-H,3-MEOB	502.51	4.94	0.44	-5.62	122.52	93.44	89.76	3	8	1	1822.88	Good	Good	Intermediate	non-mutagen
21.	2,4,6-TMB	498.57	6.42	2.64	-6.58	93.06	95.94	90.79	2	6	1	691.58	Good	Good	Intermediate	non-mutagen
22.	3-F,2-MB	488.5	5.6	1.23	-5.96	93.06	95.51	93.78	2	6	1	1266.51	Good	Good	Intermediate	non-mutagen
23.	3-F,4-MB	488.5	5.79	1.21	-6.14	93.06	95.68	92.65	2	6	1	1051.86	Good	Good	Intermediate	non-mutagen
24.	4-MTB	502.58	5.84	0.12	-6.19	118.36	95.84	95.84	2	6	2	1030.17	Good	Good	Intermediate	non-mutagen
25.	3-NB	502.49	4.53	0.01	-5.29	135.72	95.20	89.94	2	9	1	2521	Good	Good	Intermediate	mutagen
26.	4-NB	501.48	5.15	0.25	-5.76	138.88	54.07	85.19	2	9	2	1587.04	Good	Good	Intermediate	non-mutagen
27.	4-EB	484.54	6.12	1.60	-6.25	93.06	95.80	92.51	2	6	1	937.38	Good	Good	Intermediate	non-mutagen
28.	2,4,5-TMB	498.57	6.42	2.61	-6.58	93.06	95.94	90.81	2	6	1	691.58	Good	Good	Intermediate	non-mutagen
29.	4-H,3,5-DMB	500.55	4.86	2.87		113.29	93.76	89.62	3	7	1		Good	Good	Intermediate	mutagen
30.	3-MEOB	486.51	5.30	0.21	-5.75	102.29	95.45	90.51	2	7	1	1548.13	Good	Good	Intermediate	non-mutagen
31.	4-ISOB	498.57	6.45	2.08	-6.53	93.06	95.94	93.54	2	6	1	724.93	Good	Good	Intermediate	non-mutagen
32.	4-EOB	500.54	5.66	0.33	-5.99	102.30	95.56	90.60	2	7	1	1255.61	Good	Good	Intermediate	non-mutagen
33.	2,4-DNB	546.49	3.99	0.05		184.71	14.41	54.23	2	12	2		Good	Good	Intermediate	non-mutagen

115.4, 111.9, 116.8, 130.4, 122.9, 116.8, 183.7, 59.1, 165.8, 142.2, 146.9, 125.4. **MS (EI, m/z):** 474 (M-60+).

(1E,6E)-1,7-*bis*(4-hydroxy-3-methoxyphenyl)-4-(2-hydroxybenzylidene) hepta-1,6-diene-3,5-dione (2-HB): TLC: toluene/methanol (9:1). Rf=0.60, yield: 65%, mp: 93-96 °C. **IR (cm^{-1}):** 3645, 1700, 1645, 1250. **^{1}H NMR ($CDCl_3$, 400 MHz):** δ 9.43 (s, 1H), 10.27 (s, 1H), 7.22 (d, 2H), 7.10 (d, 2H), 6.72 (m, 2H), 6.85 (d, 2H), 7.49 (d, 2H), 7.06 (d, 2H), 6.90 (d, 2H), 3.83 (s, 3H), 7.82 (d, 2H), 7.03 (d, 2H). **^{13}C NMR ($CDCl_3$, 400 MHz):** δ 149.1, 147.9, 157.1, 120.0, 127.6, 111.9, 116.8, 117.6, 122.9, 132.9, 129.3, 121.2, 183.7, 55.1. MS (EI, m/z): 472 (M-60+).

(1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-4-(4-methylbenzylidene) hepta-1,6-diene-3,5-dione (4-MB): TLC: toluene/methanol (7:3). Rf = 0.58, Yield: 59%, mp: 82–87 °C. **IR (cm^{-1}):** 3639, 1695, 1659, 1240. **^{1}H NMR ($CDCl_3$, 400 MHz):** δ 9.49 (s, 1H), 7.11 (d, 2H), 6.99 (d, 2H), 6.79 (d, 2H), 7.59 (d, 2H), 7.39 (d, 2H), 3.83 (s, 3H), 2.41 (s, 3H), 8.48 (s, 1H), 7.82 (d, 2H), 7.03 (d, 2H). **^{13}C NMR ($CDCl_3$, 400 MHz):** δ 150.2, 146.8, 127.6, 129.9, 137.6, 109.9, 122.9, 134.4, 128.9, 183.7, 59.1, 165.8, 142.2, 21.3, 145.9, 123.4. MS (EI, m/z): 470 (M-60+).

