



ORIGINAL RESEARCH

Antimalarial activity of phytol derivatives: in vitro and in vivo study

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Abstract

Severe malaria is one of the leading causes of mortality among children and pregnant woman globally. Resistance development against the frontline antimalarial drugs has created an alarming situation, which requires intensive drug discovery to develop effective, affordable, and accessible antimalarial agents possessing novel modes of action. As a part of our drug discovery program for antimalarial agents from plants, phytol a very commonly occurring diterpene alcohol in the plant was investigated for its antimalarial potential. In vitro antiplasmodial activity against the chloroquine-sensitive *Plasmodium falciparum* NF54 by measuring the parasite specific lactate dehydrogenase (pLDH), showed moderate activity (IC_{50} $211.5 \pm 0.93 \mu M$). Further, phytol was chemically converted into thirteen derivatives, which were evaluated for their antiplasmodial potential. All the derivatives were moderate active, but among all the derivatives palmitoyl (*PhY-3*; IC_{50} $12.13 \pm 0.31 \mu M$) has found most active without any cytotoxic effect on macrophage cells. *PhY-3* was further validated in an in vivo system for its efficacy and safety profile in mice. Oral administration of *PhY-3* showed significant reduction of parasitemia and increased mean survival time. It also attributed significant increase in blood glucose and hemoglobin level, when compared with vehicle-treated *P. berghei* infected mice without any toxic effect on normal mice at a higher dose. These findings confirm suitability of the phytol derivatives as new chemical entities for further investigation towards the management of malaria.

Keywords Phytol · In vitro · In vivo · *Plasmodium* · Malaria · Mice

Introduction

Malaria is a major public-health problem associated with high morbidity and mortality in many countries affecting

approximately 350–500 million clinical cases every year with a corresponding mortality rate of 2–3 million deaths each year, including young children under the age of five and pregnant women (WHO 2012). Malaria is also an enormous burden to the socioeconomic progress of people in endemic areas, where most people suffer from poverty, malnutrition and infectious diseases (Snow et al. 2005). So far, no preventative vaccination against malaria exists, and its control depends heavily upon antimalarial drugs that kill parasites inside the human body (Snounou et al. 2005). The World Health Organization (WHO) currently promotes artemisinin-based combination therapy (ACT) as the first-line treatment for management of *falciparum* malaria and to reduce the risk of resistance. Although the standard antimalarial drugs are available, the burden of this disease is getting worse, mainly due to the increasing resistance of *Plasmodium falciparum* against the widely available antimalarial drugs, including emerging resistance to the artemisinin component of artemisinin-based combination therapies (Ariey et al. 2014). Therefore, there is an urgent need to discover new, highly effective antimalarial drug candidates with new mechanisms of action to overcome the

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problem of the rapid emergence of drug resistance and to achieve long-term clinical efficacy (Silva et al. 2011).

Artemisinin, a sesquiterpene lactone and its derivatives are being used as antimalarial agents, which encouraged us to investigate antimalarial activity in other terpenoids. As part of our antimalarial drug discovery programs, we recently reported significant antimalarial activity in pentacyclic triterpenoids glycyrrhetic acid (Kalani et al. 2013) and ursolic acid (Kalani et al. 2015). This encouraged us to investigate antimalarial activity in other terpenoids. A literature search revealed that phytol was another such acyclic terpenoid, occurring as major constituent in several Indian medicinal plants. Recent reports have shown that phytol possesses antimalarial activity (da Silva et al. 2004; Grace et al. 2012) prompted us to carry out chemical transformation of phytol into several derivatives, evaluation of their antimalarial activity and study their structure–activity relationship (SAR). As most of the time, this strategy has provided exciting results with high bioactivity and low toxicity (Upadhyay et al. 2014).

Materials and methods

General experimental procedure

Chemistry

All the chemicals and reagents including phytol (procured as cis–trans mixture) were obtained from Sigma-Aldrich Pvt. Ltd., India and were used without further purification. The solvents used in chromatographic separations were purchased from Merck India Pvt. Ltd. The 300 MHz nuclear magnetic resonance (NMR) (Avance, Bruker, Switzerland) was used to record ^1H and ^{13}C NMR with tetramethylsilane (TMS) as internal standard. Hyphenated LC-PDA-MS (Prominence LC and mass MS-2010EV, Shimadzu, Japan) was used for mass spectra. Infrared (IR) spectra were recorded in CCl_4 using Spectrum BX FTIR spectrometer (Perkin Elmer, USA). The progress of the reaction was monitored by thin layer chromatography on silica gel 60F254 (Merck, Germany) readymade aluminum sheets, first examined under ultraviolet (UV) illumination at 254 and 365 nm and then sprayed with vanillin-sulfuric acid (1:5, w/v) solution in ethanol followed by heating at 95 °C for 5 min. Chromatographic purifications were performed on silica gel (60–120 Mesh) procured from Merck (India).

