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Synthesis of thymol-based pyrazolines: An effort to perceive novel potent-antimalarials

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ABSTRACT

A series of thymol based substituted pyrazolines and chalcones was synthesized and evaluated for antimalarial activity, using *in-vitro* and *in-vivo* malaria models. All the target compounds (**5a-k** and **6a-j**) were found to be active against human malaria parasite strain *Plasmodium falciparum* NF54. Among all, compounds **5e** and **5f** of chalcone series and **6c** and **6f** of pyrazoline series exhibited prominent antimalarial activity with IC_{50} less than 3 and 2 μM respectively, while other pyrazolines also significantly inhibited the *P. falciparum* with IC_{50} less than 10 μM. The designed pharmacophores were found to be effective against *P. falciparum*. Compound **6f** was found to be able to retard malaria progression in mice. This was evident through decreased parasitemia, increased mean survival time and hemoglobin content in the treated animals. Moreover, **6f** was observed as an inhibitor of heme polymerization pathway of the malaria parasite. It also inhibited free heme degradation, which could be possibly responsible for higher reactive oxygen species (ROS) in parasite, thus inhibiting the rapid proliferation of the parasite. In addition to this, compound **6f** was found to be non-toxic with a good selectivity index. Based on these observations, the compound **6f** could be taken up for further antimalarial lead optimization studies.

1. Introduction

Malaria is one of the most important life-threatening diseases in the world apart from cancer, tuberculosis and AIDS. Despite comprehensive global efforts for eradication of malaria, latest WHO reports in 2016 revealed that there were 216 million cases of malaria and 0.4 million deaths [1]. Surprisingly, malaria still remains a major killer of children especially under five years old [1]. Recently the arsenal of antimalarial drugs viz. quinine, chloroquine, artemisinins along with newer chemotherapeutic agents artesunate and arteether is at high risk due to bioavailability issues and have also got trapped by the resistance problem [2–12]. Nowadays, artemisinins are used as most potent antimalarial drugs for curing chloroquine-resistant *P. falciparum* infections [13–16]. The dearth of new affordable drugs has complicated the clinical management of malaria in endemic areas resulting in an increased mortality rate necessitating the discovery of new natural product inspired chemical entities. It is important to mention that most of the anti-malarial drugs are from natural sources or natural product inspired *viz*, artemisinin, quinine, chloroquine, atovaquone etc. (Fig. 1) [17].

Thymol is a major natural monoterpene present in the essential oil of various plants, such as *Trachyspermum ammi* and *Thymus vulgaris, etc.* [18,19] possessing broad spectrum antimicrobial, anti-oxidant and anti-inflammatory activity along with antimalarial activity [19–21]. In earlier reports, pyrazolines have been possess a broad range of biological activities such as antibacterial [22,23], antidepressant [24], anticancer activities and antiviral activity etc. [25,26]. Recent literature revealed that these classes of compounds are not very much explored for their possible antimalarial activities [27–30]. To the best of our knowledge thymol based pyrazolines have not been reported previously for antimalarial activity.

Stupendous role of natural products in antimalarial drug design and as a part of our endeavor to develop highly efficient methods and designing of bioactive pharmacophores [31–34], we are reporting a synthesis of a new series of thymol derived pyrazoline derivatives through Huisgen zwitterion mechanism [35], their antimalarial activity and toxicity studies.

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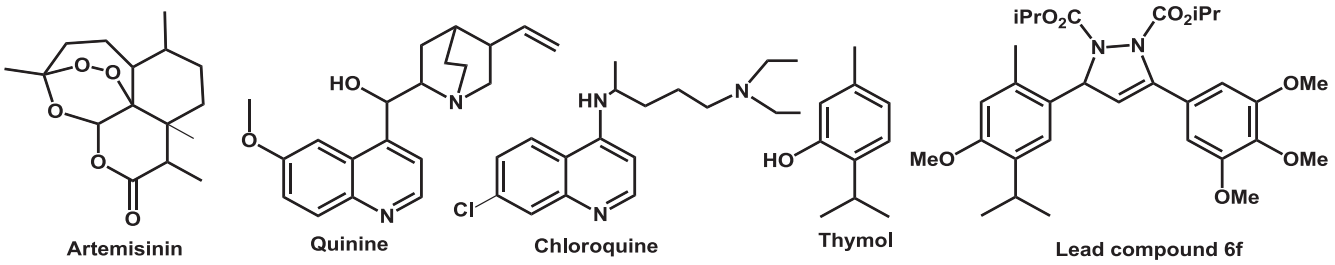
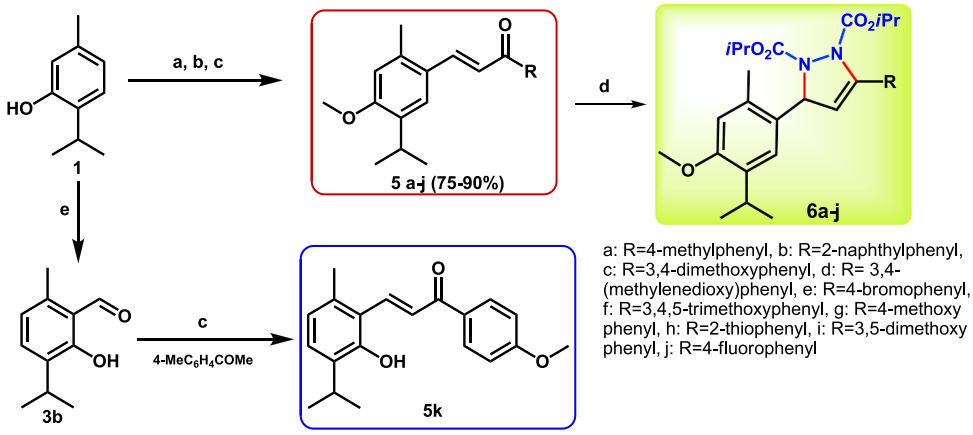


Fig. 1. Figure represents the structure of natural or natural product inspired antimalarials, thymol and lead molecule.



Reagents/conditions: (a) Dry acetone, K_2CO_3, $(CH_3)_2SO_4$ for **1** to **2**; (b) $POCl_3$, DMF, 0°C-80°C, 3h for **2** to **3**; (c) CH_3OH, KOH, 40°C, 4h for **3** to **5**; (d) Microwave reaction condition: PPh_3, Diisopropyl azodicarboxylate (DIAD), toluene, 60 watt, 100°C, 15 Min.; (e) Dry MeCN, $MgCl_2$, NEt_3, paraformaldehyde, Reflux

Scheme 1. Schematic representation of novel pyrazolines synthesis.

2. Results and discussion

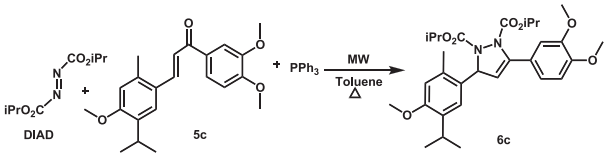
2.1. Chemistry

The synthesis of pyrazoline analogues was outlined in Scheme 1.

The chalcones were synthesised according to given method in Scheme 1 (Supplementary information Table 2). The studies were initiated to optimize the reaction conditions for a model reaction of chalcone **5c** with diisopropyl azodicarboxylate (DIAD) in the presence of a stoichiometric amount of triphenylphosphine under microwave (MW) irradiation conditions and the overall findings are given in Table 1. Interestingly, it was found that the reaction gave promising results in the toluene as well as 1,2-dimethoxyethane at 100 °C (Table 1, entries 5 and 6) under microwave conditions. To screen the effect of solvent and temperature, the model reaction was performed under microwave irradiation conditions at different temperatures in various solvents. The optimum conversion was achieved in toluene or 1,2-dimethoxyethane at 100 °C. In order to optimize the microwave power, the above reaction was also studied by varying microwave power (60 and 80 W) and it was found that 60 Watt (W) power at 100 °C was necessary to achieve maximum conversion of product **6c**.

Under the optimized set of MW reaction conditions (60 W, 100 °C), a number of chalcones **5** were subsequently allowed to react with DIAD and triphenylphosphine to afford various pyrazoline derivatives (**6a–6j**) in reasonably excellent yields in 15 min (Table 2). Interestingly, different functional groups are well tolerated in this reaction system.

Table 1
Optimization of reaction conditions for pyrazolines.[a]



S. No.	Solvent	Power (W)	Temp (°C)	Time (min.)	Yield[b] (%)
1.	PEG 400	60	100	15	nr[c]
2.	Nitromethane	60	100	15	nr
3.	Nitrobenzene	60	100	15	nr
4.	Benzene	60	100	15	55
5.	1,2-Dimethoxyethane	60	100	15	74
6.	**Toluene**	**60**	**100**	**15**	**75**
7.	Toluene	80	100	15	73
8.	Toluene	60	120	15	70
9.	Toluene	60	80	15	66
10.	Toluene	60	100	20	68
11.	Diglyme	60	100	15	23

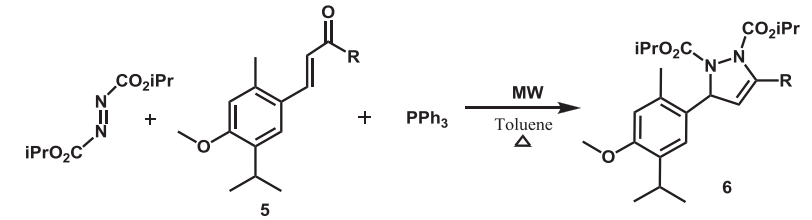
[a] Reaction conditions: **5c** (1 mmol), DIAD (1.5 mmol), PPh_3 (1.5 mmol), 15 min. 60 W, 100 °C.
[b] Isolated yield based on **5c**.
[c] nr = no reaction.