(1E,6E)-4-benzylidene-1,7-*bis(*4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione (B): TLC: Toluene/Methanol (7:1). Rf=0.63, yield: 53%, mp: 118-121 °C. **IR (cm^{-1}):** 3646, 1687, 1651, 1249. **^{1}H NMR ($CDCl_3$, 400 MHz):** δ 9.55 (s, 1H), 7.44 (d, 2H), 6.99 (d, 2H), 7.11 (d, 2H), 6.79 (d, 2H), 7.60 (d, 2H), 7.39 (d, 2H), 7.33 (d, 2H), 3.83 (s, 3H), 8.48 (s, H), 7.82 (d, 2H), 7.03 (d, 2H). **^{13}C NMR ($CDCl_3$, 400 MHz):** δ 149.8, 145.9, 128.2, 132.6, 110.9, 116.8, 122.9, 129.5, 128.6, 127.9, 183.7, 55.3, 165.8, 142.2, 146.9, 123.4. MS (EI, m/z): 456 (M-60+).

(1E,6E)-1,7-*bis*(4-hydroxy-3-methoxyphenyl)-4-(3-hydroxybenzylidene) hepta-1,6-diene-3,5-dione (3-HB): TLC: toluene/methanol (8:1). Rf=0.61 Yield: 63%, mp: 79–81 °C. **IR (cm^{-1}):** 3642, 1695, 1643, 1251. **^{1}H NMR ($CDCl_3$, 400 MHz):** δ 9.61 (s, 1H), 9.45 (s, 1H), 7.11 (d, 2H), 6.99 (d, 2H), 6.83 (d, 2H), 6.70 (s, 1H), 6.79 (d, 2H), 7.16 (d, 2H), 7.31 (d, 2H), 3.83 (s, 3H), 8.48 (s, H), 7.82 (d, 2H), 7.03 (d, 2H). **^{13}C NMR ($CDCl_3$, 400 MHz):** δ 150.2, 147.1, 158.4, 135.6, 124.6, 110.5, 116.8, 115.1, 112.1, 122.9, 121.9, 130.0, 183.7, 56.1, 165.8, 142.2, 146.9, 125.4. MS (EI, m/z): 472 (M-60+).

(1E,6E)-1,7-*bis*(4-hydroxy-3-methoxyphenyl)-4-(4-nitrobenzylidene)hepta-1,6-diene-3,5-dione (4-NB): TLC: toluene/methanol (5:1). Rf=0.49, Yield: 70%, mp: 104–106 °C. **IR (cm^{-1}):** 3639, 1682, 1647, 1244. **^{1}H NMR ($CDCl_3$, 400 MHz):** δ 9.55 (s, 1H), 7.11 (d, 2H), 6.99 (d, 2H), 8.37 (d, 2H), 6.79 (d, 2H), 8.03 (d, 2H), 6.99 (d,2H), 3.83 (s, 3H), 8.62 (s, 1H), 7.82 (d, 2H), 7.03 (d, 2H). **^{13}C NMR ($CDCl_3$, 400 MHz):** δ 151.1, 148.2, 146.7, 126.6, 139.0, 112.8, 115.8, 122.8, 120.9, 133.2, 184.7, 58.1, 164.8, 141.2, 145.9, 124.4. MS (EI, m/z): 501 (M-60+).

DISCUSSION

Artemisinin has long been the backbone of antimalarial studies but of late concerns over its resistance in south-east Asian countries and Greater Mekong areas have led to newer combinations of antimalarial agents being employed. Failure in rapid clearance of parasites compromises the use of artemisinin for the treatment of severe malaria, which in turn leads to an increased risk of resistance coupled with treatment failure. As a result of which newer antimalarial agents with substantial activity and less toxicity are the need of the hour. Studies have shown that *Pf*ATP6 protein, the major target of artemisinin is also a molecular drug target of curcumin obtained from *Curcuma longa*. The efficacy of curcumin as an antimalarial agent has already been established (Cheng *et al.*., 2001). Moreover, curcumin has been found to be nontoxic in nature over very high doses (Hatcher *et al.*., 2008). The study was thereby undertaken to assess the antimalarial activity of curcumin derivatives considering *Pf*ATP6 as the drug target and was principally aimed at correlating the *in silico* docking outcome with *in vitro* anti malarial screening results in order to develop a series of curcumin derivatives with cogent antimalarial activity. 4-FB emerged as the best dock compound among the selected compounds with better binding energy than artemisinin and curcumin. At a dosage of 5µg/ml 4-FB inhibited 97.8% schizonts as compared to 100% inhibition by artemisinin which indicated its high schizonticidal activity. Similarly, 2-HB, 4-MB, 3-HB, 4-FB, B showed higher binding potential than curcumin and better inhibitory action against *Pf* cultures as evidenced through *in vitro* assay. While only eight compounds were synthesized, the binding energies of 3-NB, 4-ISOB, 2, 4-DMB, 4-HB, 3,4-DMB, 3,4,5-THB, 4-H-3,5-DMB, 4-H,3-MEOB showed promising results and are likely to be synthesized and screened for *in vitro* antimalarial activity.