Semi-synthesis of phytol derivatives

Phytol was chemically modified into thirteen derivatives. To perform the blind pharmacological antimalarial screening to avoid the biases, phytol was coded as *PhY*.

General procedure for synthesizing phytol (PhY) derivatives 1–10

Phytol (592 mg, 2 mmol) was dissolved in dry pyridine, and the respective acyl/aryl chloride (3 mmol) was added. After adding a catalytic amount of 4-dimethylaminopyridine (DMAP), the reaction mixture was stirred at 30–35 °C for 14 h. After completion of the reaction, crushed ice was added, and the reaction mixture was extracted with chloroform (3×25 mL). The combined chloroform extract was washed with 6% aqueous HCl solution to remove the pyridine. Finally, the combined CHCl_3 extract was washed with distilled water and dried over anhydrous Na_2SO_4 , and the solvent was removed under vacuum. The resulting crude products were separately purified by column chromatography (silica gel, 60–120 mesh, 24 g, column diameter 2×30 cm) to afford the respective derivatives, 1–10, in high purity (>95%).

PhY-11(1-bromo-3, 7, 11, 15-tetramethyl-2-hexadecene)

PBr_3 (3 mmol) was added to solution of phytol (2 mmol) in dry benzene. The reaction mixture was left at 4 °C for 30 min, followed by stirring at room temperature (30–35 °C) for 3 h. After completion, the solvent was removed by evaporation, and the residue was extracted with chloroform. The combined organic extracts were dried and evaporated to give crude products, which on purification by column chromatography on silica gel, afforded pure product 11 (*PhY*-11) as a viscous dirty-white liquid in 86% yield (619.5 mg) (purity >95%).

PhY-12(3, 7, 11, 15-tetramethyl-2-hexadecenal)

Pyridinium chlorochromate (3 mmol) was added to a solution of phytol (2 mmol) in dry CH_2Cl_2 . The reaction mixture was reflux for 6 h, and then the solvent was removed by evaporation. The residue was extracted with chloroform in excess water. The combined organic extracts were dried and evaporated to give crude products which, after purification by column chromatography on silica gel, afforded pure product 12 (*PhY*-12) as a viscous colorless liquid in 74% yield (435.5 mg) (purity >95%).

3, 7, 11, 15-Tetramethyl-2'-phenyloctadec-1(1'), 2(3)-diene-2'-one (13)

$\text{BF}_3 \cdot \text{Et}_2\text{O}$ (5 mmol) was gradually added to a stirred solution of 12 (2 mmol) and acetophenone (2 mmol). The reaction mixture was reflux for 1 h and then left at room temperature overnight (14 h). After completion of the reaction, the mixture was diluted with diethyl ether (50 mL), followed by washing with distilled water (5×100 mL) to discharge the

BF₃.Et₂O complex. The combined organic extract was dried over anhydrous Na₂SO₄ and evaporated to give crude products which, after purification by column chromatography on silica gel, afforded pure product 13 as a reddish viscous liquid in 62% yield (491 mg) (purity >95 %).