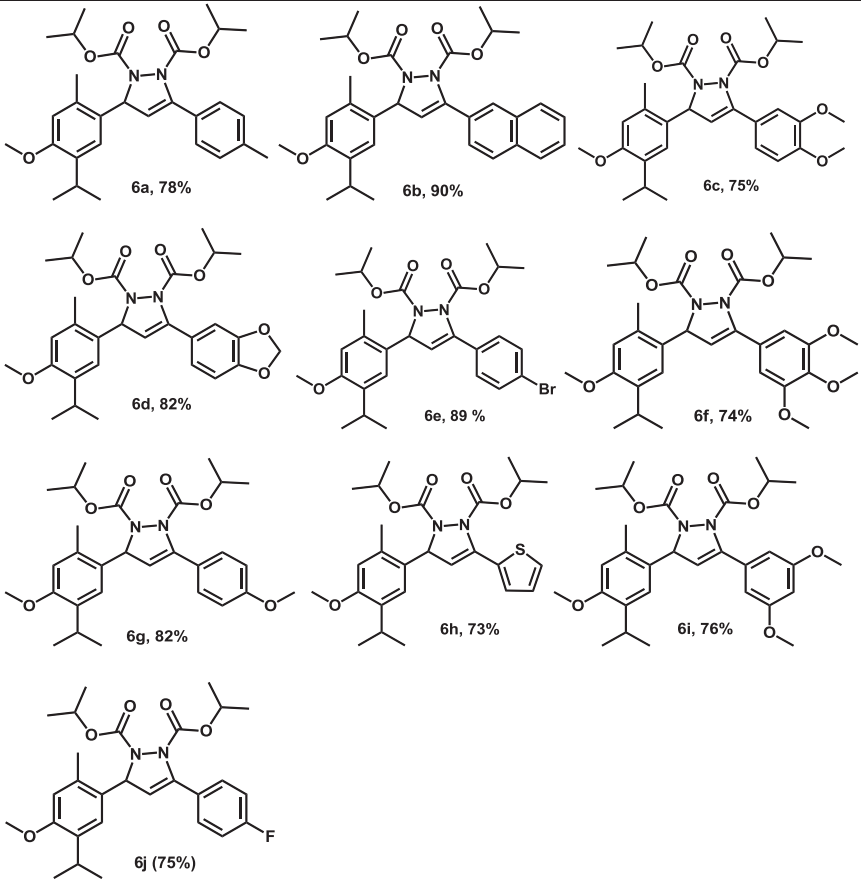
2.2. Biology

2.2.1. Antiplasmodial activity and cytotoxicity

2.2.1.1. Thymol based chalcones. In our preliminary *in-vitro* testing, thymol isolated from *Trachyspermum ammi,* exhibited a promising IC_{50} (85.87 ± 12.70 μM; Table 3) against human malaria parasite strain *P. falciparum* NF54 (chloroquine sensitive strain-CQ sensitive). This initial finding prompted us for further chemical modification of the thymol. Furthermore, thymol based chalcones (intermediates) were also tested *in-vitro*, and found to be as promising antiplasmodial agents (Table 3), hence, we selected these intermediates for further chemical

Table 2
Synthesis of functionalized pyrazolines.[a,b]





[a] Microwave reaction conditions: **5** (1 mmol), DIAD (1.5 mmol), PPh_3 (1.5 mmol), Toluene (2 mL), 15 min, 60 W, 100 °C.
[b] Isolated yield based on 5.

Table 3
Antiplasmodial activity of thymol and its chalcone analogues.

S. NO.	Compounds	IC_{50} (μM)
1.	Thymol	85.87 ± 12.70
2.	5a	24.49 ± 5.70
3.	5b	17.60 ± 3.25
4.	5c	21.93 ± 4.61
5.	5d	23.71 ± 8.95
6.	**5e**	**2.80 ± 0.22**
7.	**5f**	**2.25 ± 0.17**
8.	5 g	40.96 ± 10.92
9.	5 h	35.58 ± 10.12
10.	5i	20.41 ± 3.31
11.	5j	27.51 ± 5.56
12.	5 k	15.78 ± 6.20

Data are the mean ± SD of three experiments in triplicate; values are in μM.

Table 4
Antiplasmodial activity of the pyrazoline derivatives of thymol and their selectivity index.

Compounds	Inhibitory concentration (IC_{50})	Cytotoxic concentration (CC_{50})	Selectivity index (SI)
6a	2.32 ± 0.22	101.18 ± 3.95	43.61
6b	2.86 ± 0.27	113.61 ± 8.14	39.72
6c	1.75 ± 0.18	147.46 ± 4.67	84.26
6d	9.84 ± 0.42	177.48 ± 4.44	14.98
6e	3.61 ± 0.26	137.64 ± 3.69	38.12
6f	**1.05 ± 0.11**	**103.19 ± 2.54**	**98.27**
6 g	3.61 ± 0.31	120.85 ± 8.47	33.47
6 h	5.75 ± 1.17	140.91 ± 7.63	24.50
6i	3.11 ± 0.16	123.59 ± 5.61	39.73
6j	ND	ND	ND
Chloroquine	0.0174 ± 0.005	148.17 ± 23.52	8515.51

Data are the mean ± SD of three experiments in triplicate; values are in μM.

modification to perceive more active antiplasmodial agents.

2.2.1.2. Thymol based pyrazolines. Interestingly, upon chemical alteration and compared to thymol based chalcones, all the pyrazoline derivatives were proved to be potential growth inhibitors (IC_{50} value < 10 μM) of human malaria parasite *P. falciparum* (NF54) (Table 4). Amongst all the derivatives, compound **6f** was found to be most active, with an IC_{50} value of 1.05 ± 0.11 μM. We have also evaluated the antiplasmodial activity of most active lead **6f**, against resistant strain of human malaria parasite (*P. falciparum*-K1) and was recorded to be 3.07 ± 0.65 μM. Additionally, all the synthesized derivatives were found to have good selective index against murine peritoneal macrophages, exhibited SI values being higher than 10 (Table 4). Chloroquine used as a reference antimalarial exhibited an IC_{50} value of 0.0174 ± 0.005 μM against *P. falciparum* NF54 strain (CQ-sensitive). Compound **6f** was found as the most active among the all pyrazolines, therefore selected as a lead for further studies (*in-vivo* and mechanism based studies).

2.2.2. Selectivity towards host erythrocytes (RBCs)

The synthesized pyrazolines were also tested for their hemolytic potential to examine their selectivity against human B^{+ve} erythrocytes (separated using standard protocol). Upon 48 h incubation, none of the derivatives exhibited lytic potential upto 50 μM concentration. These results are indicative for the selective antiplasmodial activity of the synthesized pyrazolines against human malaria parasite (*P. falciparum*) in the host erythrocytes.

2.2.3. Compound 6f inhibited the hemozoin formation in vitro

Hemozoin formation is a key metabolism of malaria parasite and a well known chemotherapeutic target for many antimalarials, especially the quinoline derivatives viz., chloroquine [36]. As a lead antiplasmodial agent of the present work, we have also investigated the effect of **6f** on hemozoin formation through *in-vitro* β-hematin (synthetic analog of hemozoin) formation assay. In our results, **6f** significantly inhibited this polymerization in a concentration dependent manner (Fig. 2) with an IC_{50} value of 38.79 ± 0.50 μM. Chloroquine as standard inhibitor of hemozoin formation exhibited an IC_{50} value of 17.25 ± 1.60 μM.

2.2.4. Compound 6f inhibited the free heme degradation

The effect of compound **6f** on H_2O_2 mediated free heme degradation was also monitored since it has shown to have inhibitory effect on heme polymerization (hemozoin formation). As compared to negative control, H_2O_2 degraded free hemin at 20 mM concentration in positive controls (Fig. 3). Furthermore, at the highest tested concentration of 100 μM, compound **6f** significantly ($p < 0.001$) inhibited H_2O_2 mediated free heme degradation (Depicted in figure).

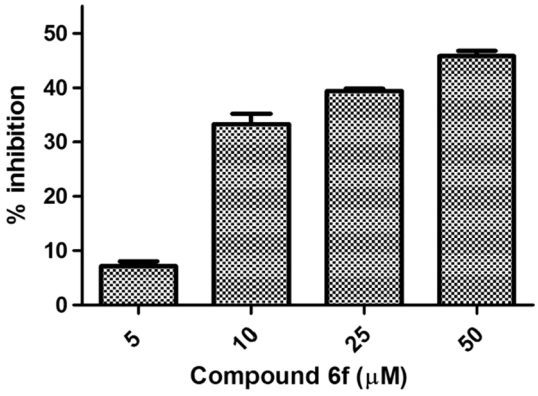


Fig. 2. Effect of compound **6f** on *in vitro* β-hematin (hemozoin) formation. Results are represented as mean percentage ± SD.