In silico ADME/Tox study provided substantial information about the selected compounds regarding their pharmacokinetic and toxicity profiles. 32 compounds along with the secondary standard curcumin were screened for their pharmacokinetic, pharmacodynamic and toxicity properties.

Blood Brain Barrier (BBB) Penetration is the capacity of a compound to penetrate the endothelial cells in CNS vessels that usually restricts the passage of

solutes. 2,3-DCLB; 2,4,6-TMB; 2,4,5-TMB; 4-H,3,5-DMB; 4-ISOB showed slightly high BBB penetration values (2.32, 2.64, 2.61, 2.87, 2.08 respectively) which led to the rejection of the candidates as prospects of drug development.

Plasma protein binding of a drug influences not only on the drug's action but also its disposition and efficacy as only the unbound drug is available for diffusion or transport across cell membranes, and interaction with a pharmacological target. Through PreADMET predictions it was observed that all the compounds except 2,4-DNB demonstrated a considerable amount of binding to the plasma proteins, indicating improved attachment to the target site.

Ames test determines the mutagenicity of a compound using several strains of the bacterium *Salmonella typhimurium* that carry mutations in genes involved in histidine synthesis. The variable being tested is the mutagen's ability to cause a reversion to growth on a histidine-free medium (Ames *et al.*., 1972). TA98, TA100 and TA1535 strains are used in PreADMET toxicity predictions which are often used in Ames test. The results can be estimated both with the application of metabolite (Metabolic activation by rat liver 10% homogenate, +S9) and without application of the metabolite (No metabolic activation, -S9). Among the selected compounds only 3-NB showed chances of mutating while the rest were classified as nonmutagens.

Predicting human intestinal absorption of drugs is an important criterion for identifying potential drug candidates. It is considered the sum of bioavailability and absorption evaluated from ratio of cumulative excretion in urine, bile and feces (Yee, 1997). Through PreADMET analysis it was observed that out of the selected compounds 4-NB and 2, 4-DNB showed poor intestinal absorption than the rest indicating a lower rate of absorption of nitrogen containing compounds.

Structure activity relationship

The phenolic groups in the curcumin nucleus are generally considered for exerting their antimalarial activity. Any change in these groups has been reported to have decreased the antimalarial efficacy of the compound (Mishra *et al.*, 2008). Any changes in the parent molecule thereby would have to be made either by condensing the diketo groups or attacking the active methylene bridge of the curcumin scaffold. The latter step was chosen, and the active methylene bridge was targeted with aryl benzaldehydic groups through Knoevenagel condensation and the results were analyzed to determine increase/decrease in antimalarial activity as compared to the parent molecule. A detailed SAR analysis revealed important structural points responsible for variation in antimalarial activity. A fluorine group in the *para* position of the substituent increases activity of the compound. The presence of a hydroxyl group enhances the activity of the compound as evidenced by its binding energy and schizonticidal activity (2-HB, 3-HB). Apart from the synthesized compounds, 4-HB, 3,4,5-THB, 2,4-DHB also showed fair binding energies, thereby validating the theory. The position of the hydroxyl group in the compound does not produce any large scale differences to the results. Increasing the number of hydroxyl groups decreases the score of the compounds, which can be attributed to steric hindrance. A nitro group at the para and meta position shows moderate activity. Toxicity and plasma protein binding capacities of nitro group compounds appear to be governed by the number and position of the nitro substituents. The addition of a methyl group augments the activity of the compound. Electron donating groups enhance the activity of the compounds. Increase in the number of nitro and halogen substituent's leads to a decrease in the binding energy which can be associated to the bulkiness of the compound leading to steric hindrance. As can be seen from Figures 7 and 8 using LigPlus, both artemisinin and the synthesized curcumin derivatives were observed to fit in the exact docking pocket (Lys 1213, Leu 1044, Asn 1042, Tyr 966, Thr 1045, Lys 970, Arg 1034, Asn 967, Glu 1142) through a series of hydrophilic and hydrophobic interactions. The methoxy oxygen in the 3rd position of 4-FB (bond length 2.65 Å) and the keto group of artemisinins (bond length 2.83 Å) both show a strong hydrogen bonded interaction with the Lys 1213 residue of *Pf*ATP6. This asserts the fact that the methoxy and hydroxyl groups of the phenolic ring systems in the curcumin molecule are required for proper binding to the *Pf*ATP6 protein. Spikes indicate favorable hydrophobic interactions between 4-FB and *Pf*ATP6 which conclude that hydrophobic force is the main interaction force in the binding of the curcumin derivatives to the PfATP6 protein. Analogous binding site of curcumin and artemisinin enabled us to consider artemisinin as the primary standard for our study. The enhanced properties of the compounds maybe attributed to stronger binding affinities of the designed compounds to the *Pf*ATP6 protein then the parent molecule. Further *in vivo* studies would be required to determine the efficacy of the compounds, their toxicities and the effect of biotransformation on the compounds. Though there are reports on *in silico* studies of curcumin on *Pf*ATP6 [24] as well as the synthesis of different curcumin analogues/derivatives (Manohar *et al*., 2013; Mishra *et al*., 2008; Sahu *et al*., 2012; Zambre *et al*., 2007; Padhye *et al*., 2009; Liang *et al*., 2009; Selvam *et al*.,