In vitro antiplasmodial activity

Chloroquine-sensitive *P. falciparum* (NF-54) was cultivated in human B+ red blood cells using RPMI-1640 medium supplemented with 25 mM HEPES, 0.2% NaHCO₃, 370 μ M hypoxanthine, 40 μ M gentamycin, 0.25 μ M fungizone, and 0.5% albumax at 37 °C (Butler and Buss 2006). Culture was maintained in a standard gas mixture consisting of 1% O₂, 5% CO₂, 94% N₂, and culture medium was changed after every 24 h. The culture was routinely monitored through Giemsa staining of thin smears. The culture was synchronized by 5% sorbitol treatment to obtain ring-stage parasites (Lambros and Vanderberg 1979). Antiplasmodial activity of phytol and its derivatives was estimated spectrophotometrically in control and drug-treated cultures by measuring the parasite specific lactate dehydrogenase activity (Trager and Jensen 1976). Briefly, a synchronous ring stage culture with 1.2% parasitemia and 2% hematocrit was incubated in 96-well microtiter plate in different concentrations of phytol and its derivatives (μ M) at 37 °C for 72 h. Chloroquine was used as positive control. After incubation, plates were subjected to three 20-min freeze-thaw cycles to release the cell content, then cultures were mixed carefully and aliquots of 20 μ L were transferred to another microtiter plate containing 100 μ L of Malstat reagent (0.125% Triton X-100, 130mM L-lactic acid, 30 mMTris buffer and 0.62 μ M 3-acetylpyridine adenine dinucleotide) and 25 μ L of NBT-PES (1.9 μ M nitro blue tetrazolium and 0.24 μ M phenazine ethosulphate) solution per well. The plate was incubated in the dark for 30 min and absorbance was measured at 650 nm using a microplate reader (BMG Labteck Pvt. Ltd, Germany). The absorbance values of test compounds and standard were converted to percentage inhibition using absorbance of control wells. Antiplasmodial activity of the test compounds were expressed as IC₅₀ (Mean \pm SEM) of the three separate experiments performed in triplicate. IC₅₀ responses were compared with drug-free controls.

Ethics statement

In vitro cytotoxicity study on isolated peritoneal macrophages cells from healthy mice and In vivo animals experimentation on mice was performed as per the approved protocol by the Institutional Animal Ethics Committee (IAEC) of CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow followed by the Committee for the

Purpose of Control and Supervision of Experimental Animals (CPCSEA), New Delhi, Government of India (Registration No: 400/01/AB/CPCSEA).

In vitro cytotoxicity study

The most active derivative of phytol in in vitro antimalarial screening was further taken up for its cytotoxicity profile in normal mammalian cells. In brief, the macrophage cells were isolated from the peritoneal cavities of mice (8-week-old female BALB/C mice) after an intra-peritoneal injection of 1.0 mL of 1% peptone (BD Biosciences, USA) 3 days before harvesting. Mice were euthanized by cervical dislocation under ether anesthesia and peritoneal macrophages were obtained by intra-peritoneal (i.p.) injection of phosphate buffer saline (PBS), pH-7.4. Membrane debris was removed by filtering the cell suspensions through sterile gauze. Macrophage cells were treated with *PhY*-3 (10, 30, and 100 μ g/mL) for 24 h. The MTT (stock solution 5 mg/ml) was added to a final concentration of 0.5 mg/mL and cells were incubated for an additional 4 h. The medium was removed and formazon precipitate was solubilized in 100 μ L dimethyl sulfoxide (DMSO) for 10 min and absorbance was measured at 570 nm (molecular device, USA). The amount of color produced is directly proportional to the number of viable cells. Cell cytotoxicity was calculated as the percentage of MTT absorption as follows: Percentage (%) of survival = (mean experimental absorbance/mean control absorbance \times 100).

In vivo study

Experimental animals

BALB/C female mice obtained from the animal house of the institute were used for the antimalarial and toxicity studies. The animals were housed in plastic polypropylene cages under standard animal house conditions with a 12 h light/dark cycle and temperature of 25 \pm 2 °C. The animals had ad libitum access to drinking water and food.

In vivo antimalarial activity

The in vivo antimalarial activity of in vitro potentially active palmitoyl derivatives of phytol (*PhY*-3) was carried out on *Plasmodium berghei* infected mice, based on percent parasitemia, hemoglobin, survival kinetics, and mean survival time. The schizontocidal activity of *PhY*-3 was evaluated using the method described by Knight and Peters (Makler et al. 1993). Briefly, in the experiments; the animals were infected intraperitoneally with inoculum of 1 \times 10⁶ *Plasmodium berghei* K-173 infected erythrocytes. Following the infection, the animals were orally