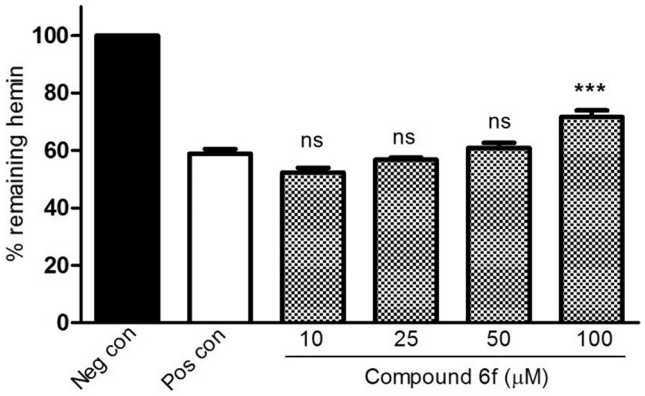


Fig. 3. Effect of compound **6f** on peroxidative hemin degradation. Results are represented as mean percentage ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, ns- non significant.

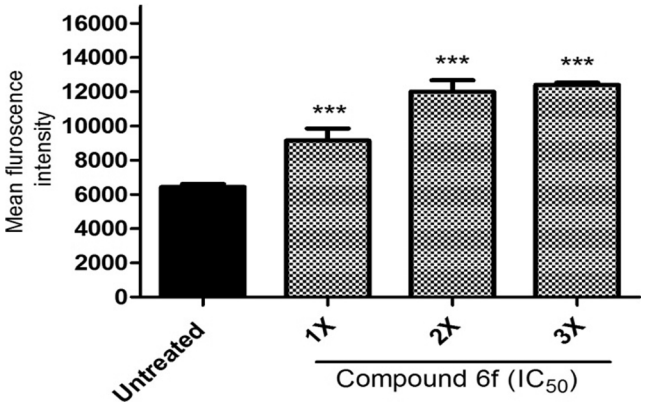


Fig. 4. Effect of compound **6f** on total ROS generation in the treated parasites. 1X, 2X, and 3X are the times concentration of IC_{50} value of compound 6f (1.05 ± 0.11 μM). Results are represented as mean percentage ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

2.2.5. Effect on total ROS

Compound **6f** treatment caused generation of free radicals in the malaria parasite. The mean fluorescence intensity values were recorded higher in the treated (in multiple of its IC_{50}) parasite as compared to untreated parasite (Fig. 4). Since compound **6f** was found to be an inhibitor of free heme polymerisation and degradation, so it might be responsible for free heme auto-oxidation which consequently caused ROS generation in parasite, as also observed augmented here.

Table 5
In-vivo antimalarial activity of compound **6f** against rodent malaria parasite *P. berghei* in Swiss mice.

Doses	Percent parasitemia		Percent chemosupression	Mean survival time (Days)
	Day 4	Day 6		
Vehicle	2.14 ± 0.36	10.41 ± 0.78	–	8.80
50 mg/kg	2.06 ± 0.55	8.03 ± 1.09	25.29 ± 5.14*	11.60
100 mg/kg	0.59 ± 0.17	4.62 ± 1.01	55.60 ± 9.71**	16.40
Chloroquine (10 mg/kg)	0.0	0.0	100***	> 28

Data are the mean ± SD of six animals in each group.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$ vehicle vs treatment.

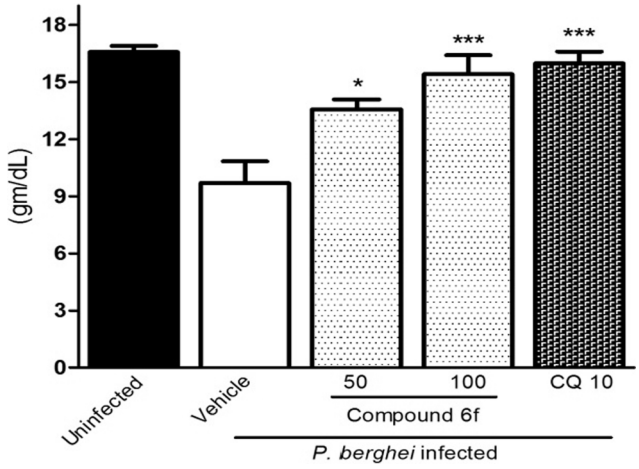


Fig. 5. At peak parasitemia on day 6, level of hemoglobin in the experimental animals treated with **6f** at the doses of 50 and 100 mg/kg body weight. Results are represented as mean percentage ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, Infected vs. treatment, n = 6.

2.2.6. Compound 6f inhibited malaria progression in mice

Under *in vivo* conditions, the compound **6f** significantly inhibited *P. berghei* growth, exhibiting a chemo-suppression of 25.29 ± 5.14% and 55.60 ± 9.71% at 50 and 100 mg/kg body weight, respectively (Table 5). Additionally, **6f** treated mice were found to exhibit a better lifespan (MST, 16.40 days), as compared to vehicle control group (MST, 8.80 days) (Table 5). These results are also corroborated with the hemoglobin level of **6f** treated animals estimated at peak parasitemia on day 6, where **6f** significantly ($p < 0.001$) restored the hemoglobin levels deprivation in mice (Fig. 5).

2.2.7. Acute oral toxicity

The most active compound **6f** was also studied for its safety profile for its further selection for a lead optimization particular to malaria. No significant changes in the serum and biochemical parameters of the treated animals were recorded as compared to vehicle received control animals (Table 6). Animals on gross pathological study showed no changes in any of the major organs studied including their absolute weight (Fig. 6). These results of acute oral toxicity clearly revealed that the compound **6f** has been found to be safe up to the single dose of 2000 mg/kg body weight.

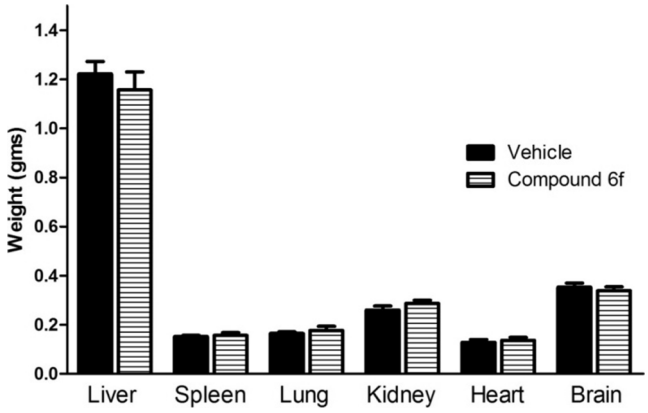


Fig. 6. Acute effect of the compound **6f** at the dose of 2000 mg/kg bd. wt. on the absolute organ weight of the experimental animals.

3. Discussion

Medicinal plants are a rich source of diverse pharmacologically active secondary metabolites especially in case of antimalarial drug development. This has already been confirmed by the developments of the new natural product derived antimalarials. Thymol is a natural monoterpenoid phenol widely distributed in nature and isolated from the *Trachyspermum ammi* (Ajwain seeds) and others. Earlier thymol showed significant antimalarial activity against *P. falciparum* (IC_{50} ~ 5 μg/mL) [19]. In recent years, pyrazoline nucleus has received significant attention in the area of malaria. Acharya *et al.,* reported the *in-vivo* antimalarial activity of tri-substituted pyrazolines against *P. berghei* strain and showed 68.93% chemosuppression at 50 mg/kg at 4th day of experiment [29]. This novel finding provoked us to design thymol based pyrazolines, and herein our report showed significant *in-vitro* antimalarial activity against *P. falciparum* (IC_{50} ~ 1.05 μM) and *in-vivo* against *P. berghei* showed significant chemosuppression 55.60% at 100 mg/kg body weight dose on day 4, by the lead compound **6f**. Additionally, pyrazoline analogue **6f** also exhibited potent antimalarial activity against CQ resistant strain of *P. falciparum*, IC_{50} (3.07 μM). The antimalarial activity of designed pharmacophore reveals that the phenyl ring with methoxy and bromo substituent was found to be very much favourable (Tables 3 and 4). This SAR is in full agreement with published antimalarial report [37]. The compound **6f** was found to be safe up to 2000 mg/kg bd.wt. under *in-vivo* condition [19,29].

Our study was initiated to achieve a natural product derived novel antimalarial agent. To accomplish the objective, thymol was isolated

Table 6
Effect of **6f** as a single acute oral dose at 2000 mg/kg body weight on hematological and serum biochemical parameters in Swiss albino mice.