2005; Ali *et al*., 2013) the two have not been interlinked in relation to antimalarial *in vitro* studies. The originality of the work lies in the fact that design and *in silico* studies on an entire series of Knoevenagel condensates of curcumin and their correlation with *in vitro* antimalarial activity have been discussed for the first time.

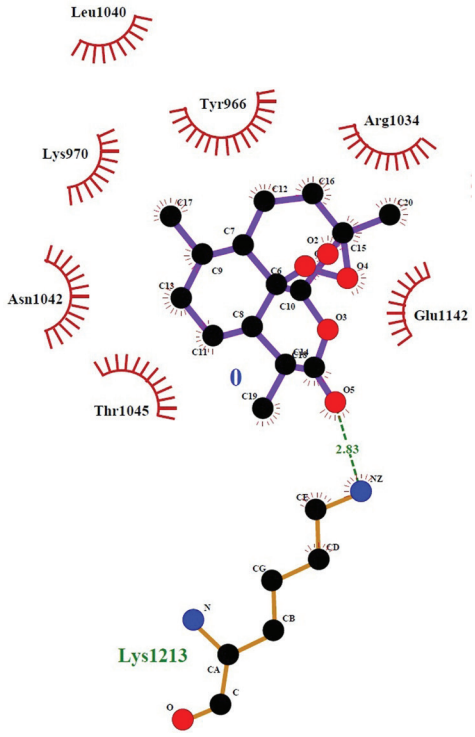


FIGURE 7 - Artemisinin docking pocket.

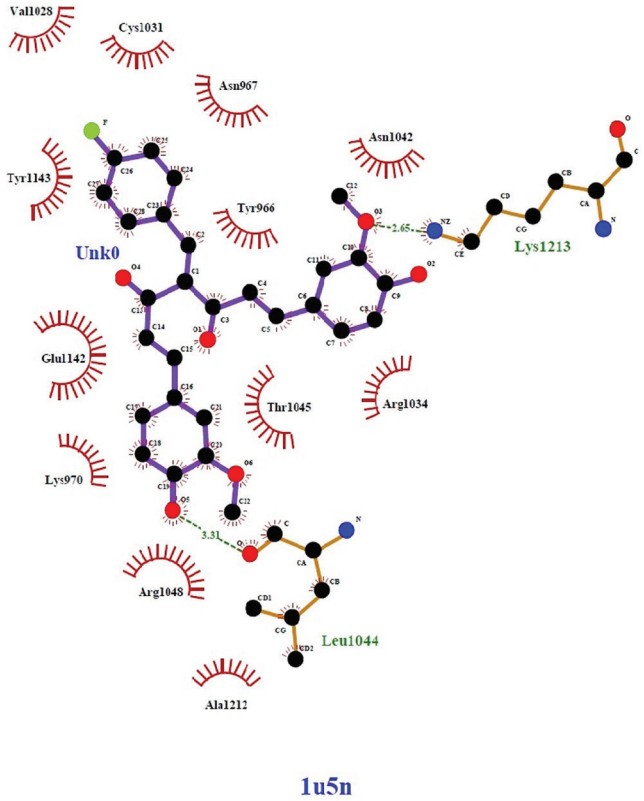


FIGURE 8 - 4-FB docking pocket.

CONCLUSION

The high binding energy of the synthesized derivatives showed that the designed curcumin analogues have good affinities for the PfATP-6 protein which is likely to be the reason for its significant antimalarial activity against the *Plasmodium falciparum* species. Though this might not be the only way in which the compounds act against the parasites, it paves the way for future molecular level studies to ascertain the mechanism. The study is likely to provide valuable insight and in-depth assessment to researchers in the design and development of curcumin derivatives/analogues as promising drug candidates in the battle against malaria.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

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