Parameters	Effect of 6f in various parameters as a single oral dose in Swiss albino mice		
		Vehicle	Treatment @ 2000 mg/kg
Body weight (g)	Body weight (g)	20.49 ± 2.35	21.25 ± 2.32
Hematological (Unit)	Hemoglobin (g/dL)	11.58 ± 1.34	11.08 ± 0.28
	RBC count (cells × 10^6/μL)	8.62 ± 0.91	7.99 ± 1.19
	WBC count (cells × 10^4/μL)	12.10 ± 1.54	12.07 ± 0.51
Serum biochemical (Unit)			
Cholesterol profile	Total cholesterol (mg/dL)	109.65 ± 14.85	108.51 ± 29.59
	Triglycerides (mg/dL)	201.32 ± 61.75	204.70 ± 39.85
Kidney Function Test	Serum creatinine (mg/dL)	0.10 ± 0.06	0.07 ± 0.04
	Albumin (g/dL)	2.33 ± 0.13	2.31 ± 0.09
Liver Function Test	Alkaline phosphatase (U/dL)	25.35 ± 9.54	33.56 ± 8.54
	SGPT (U/L)	16.07 ± 6.05	21.28 ± 5.42
	SGOT (U/L)	30.34 ± 9.43	28.82 ± 4.77

Data are mean ± SD of six animals in each group.

from the seeds of *Trachyspermum ammi* (Supplementary data: Table 1) and was further modified. Thymol (**1**) and 2-*O*-alkylthymol (**2**) was converted into corresponding aldehydes (**3**) by Vilsmeier-Haack or $MgCl_2$/HCHO reaction. Compound **3** was further converted into the desired chalcones **5** (Supplementary data: Table 2) Finally, substituted pyrazolines (**6**) were synthesised by the reaction of chalcones and Diisopropyl azodicarboxylate through Huisgen Zwitterion mechanism (Scheme 1). All the studied compounds were characterized using ^{1}H and ^{13}C NMR and mass spectrometry (Supplementary data).

All the thymol derived molecules including chalcone and pyrazoline scaffolds in one frame (**5 & 6**) were evaluated for their antiplasmodial activity against CQ sensitive (NF54) strain of *P. falciparum*. Among the tested thymol based chalcones (**5**), compounds **5e** and **5f** exhibited promising antiplasmodial activity with IC_{50} values of 2.80 ± 0.22 and 2.25 ± 0.17 μM respectively. Interestingly, thymol based pyrazolines **6**, which were synthesised from chalcones (**5**), exhibited an IC_{50} value of < 10 μM. In pyrazoline series (**6a-j**), compound **6f** exhibited better antiplasmodial activity against human malaria parasite *P. falciparum* (IC_{50}; 1.05 ± 0.11 μM). The difference in the IC_{50} values can be attributed to the factors such as number of carbon atoms, nitrogenous ring, and the substitutions on the pyrazoline ring. The significant activity of the above mentioned compounds might be due to the presence of nitrogenous ring and trimethoxy system, since both trimethoxy containing molecule **5f** and **6f** exhibited potential antiplasmodial activity against *P. falciparum*. To elucidate the role of thymol based chalcones and pyrazolines in antimalarial activity, we derivatized the compounds (**5a-k** & **6a-j**) as outlined in Fig. 2. Additionally, all the synthesized derivatives were found to have good selective index against murine peritoneal macrophages, thus exhibited an acceptable selectivity index. In order to find out the possible mode of action of lead compound **6f**, we have studied its effect on heme polymerization and degradation pathway of malaria parasite. The compound **6f** exhibited promising inhibitory activity, hence could be responsible for the generation of heme mediated toxicity towards malaria parasite, and therefore for the delayed progression of parasite, as observed in our result also. As a lead compound, **6f** was selected for further detailed study under *in-vivo* conditions against rodent malaria parasite *P. berghei* in a dose dependent manner. Compound **6f** significantly inhibited the growth of malaria parasite *in vivo*, whereas the level of hemoglobin in the treated animals was also found to be higher, as compared the vehicle received animals. The increased level of hemoglobin in the treated parasite is indicative for the growth inhibition of malaria parasite, since hemoglobin is used as nutrient source by the parasite. We have also assessed the antiplasmodial activity of compound **6f** against the resistant strain of malaria parasite, where it exhibited significant inhibition of resistant parasite, which makes it a dual action molecule for antimalarial chemotherapy. Overall, the above observations are indicative for further detailed prospection of compound **6f** in antimalarial combination therapy.

4. Conclusion

In conclusion, a series of thymol based chalcones and novel pyrazolines were synthesized with highly efficient methods and evaluated for their antimalarial efficacy. Compound **6f**, a pyrazoline derivative of thymol was found to be the most active antimalarial lead, being biologically active against *P. falciparum* (*in vitro*) and *P. berghei* (*in vivo*). Furthermore, compound **6f** was found as an inhibitor of heme polymerization and degradation pathway of the malaria parasite, hence augmented total ROS in the treated parasite, which might be responsible for inhibition of parasite growth. It was also found to be non-toxic with good selectivity index. Currently, we are focusing towards the lead optimisation of these thymol derivatives towards the other aspects of pharmacology for a better *in vivo* antimalarial activity.

5. Experimental section

5.1. Chemistry

5.1.1. General experimental

The reactions were performed in round bottom flask and microwave vial using Teflon-coated magnetic stirring bar with constant stirring. Column chromatography was performed on silica gel (Merck 100–200 mesh) column. Merck GF_{254} plates (thickness 0.25 mm) were used for thin layer chromatography (TLC). TLC plate spots visualization was accomplished with UV light and staining in I_2 chamber. ^{1}H and ^{13}C NMR were recorded on 300/500 MHz and 75/125 MHz Bruker FTNMR spectrometer. Chemical shifts in NMR are expressed in δ values and tetramethylsilane (TMS) used as internal standard.

5.1.2. Reagents

All the chemicals including triphenylphosphine, DIAD, and other solvents and reagents were procured from Sigma-Aldrich and Alfa-aesar and were used as received. Thymol was isolated from *trachyspermum ammi* and used after purification.

5.1.3. Conversion of thymol to thymol methyl ether (2)

A 250 mL round bottom flask was charged with potassium carbonate (6 g) and dry acetone (30 mL), thymol **1a** (2 g) and dimethyl sulphate (3 mL) were added to this mixture and the reaction mixture was stirred for 5 h at room temperature. On completion, the reaction mixture was filtered and washed with acetone and distilled off to get thymol ether **2** (2.1 g).

5.1.4. Synthesis of 5-isopropyl-4-methoxy-2-methylbenzaldehyde (3a)

In a round bottom flask, thymol methyl ether **2** (4 g) was stirred in dimethylformamide (20 mL) in ice bath having temperature range between 0 to −10 °C, subsequently $POCl_3$ (12 mL) was added to the mixture and temperature of the reaction was maintained below 0 °C. After complete addition of $POCl_3$, reaction was heated at 80 °C for 2–3 h. Upon completion, the reaction mixture was poured into cold water, and extracted with ethyl acetate. The organic layer was washed with water, dried over anhydrous sodium sulphate, evaporated and purified by column chromatography to get the desired compound of formula **3a** (3.6 g).

5.1.5. Synthesis of 2-hydroxy-3-isopropyl-6-methylbenzaldehyde (3b)

In a100-mL three-necked round-bottomed flask anhydrous magnesium dichloride (4.76 g, 50 mmol) and solid paraformaldehyde (2.25 g, 75 mmol) were added in dry CH_3CN (125 mL) in an inert atmosphere. Triethylamine (5.06 g, 50 mmol) was added drop wise to the above mixture and stirred for 10 min, subsequently, thymol (3.75 g, 25 mmol) was added and refluxed for 4 h. After completion of the reaction, flask was cooled to room temperature and quenched with 10% HCl (aq) solution. The biphasic reaction was extracted with 3 × 50 mL ethyl acetate. The combined extracts were washed with saturated sodium chloride. The organic phase was dried over anhydrous Na_2SO_4, and was purified by column chromatography to get the desired compound of formula **3b** (70%).

5.1.6. General procedure for the preparation of chalcones (5)

To a solution of **3a**/**3b** (1.5 mmol) in methanol (5 mL), acetophenones **4a-j** (1 mmol) and KOH (10 mmol) were added. The reaction mixture was allowed to stirrer for 4 h at 40 °C. Upon completion of reaction (monitored through TLC), the mixture was cooled to room temperature by treating with cold water (15 mL). The precipitate was filtered and purified by recrystallization or column chromatography using a mixture of ethyl acetate/*n*-hexane as eluent to afford the desired thymol based chalcones **5**.

5.1.6.1. (E)-3-(5-isopropyl-4-methoxy-2-methylphenyl)-1-p-tolylprop-2-en-1-one (5a). ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 1.25 (d, J = 7.2 Hz, 6H, 2 × CH_3), 2.45 (s, 3H, CH_3), 2.48 (s, 3H, CH_3), 3.27–3.34 (m, 1H,

CH), 3.87 (s, 3H, OCH_3), 6.69 (s, 1H, ArH), 7.27–7.40 (m, 3H, CH, ArH), 7.57 (s, 1H, ArH), 7.94 (d, J = 8.4 Hz, 2H, ArH), 8.07 (d, J = 15.6 Hz, 1H, CH).

^{13}C NMR (75 MHz, $CDCl_3$, δ ppm): 20.28, 22.05, 23.03, 27.18, 55.81, 113.00, 120.83, 124.86, 126.38, 129.00, 129.37, 129.64, 135.60, 136.53, 138.30, 142.69, 143.61, 159.15, 190.71.

ESI-MS ($C_{21}H_{24}O_2$): 309 $[M+H]^+$.

*5.1.6.2. (E)-3-(5-isopropyl-4-methoxy-2-methylphenyl)-1-(naphthalen-2-yl)prop-2-en-1-one (**5b**).* ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 1.26 (d, J = 6.3 Hz, 6H, 2 × CH_3), 2.48 (s, 3H, CH_3), 3.28–3.32 (m, 1H, CH), 3.87 (s, 3H, OCH_3), 6.69 (s, 1H, ArH), 7.47–7.62 (m, 4H, CH, ArH), 7.89–8.02 (m, 3H, CH, ArH), 8.09–8.18 (m, 2H, ArH), 8.54 (s, 1H, ArH).

^{13}C NMR (75 MHz, $CDCl_3$, δ ppm): 20.33, 23.07, 27.25, 55.83, 113.04, 120.87, 124.95, 125.07, 126.35, 127.09, 128.22, 128.50, 128.59, 128.85, 129.92, 130.12, 133.04, 135.67, 135.78, 136.47, 138.48, 143.14, 159.29, 191.07.

ESI-MS ($C_{24}H_{24}O_2$): 345 $[M+H]^+$.

*5.1.6.3. (E)-1-(3,4-dimethoxyphenyl)-3-(5-isopropyl-4-methoxy-2-methylphenyl)prop-2-en-1-one (**5c**).* ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 1.25 (d, J = 6.9 Hz, 6H, 2 × CH_3), 2.48, (s, 3H, CH_3), 3.25–3.34 (m, 1H, CH), 3.87 (s, 3H, OCH_3), 3.98 (s, 6H, 2 × OCH_3), 6.69 (s, 1H, ArH), 6.94 (d, J = 8.4 Hz, 1H, ArH), 7.35 (d, J = 15.6 Hz, 1H, CH), 7.56 (s, 1H, ArH), 7.64–7.72 (m, 2H, ArH), 8.06 (d, J = 15.6 Hz, 1H, CH).

^{13}C NMR (75 MHz, $CDCl_3$, δ ppm): 20.28, 23.02, 27.18, 55.80, 56.44, 110.41, 111.35, 113.00, 120.55, 123.24, 124.83, 126.47, 132.19, 135.57, 138.19, 142.30, 149.61, 153.43, 159.09, 189.38.

ESI-MS ($C_{22}H_{26}O_4$): 355 $[M+H]^+$.

*5.1.6.4. (E)-1-(benzo[d][1,3]dioxol-5-yl)-3-(5-isopropyl-4-methoxy-2-methylphenyl)prop-2-en-1-one (**5d**).* ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 1.24 (d, J = 6.9 Hz, 6H, 2 × CH_3), 2.47 (s, 3H, CH_3), 3.23–3.32 (m, 1H, CH), 3.86 (s, 3H, OCH_3), 6.06 (s, 2H, CH_2), 6.68 (s, 1H, ArH), 6.89 (d, J = 8.4 Hz, 1H, ArH), 7.29 (d, J = 15.6 Hz, 1H, CH), 7.54 (s, 2H, ArH), 7.64–7.67 (m, 1H, ArH), 8.05 (d, J = 15.3 Hz, 1H, CH).

^{13}C NMR (75 MHz, $CDCl_3$, δ ppm): 20.28, 23.03, 27.18, 55.81, 102.18, 108.27, 108.88, 113.00, 120.34, 124.81, 124.87, 126.36, 133.86, 135.60, 138.31, 142.56, 148.60, 151.82, 159.14, 188.90.

ESI-MS ($C_{21}H_{22}O_4$): 339 $[M+H]^+$.

*5.1.6.5. (E)-1-(4-bromophenyl)-3-(5-isopropyl-4-methoxy-2-methylphenyl)prop-2-en-1-one (**5e**).* ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 1.24 (d, J = 7.0 Hz, 6H, 2 × CH_3), 2.46, (s, 3H CH_3), 3.26–3.29 (m, 1H, CH), 3.86 (s, 3H, OCH_3), 6.68 (s, 1H, ArH), 7.28 (d, J = 15.5 Hz, 1H, CH), 7.55 (s, 1H, ArH), 7.62–7.65 (m, 2H, ArH), 7.87–7.90 (m, 2H, ArH), 8.08 (d, J = 15.5 Hz, 1H, CH).

^{13}C NMR (125 MHz, $CDCl_3$, δ ppm): 19.88, 22.61, 26.78, 55.43, 112.64, 119.66, 124.49, 125.65, 127.47, 130.00, 131.83, 135.32, 137.47, 138.25, 143.28, 159.05, 189.62.

ESI-MS ($C_{20}H_{21}BrO_2$): 371 $[M-H]^+$.

*5.1.6.6. (E)-3-(5-isopropyl-4-methoxy-2-methylphenyl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (**5f**).* ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 1.24 (d, J = 7.0 Hz, 6H, 2 × CH_3), 2.47 (s, 3H CH_3), 3.26–3.29 (m, 1H, CH), 3.86 (s, 3H, OCH_3), 3.93 (s, 3H, OCH_3), 3.94 (s, 6H, 2 × OCH_3), 6.69 (s, 1H, ArH), 7.26–7.29 (m, 3H, CH, ArH), 7.54 (s, 1H, ArH), 8.05 (d, J = 15.5 Hz, 1H, CH).

^{13}C NMR (125 MHz, $CDCl_3$, δ ppm): 19.91, 22.60, 26.83, 55.44, 56.42, 60.98, 106.18, 112.62, 120.54, 124.53, 125.91, 134.06, 135.26, 137.92, 142.26, 142.73, 153.12, 158.88, 189.91.

ESI-MS ($C_{23}H_{28}O_5$): 385 $[M+H]^+$.

*5.1.6.7. (E)-3-(5-isopropyl-4-methoxy-2-methylphenyl)-1-(4-methoxyphenyl)prop-2-en-1-one (**5g**).* ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 1.25 (d, J = 7.0 Hz, 6H, 2 × CH_3), 2.47 (s, 3H CH_3), 3.27–3.30 (m, 1H, CH), 3.86 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 6.68 (s,1H, ArH), 6.97–7.00 (m, 2H, ArH), 7.36 (d, J = 15.5 Hz, 1H, CH), 7.56 (s, 1H, ArH), 8.03–8.10 (m, 3H, CH, ArH).

^{13}C NMR (75 MHz, $CDCl_3$, δ ppm): 20.30, 23.04, 27.16, 55.81, 55.87, 112.98, 114.17, 120.57, 124.79, 126.44, 131.12, 131.75, 131.95, 135.55, 138.24, 142.27, 159.06, 163.60, 189.41.

ESI-MS ($C_{21}H_{24}O_3$): 325 $[M+H]^+$.

*5.1.6.8. (E)-3-(5-isopropyl-4-methoxy-2-methylphenyl)-1-(thiophen-2-yl) prop-2-en-1-one (**5h**).* ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 1.24 (d, J = 6.9 Hz, 6H, 2 × CH_3), 2.47 (s, 3H, CH_3), 3.23–3.33 (m, 1H, CH), 3.86 (s, 3H, OCH_3), 6.68 (s, 1H, ArH), 7.16–7.28 (m, 2H, CH, ArH), 7.55 (s, 1H, ArH), 7.65 (d, J = 4.8 Hz, 1H, ArH), 7.86 (d, J = 3.6 Hz, 1H, ArH), 8.10 (d, J = 15.3 Hz, 1H, CH).

^{13}C NMR (75 MHz, $CDCl_3$, δ ppm): 20.30, 23.02, 27.20, 55.82, 113.02, 120.25, 124.88, 126.08, 128.53, 131.81, 133.76, 135.62, 138.56, 142.31, 146.37, 159.31, 182.70.

ESI-MS ($C_{18}H_{20}O_2S$): 301 $[M+H]^+$.

*5.1.6.9. (E)-1-(3,5-dimethoxyphenyl)-3-(5-isopropyl-4-methoxy-2-methylphenyl)prop-2-en-1-one (**5i**).* ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 1.24 (d, J = 7.0 Hz, 6H, 2 × CH_3), 2.46 (s, 3H CH_3), 3.25–3.30 (m, 1H, CH), 3.86 (s, 3H, OCH_3), 3.86 (s, 6H, 2 × OCH_3), 6.66–6.68 (m, 2H, ArH), 7.15 (d, J = 2.5 Hz, 2H, ArH), 7.27 (d, J = 15.5 Hz, 1H, CH), 7.54 (s, 1H, ArH), 8.07 (d, J = 15.5 Hz, 1H, CH).

^{13}C NMR (125 MHz, $CDCl_3$, δ ppm): 19.90, 22.63, 26.78, 55.42, 55.63, 104.62, 106.39, 112.60, 120.40, 124.53, 125.83, 135.29, 138.05, 140.79, 142.88, 158.89, 160.86, 190.45

ESI-MS ($C_{22}H_{26}O_4$): 355 $[M+H]^+$.

*5.1.6.10. (E)-1-(4-flurophenyl)-3-(5-isopropyl-4-methoxy-2-methylphenyl)prop-2-en-1-one (**5j**).* ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 1.25 (d, J = 6.5 Hz, 6H, 2 × CH_3), 2.47 (s, 3H CH_3), 3.26–3.31 (m, 1H, CH), 3.86 (s, 3H, OCH_3), 6.69 (s, 1H, ArH), 7.15–7.19 (m, 2H, ArH), 7.32 (d, J = 15.5 Hz, 1H, CH), 7.56 (s, 1H, ArH), 8.04–8.12 (m, 3H, ArH, CH).

^{13}C NMR (125 MHz, $CDCl_3$, δ ppm): 19.90, 22.64, 26.77, 55.43, 112.61, 115.55 (d, $J_{C\text{-}C\text{-}F}$ = 21.25 Hz, CH), 119.72, 124.43, 125.71, 130.97 (d, $J_{C\text{-}C\text{-}C\text{-}F}$ = 8.75 Hz, CH), 135.01 (d, $J_{C\text{-}C\text{-}C\text{-}C\text{-}F}$ = 2.50 Hz, C), 135.27, 138.17, 142.91, 158.95, 164.43 (d, $J_{C\text{-}F}$ = 252.50 Hz, C), 189.06.

ESI-MS ($C_{20}H_{21}FO_2$): 313 $[M+H]^+$.

*5.1.6.11. (E)-3-(2-hydroxy-3-isopropyl-6-methylphenyl)-1-(4-methoxyphenyl)prop-2-en-1-one (**5k**).* ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 1.26 (d, J = 7.0 Hz, 6H, 2 × CH_3), 2.36, (s, 3H CH_3), 3.19–3.22 (m, 1H, CH), 3.89 (s, 3H, OCH_3), 5.54 (s, 1H, OH), 6.79 (d, J = 8.0 Hz, 1H, ArH), 6.96–6.98 (m, 2H, CH, ArH), 7.10 (d, J = 7.5 Hz 1H, ArH), 7.59 (d, J = 16 Hz, 1H, CH), 7.94–7.97 (m, 1H, ArH), 8.01–8.03 (m, 2H, CH, ArH).

^{13}C NMR (125 MHz, $CDCl_3$, δ ppm): 19.51, 22.37, 26.63, 55.23, 112.51, 115.27, 119.89, 124.25, 125.76, 130.08, 130.97, 135.08, 137.88, 142.06, 158.71, 161.71, 189.99.

ESI-MS ($C_{20}H_{22}O_3$): 311 $[M+H]^+$.

5.2. Synthesis of functionalized pyrazolines (6)

Diisopropyl azodicarboxylate (1.5 mmol), thymol based chalcones **5** (1.0 mmol), PPh_3 (1.5 mmol) and toluene (2 mL) were put in a pressure regulation 10 mL pressurized vial with 'snap-on' cap and flushed with nitrogen before closing the vial. The reaction mixture was irradiated in a single-mode microwave system at 60 W power and 100 °C for 15 min. After completion of the reaction (checked by TLC), reaction mixture was cooled at room temperature and the solvent was removed under reduced pressure. The residue was subjected to column chromatography embedded with silica gel. Hexane/ethyl acetate was used as eluent to afford the functionalized pyrazolines **6**.

*5.2.1. Diisopropyl 3-(5-isopropyl-4-methoxy-2-methylphenyl)-5-p-tolyl-1H-pyrazole-1,2(3H) dicarboxylate (**6a**)*

^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 0.99 (d, J = 6.0 Hz, 3H, CH_3), 1.07 (d, J = 6.5 Hz, 3H, CH_3), 1.12–1.16 (m, 6H, 2 × CH_3), 1.30–1.34 (m, 6H, 2 × CH_3), 2.36 (s, 3H, CH_3), 2.50 (s, 3H, CH_3), 3.17–3.22 (m, 1H, CH), 3.81 (s, 3H, OCH_3), 4.81–4.86 (m, 1H, CH), 5.03–5.08 (m, 1H, CH), 5.60 (d, J = 3.0 Hz, 1H, CH), 6.01 (d, J = 3.0 Hz, 1H, CH), 6.66 (s, 1H, ArH), 7.13–7.17 (m, 3H, ArH), 7.40 (d, J = 8.5 Hz, 2H, ArH).

^{13}C NMR (125 MHz, $CDCl_3$, δ ppm): 19.25, 21.35, 21.62, 21.75, 22.08, 22.14, 22.56, 22.70, 26.94, 55.44, 63.87, 70.32, 70.44, 111.32, 112.78, 124.92, 127.11, 128.64, 128.78, 129.17, 133.48, 134.92, 138.49, 142.93, 155.29, 156.32.

ESI-MS ($C_{29}H_{38}N_2O_5$): 495 $[M+H]^+$.

*5.2.2. Diisopropyl3-(5-isopropyl-4-methoxy-2-methylphenyl)-5-(naphthalen-2-yl)-1H pyrazole-1,2(3H)-dicarboxylate (**6b**)*

^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 0.95 (d, J = 6.0 Hz, 3H, CH_3), 1.02 (d, J = 6.5 Hz, 3H, CH_3), 1.16–1.21(m, 6H, 2 × CH_3), 1.35–1.39 (m, 6H, 3 × CH_3), 2.54 (s, 3H, CH_3), 3.22–3.25 (m, 1H, CH), 3.84 (s, 3H, OCH_3), 4.83–4.88 (m, 1H, CH), 5.09–5.14 (m, 1H, CH), 5.79 (d, J = 3.0 Hz, 1H, CH), 6.08 (d, J = 3.0 Hz, 1H, CH), 6.70 (s, 1H, ArH), 7.22 (s, 1H, ArH), 7.46–7.49 (m, 2H, ArH), 7.64–7.66 (m, 1H, ArH), 7.82–7.85 (m, 3H, ArH), 7.99 (s, 1H, ArH).

^{13}C NMR (125 MHz, $CDCl_3$, δ ppm): 19.26, 21.58, 21.72, 22.15, 22.60, 22.71, 27.00, 29.70, 30.87, 55.45, 64.13, 70.44, 70.61, 112.52, 112.84, 124.87, 125.27, 126.16, 126.26, 126.33, 127.43, 127.69, 128.22, 128.76, 129.53, 132.95, 133.34, 133.40, 135.05, 142.87, 155.27, 156.39.

ESI-MS ($C_{32}H_{38}N_2O_5$): 531 $[M+H]^+$.

*5.2.3. Diisopropyl 5-(3,4-dimethoxyphenyl)-3-(5-isopropyl-4-methoxy-2-methylphenyl)-1H pyrazole-1,2(3H)-dicarboxylate (**6c**)*

^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 1.01 (d, J = 6.5 Hz 3H, CH_3), 1.07 (d, J = 6.5 Hz, 3H, CH_3), 1.13–1.16 (m, 6H, 2 × CH_3), 1.26–1.34 (m, 6H, 2 × CH_3), 2.49 (s, 3H, CH_3), 3.18–3.23 (m, 1H, CH), 3.81 (s, 3H, OCH_3), 3.87 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3), 4.82–4.87 (m, 1H, CH), 5.03–5.08 (m, 1H, CH), 5.57 (d, J = 3.0 Hz, 1H, CH), 6.00 (d, J = 2.5 Hz, 1H, CH), 6.66 (s, 1H, ArH), 6.85 (d, J = 8.0 Hz, 1H, ArH), 7.03 (d, J = 1.5 Hz, 1H, ArH), 7.09–7.11 (m, 1H, ArH), 7.15 (s, 1H, ArH).

^{13}C NMR (125 MHz, $CDCl_3$, δ ppm): 19.24, 21.69, 21.76, 21.95, 22.10, 22.12, 22.56, 22.71, 26.95, 55.43, 55.90, 55.94, 63.96, 70.31, 70.47, 110.50, 110.59, 110.88, 112.72, 120.05, 124.80, 124.92, 128.85, 133.31, 134.97, 142.59, 148.44, 149.47, 155.26, 156.31.

ESI-MS ($C_{30}H_{40}N_2O_7$): 541 $[M+H]^+$.

*5.2.4. Diisopropyl 5-(benzo[d][1,3]dioxol-5-yl)-3-(5-isopropyl-4-methoxy-2-methylphenyl)-1H pyrazole-1,2(3H)-dicarboxylate (**6d**)*

^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 1.04 (d, J = 6.0, Hz, 3H, CH_3), 1.13–1.18 (m, 9H, 3 × CH_3), 1.31–1.36 (m, 6H, 2 × CH_3), 2.52 (s, 3H, CH_3), 3.19–3.24 (m, 1H, CH), 3.83 (s, 3H, OCH_3), 4.84–4.89 (m, 1H, CH), 5.05–5.10 (m, 1H, CH), 5.59 (d, J = 3.0 Hz, 1H, CH), 5.96 (s, 2H, CH_2) 6.00–6.03 (m, 1H, CH), 6.68 (s, 1H, ArH), 6.81 (d, J = 8.0 Hz, 1H, ArH), 7.01 (d, J = 2.0 Hz, 1H, ArH), 7.05–7.07 (m, 1H, ArH), 7.12 (s, 1H, ArH).

^{13}C NMR (125 MHz, $CDCl_3$, δ ppm): 19.25, 21.64, 21.67, 21.80, 22.07, 22.14, 22.57, 22.69, 26.94, 55.44, 63.81, 70.37, 70.52, 101.20, 107.81, 107.96, 111.21, 112.80, 121.14, 124.87, 126.13, 128.64, 133.54, 134.91, 142.58, 147.31, 147.93, 155.32, 156.35.

ESI-MS ($C_{29}H_{36}N_2O_7$): 525 $[M+H]^+$.

*5.2.5. Diisopropyl 5-(4-bromophenyl)-3-(5-isopropyl-4-methoxy-2-methylphenyl)-1H-pyrazole-1,2(3H)-dicarboxylate (**6e**)*

^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 1.01 (d, J = 6.5 Hz, 3H, CH_3), 1.08 (d, J = 6.0 Hz, 3H, CH_3), 1.12–1.15 (m, 6H, 2 × CH_3), 1.30–1.34 (m, 6H, 2 × CH_3), 2.49 (s, 3H, CH_3), 3.13–3.25 (m, 1H, CH), 3.81 (s, 3H, OCH_3), 4.82–4.87 (m, 1H, CH), 5.03–5.08 (m, 1H, CH), 5.67 (d, J = 3.0 Hz, 1H, CH), 6.01 (d, J = 3.0 Hz, 1H, CH), 6.66 (s, 1H, ArH), 7.10 (s, 1H, ArH), 7.37 (d, J = 8.5 Hz, 2H, ArH), 7.48 (d, J = 8.5 Hz, 2H, ArH).

^{13}C NMR (125 MHz, $CDCl_3$, δ ppm): 19.23, 21.46, 21.75, 22.07, 22.13, 22.56, 22.68, 26.93, 55.44, 64.03, 70.51, 70.82, 112.80, 122.56, 124.75, 128.37, 128.72, 131.00, 131.16, 133.40, 135.03, 141.86, 155.24, 156.41.

ESI-MS ($C_{28}H_{35}BrN_2O_5$): 559 $[M+H]^+$.

*5.2.6. Diisopropyl 3-(5-isopropyl-4-methoxy-2-methylphenyl)-5-(3,4,5-trimethoxyphenyl)-1H-pyrazole-1,2(3H)-dicarboxylate (**6f**)*

^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 1.02 (d, J = 6.5 Hz, 3H, CH_3), 1.08 (d, J = 6.0 Hz, 3H, CH_3), 1.14–1.17 (m, 6H, 2 × CH_3), 1.30–1.35 (m, 6H, 2 × CH_3), 2.49 (s, 3H, CH_3), 3.19–3.25 (m, 1H, CH), 3.81 (s, 3H, OCH_3), 3.85 (m, 3H, OCH_3), 3.86 (s, 6H, 2 × OCH_3), 4.83–4.88 (m, 1H, CH), 5.04–5.09 (m, 1H, CH), 5.63 (d, J = 3.0 Hz, 1H, CH), 6.01 (d, J = 3.0 Hz, 1H, CH), 6.67 (s, 1H, ArH), 6.75 (s, 2H, ArH), 7.18 (s, 1H, ArH).

^{13}C NMR (125 MHz, $CDCl_3$, δ ppm): 19.23, 21.67, 21.75, 22.09, 22.55, 22.73, 26.92, 55.39, 56.13, 60.93, 64.15, 70.37, 70.57, 104.59, 111.81, 112.68, 124.64, 127.70, 128.68, 133.18, 135.01, 138.54, 142.64, 152.86, 155.19, 156.34.

GC–MS ($C_{31}H_{42}N_2O_8$): 571 $[M+H]^+$.

*5.2.7. Diisopropyl 5-(4-methoxyphenyl)-3-(5-isopropyl-4-methoxy-2 methylphenyl)-1H-pyrazole-1,2(3H)-dicarboxylate (**6g**)*

^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 1.00 (d, J = 6.5 Hz, 3H, CH_3), 1.08 (d, J = 6.5 Hz, 3H, CH_3), 1.12–1.16 (m, 6H, 2 × CH_3), 1.30–1.34 (m, 6H, 2 × CH_3), 2.50 (s, 3H, CH_3), 3.17–3.22 (m, 1H, CH), 3.81 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3), 4.81–4.86 (m, 1H, CH), 5.03–5.08 (m, 1H, CH), 5.56 (d, J = 3.5 Hz, 1H, CH), 6.00 (d, J = 2.0 Hz, 1H, CH), 6.66 (s, 1H, ArH), 6.88 (d, J = 9.0 Hz, 2H, ArH), 7.13 (s, 1H, ArH), 7.44 (d, J = 9.0 Hz, 2H, ArH).

^{13}C NMR (125 MHz, $CDCl_3$, δ ppm): 19.26, 21.66, 21.77, 22.09, 22.15, 22.58, 22.71, 26.94, 55.32, 55.43, 63.82, 70.33, 70.43, 110.53, 112.74, 113.39, 124.61, 124.88, 128.55, 128.85, 133.46, 134.89, 142.55, 155.32, 156.29, 159.91.

ESI-MS ($C_{29}H_{38}N_2O_6$): 511 $[M+H]^+$.

*5.2.8. Diisopropyl 3-(5-isopropyl-4-methoxy-2-methylphenyl)-5-(thiophen-2-yl)-1H-pyrazole-1,2(3H)-dicarboxylate (**6h**)*

^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 0.97 (d, J = 6.5 Hz, 3H, CH_3), 1.04–1.08 (m, 9H, 3 × CH_3), 1.22–1.26 (m, 6H, 2 × CH_3), 2.42 (s, 3H, CH_3), 3.09–3.15 (m, 1H, CH), 3.74 (s, 3H, OCH_3), 4.78–4.83 (m, 1H, CH), 4.95–5.00 (m, 1H, CH), 5.62 (d, J = 3.0 Hz, 1H, CH), 5.95 (s, 1H, CH), 6.59 (s, 1H, ArH), 6.93–6.95 (m, 1H, ArH), 7.04 (s, 1H, ArH), 7.17–7.19 (m, 1H, ArH), 7.22–7.23 (m, 1H, ArH).

^{13}C NMR (125 MHz, $CDCl_3$, δ ppm): 19.32, 21.64, 21.78, 22.10, 22.57, 22.70, 26.92, 55.44, 63.69, 70.52, 70.79, 112.42, 112.73, 124.81, 125.86, 126.94, 127.17, 128.32, 133.53, 133.82, 134.92, 136.51, 155.34, 156.35.

ESI-MS ($C_{26}H_{34}N_2O_5S$): 487 $[M+H]^+$.

*5.2.9. Diisopropyl 5-(3,5-dimethoxyphenyl)-3-(5-isopropyl-4-methoxy-2-methylphenyl)-1H-pyrazole-1,2(3H)-dicarboxylate (**6i**)*

^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 1.00 (d, J = 6.0 Hz, 3H, CH_3), 1.07 (d, J = 6.5 Hz, 3H, CH_3), 1.13–1.16 (m, 6H, 2 × CH_3), 1.24–1.34 (m, 6H, 2 × CH_3), 2.49 (s, 3H, CH_3), 3.17–3.23 (m, 1H, CH), 3.79 (s, 3H, OCH_3), 3.81 (s, 6H, 2 × OCH_3), 4.82–4.87 (m, 1H, CH), 5.03–5.08 (m, 1H, CH), 5.65 (d, J = 3.5 Hz, 1H, CH), 6.01 (d, J = 3.0 Hz, 1H, CH), 6.44–6.45 (m, 1H, ArH), 6.66–6.68 (m, 3H, ArH), 7.13 (s, 1H, ArH).

^{13}C NMR (125 MHz, $CDCl_3$, δ ppm): 19.23, 21.61, 21.72, 21.77, 22.07, 22.12, 22.56, 22.70, 26.93, 29.70, 55.39, 55.43, 64.02, 70.38, 70.56, 100.86, 105.44, 112.50, 112.77, 124.81, 128.57, 133.99,

135.01, 142.75, 155.20, 156.36, 160.37.

ESI-MS ($C_{30}H_{40}N_2O_7$): 541 $[M+H]^+$.

*5.2.10. Diisopropyl 5-(4-hydroxyphenyl)-3-(5-isopropyl-4-methoxy-2-methylphenyl)-1H-pyrazole-1,2(3H)-dicarboxylate **(6j)***

^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 1.00 (d, J = 6.0 Hz, 3H, CH_3), 1.07 (d, J = 6.5 Hz, 3H, CH_3), 1.13–1.16 (m, 6H, 2 × CH_3),1.30–1.34 (m, 6H, 2 × CH_3), 2.50 (s, 3H, CH_3), 3.18–3.23 (m, 1H, CH), 3.81 (s, 3H, OCH_3), 4.81–4.86 (m, 1H, CH), 5.04–5.09 (m, 1H, CH), 5.62 (d, J = 3.0 Hz, 1H, CH), 6.02 (d, J = 3 Hz, 1H, CH), 6.66 (s, 1H, ArH), 7.03–7.07 (m, 2H, ArH), 7.12 (s, 1H, ArH), 7.48–7.51 (m, 2H, ArH).

^{13}C NMR (125 MHz, $CDCl_3$, δ ppm): 19.25, 21.63, 21.75, 22.09, 22.14, 22.58, 22.70, 26.92, 55.43, 63.96, 70.48, 70.69, 112.01, 112.77, 114.92, 115.09, 124.76, 128.21, 128.24, 128.53, 128.94, 129.01, 133.41, 134.99, 141.84, 155.23, 156.37, 161.87, 163.85.

ESI-MS ($C_{28}H_{35}FN_2O_5$): 499 $[M+H]^+$.

6. Biology

6.1. In vitro antiplasmodial activity

The *in vitro* antiplasmodial activity of synthesized compounds was evaluated against chloroquine sensitive (NF54) and resistant (K1) strains of human malaria parasite *P. falciparum*, by using the protocol described earlier in our report [33]. Compounds were dissolved in DMSO (Dimethyl sulfoxide) at stock concentration of 10 mM, and the two-fold serial dilutions of test samples were made in 96 well plates and incubated with 1.0% parasitized cell suspension containing 1% parasitemia (more than 90% ring stages). The plates were incubated at 37 °C in CO_2 incubator in an atmosphere containing 5% CO_2 and air mixture. Onwards 72 h incubation, culture supernatant was discarded gently and thin blood smears were prepared from each well. Slides were fixed in methanol and stained with *Giemsa* dye (10%) upto 30 min, the percent parasitemia in each smear was evaluated by counting a total 1000 erythrocytes from each smear. Antiplasmodial activity of the tested samples was expressed in term of concentration inhibiting 50% of parasite growth (IC_{50}). IC_{50} values were calculated using pre-programmed excel spreadsheet by Logit regression analysis. In order to assess the antiplasmodial activity three replicates were carried out, and chloroquine was used as reference antimalarial drug.

6.2. Cytotoxicity and selectivity index

In order to estimate the selectivity (therapeutic) index of the synthesized molecules, cell cytotoxicity of samples was also evaluated against murine intraperitoneal primary macrophages. Cells were cultivated in Minimum Essential Medium (Sigma, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, USA) and antibiotics. The evaluation of cell viability was performed through MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay [38]. The data was expressed as percent cell viability in term of 50% cytotoxic concentration (CC_{50}), where DMSO was taken as negative control. The selectivity index of the samples was expressed as a ratio of CC_{50} and IC_{50} value and considered safe above than 5 as per WHO guidelines [39].

6.3. Hemolytic assay

The synthesized compounds were also evaluated for their hemolytic (RBC damage) potential. Briefly, B^{+ve} blood group human erythrocytes (2% hematocrit in RPMI media) were incubated for 48 h in a 96-well plate, in the presence of varying concentrations (1–50 μM) of compounds. In each experiment 0.5% saponin (positive control) and DMSO (negative control) were used. The RBC damage was calculated by following the absorbance at 540 nm and expressed in term of hemolysis [40].

6.4. Effect on in vitro heme polymerization

In an effort to eliminate the detrimental destructions experienced during hemoglobin degradation and utilization, malaria parasite exploited its survival through heme polymerization. In the present work, the inhibitory effect of lead compound **6f** on heme polymerization pathway of the malaria parasite was evaluated using the method reported earlier [41]. Briefly, male Swiss mice, weighing 15–20 g were inoculated (i.p.) with 1 × 10^6 *P. yoelii* infected red blood cells. Blood from infected animals at approx. 50% parasitemia was collected by cardiac puncture in 2.0% citrate saline buffer and centrifuged at 2500 rpm for 15 min at 4 °C for the isolation plasma. The plasma was used for β-hematin formation inhibitory (BHIA) assay. The assay mixture contained 100 mM sodium acetate buffer pH (5.1), 100 μL plasma, 100 μM hemin as the substrate and 1–50 μM compound in a total volume of 1.0 mL. The control tubes contained all the reagents except compounds. The triplicate reaction mixture was incubated at 37 °C for 16 h in a rotary shaker. The reaction was stopped by centrifugation at 10,000 rpm for 10 min at 30 °C. The pellet was suspended in 100 mM tris-HCl buffer pH (7.4) containing 2.5% SDS for washing the pellet (three times). The pellet obtained after centrifugation was again washed with distilled water (TDW) to remove free hemin attached to polymerized β-hematin. The pellet was finally solubilized in 0.1 N NaOH. Absorbance was measured at 400 nm and the IC_{50} value was determined using non-linear regression analysis dose response curves.

6.5. Inhibition of hydrogen peroxide-mediated hemin degradation

As a part of parasite rescue mechanism(s), free heme released upon hemoglobin degradation in malaria parasite is degraded by intraparasitic H_2O_2. The effect of lead compound on peroxidative decomposition of hemin was estimated by using the protocol established earlier by Loria et al., 1999 [42]. Briefly, a hemin stock solution (1 mM in 0.1 N NaOH) was freshly prepared and 20 μL of this was distributed in each well of 96-well flat bottom microplate. Onwards this, the lead compound **6f** was added from 10 to 100 μM final concentrations, chloroquine was used as reference inhibitor of heme degradation. The plates were left to equilibrate (pre-incubated) for 30 min at room temperature after addition of 150 μL bovine serum albumin (stock 1 mg/mL) in sodium acetate 0.1 M (pH 5.1), as similar to the pH range of *P. falciparum* food vacuole. The peroxidative reaction was initiated by the addition of 20 μL H_2O_2 (2 M) and followed by measuring the decrease in absorption at the Soret band (404 nm) after 30 min of incubation, using a multiplate reader (SpectraMax plus 384). Negative control with addition of TDW instead of H_2O_2, was also included in each experiment. Results were expressed as the percentage of remained hemin in the reaction mixture.

6.6. Measurement of total ROS (oxidative stress)

The Intracellular oxidative stress (total ROS) in the treated parasite produced during the chemotherapeutic treatment of compound **6f** in malaria parasite was monitored by measuring the alteration of fluorescence resulted from the oxidation of 2′,7′-dichlorofluorescein diacetate (DCF-DA) [43]. Briefly, as mentioned above, *P. falciparum* culture at trophozoite stage (~85% synchronized) and ~5% parasitemia was treated with varying concentrations (1X, 2X and 3X of IC_{50} value) of test compound for 12 h (overnight). Upon treatment, 10 μM of DCFDA (stock in 1X PBS) was added in each well with subsequent incubation for 1 h. After incubation, pre-labeled cells were treated with saponin (0.075% final concentration) to isolate parasite which were lysed by mild sonication. The relative fluorescence intensity in parasite lysate (200 μL) was measured by using the fluorescence plate reader (FLx 800, Bio Tek, USA) and determined with an excitation and emission wavelengths of 485 ± 10 and 535 ± 10 nm respectively. The ROS level

was calculated as the mean fluorescence intensity (MFI) and expressed as percentage of the control value.

6.7. In vivo antimalarial activity

The laboratory maintained chloroquine-sensitive strain of rodent malaria parasite (*P. berghei* K-173) was used for the evaluation of antimalarial activity of lead derivative **6f**, in swiss albino mice [44]. Briefly, on day 0, malarial infection in experimental mice was induced with an intra-peritoneal injection of the parasitized RBCs (1×10^6 cells, in 0.2 mL ACD) and six mice were randomly assigned in each group. Microscopic examination of the stained (*Giemsa*) blood smears were undertaken to quantify and monitor the progression of parasitemia within the hosts. Chemotherapeutic doses were prepared in 0.1% Tween 80 (v/v in water) and orally administered (each 0.5 mL at 24 h interval), for a period of 4 days. Chloroquine was used as standard antimalarial at the dose of 10 mg/kg body weight. Control untreated group animals received equal volumes of vehicle (0.1% Tween 80). The treatment was followed up to 28 days with regular examination of the percentage parasitemia (number of infected erythrocytes per 100 erythrocytes). Clinical sign and mortality in each group was observed during the experiment for evaluation of percent survival and mean survival time (MST). Hemoglobin in the experimental animals was also estimated by using the Drabkin's reagent [45].

6.8. Acute oral toxicity

In order to explore the therapeutic value of the most active antimalarial derivative of the present work, acute oral toxicity study of the compound **6f** was also performed in Swiss albino mice. Experiment was performed in accordance with the Organization for Economic Co-operation and Development (OECD) test guideline No 423 (1987). Briefly, 12 mice (6 male and 6 female) were randomly divided into two groups comprising 3 male and 3 female mice in each group (20 ± 2 g). The animals were maintained at 22 ± 5 °C with humidity control and dark and light cycle of 12 h each. The animals were fed with standard mice feed and provided *ad libitum* drinking water. Group 1 was considered as control group (received vehicle only) while group 2 as experimental group where they received the compound **6f** at a single dose of 2000 mg/kg body weight. The experimental mice were fasted overnight before the dosing of the test compound **6f** and upon dosing the animals were monitored for mortality and clinical signs of illness (skin irritation, lachrymation, piloerection, gait, posture and diarrhea) at hourly interval on day of test sample administration and there after once in a day. In addition to observational study, body weight and major organ weight were also recorded from the experimental animals. Blood and serum samples were collected from all the animals on 7^{th} day of the experiment in acute oral toxicity. The blood was used to analyze the total RBC, WBC, differential leucocytes count, hemoglobin percentage. Serum was used for biochemical parameters like ALKP, SGPT, SGOT, total cholesterol, triglycerides, creatinine, bilirubin, serum protein and high density lipoprotein (HDL).

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.102933.

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