administered with *PhY-3* at doses 3, 10 and 30 and *PhY-3* (3 mg/kg) in combination with chloroquine at semi curative dose 2.5 mg/kg body weight for a period of 4 days after suspending them in 0.7% carboxymethyl cellulose (CMC) along with 0.1% ethyl acetate. However, chloroquine alone at 2.5 mg/kg dose was orally administered, while oral dose of 0.7% CMC was given to the vehicle group. On day 4, tail blood was used to make thin blood slides and parasitemia was counted by microscopic examination of Giemsa stained blood smears. Parasitemia was determined by counting RBCs comprising of both the parasitized RBCs and the normal RBCs. Parasitemia was counted on 4th, 6th, 8th, and onwards up to 28th day. Percentage of parasitemia was counted based on infected erythrocytes calculated per 100 erythrocytes. Throughout the test, the general condition of the animals in terms of behavior and clinical signs were also evaluated and the survival of the recovered mice was observed until day 28. Percent suppression was calculated as $100[(A-B)/A]$, where A is the mean percent parasitemia of the mice taken as vehicle control and B is the mean parasitemia in the test group. In addition, mortality in the mice was followed up to 28 days post-infection to evaluate the percent survival and mean survival time.

Estimation of hemoglobin and glucose from blood from malaria-infected mice

Hemoglobin was estimated by standard drabkin's cyanmethemoglobin method (Knight and Peters 1980). In brief, 2 μ L of whole blood was added into 500 μ L of drabkin's solution (Monozyme Ltd., India) and kept at room temperature for 5 min. Optical density of test was measured against drabkin's solution at 540 nm against blank using CMG as standard through spectrophotometer (SpectraMax, Mol Devices, USA). Blood glucose was tested using a portable glucometer (Ascensia, Bayer). In brief, blood was obtained by pricking the tail of mice on glucometer strips to analyze the blood glucose level using glucometer (Balasubramaniam and Malathi 1992). Hemoglobin and blood glucose level were estimated on the day when mortality started in vehicle treated infected mice.

Acute toxicity study

Acute oral toxicity of *PhY-3* was done on female BALB/c mice. Ten mice were taken and divided into two groups of five mice in each group. Animals of group 1 were considered as control and treated with 0.7% CMC; 10 mL/kg body weight while animals of group 2 were taken as experimental wherein the animals were treated with *PhY-3*; 300 mg/kg body weight as a single oral dose. Surviving animals were weighed and visual observations for mortality, behavioral pattern, changes in physical appearance, injury,

pain and signs of illness were conducted daily during the period of 7 days. On day 7, blood was collected from the retro-orbital plexus of the animals for hematological and biochemical parameters.

Statistical analysis

Results were presented as the mean \pm SEM and analyzed using Graph Pad Prism 4. Student's "t" test was performed to compare the results of vehicle and treatments. $P < 0.05$ was considered statistically significant.

Results

Phytol and its semi-synthetic derivatives

Phytol was procured from Sigma-Aldrich Pvt. Ltd., India. Since phytol is a diterpene primary alcohol, it was decided to prepare its simple acyl, benzoyl, and other related derivatives. The rationale for preparing these acyl and benzoyl ester derivatives was that we prepared the similar acyl and benzoyl ester derivatives for our pentacyclic triterpenoid antimalarial leads glycyrrhetic acid (Kalani et al. 2013) and ursolic acid (Kalani et al. 2015) and some of them showed significant antimalarial activity. For this purpose, a total of thirteen derivatives were synthesized for evaluation of antiplasmodial activity and SAR study. Spectral data of phytol derivatives has been depicted as supplementary data (Supplementary Fig.1).

In vitro antiplasmodial activity

The phytol derivatives (1–13) were evaluated for their in vitro antiplasmodial activity against *P. falciparum* NF-54 and the results are presented in Table 1. The results showed that although the parent compound phytol was marginally active (IC_{50} $211.5 \pm 0.93 \mu$ M), but the *PhY-3* was most active (IC_{50} $12.13 \pm 0.31 \mu$ M) followed by *PhY-13* (IC_{50} $21.2 \pm 0.29 \mu$ M) and *PhY-8* (IC_{50} $18.8 \pm 0.29 \mu$ M). Four concentrations (1, 10, 50, and 100 μ M) of the test compounds and standard drug were used for the *In vitro* screening. Chloroquine was used as standard antiplasmodial drug, which exhibited IC_{50} ($0.042 \pm 0.006 \mu$ M).

Structure–activity relationship (SAR) study

The careful analysis of the results (Table 1) revealed that antiplasmodial activity of phytol got decreased in its acetyl derivative (*PhY-1*). The antiplasmodial activity got further decreased in its trans-crotonyl derivative (*PhY-5*), which consisted of a four carbon chain with olefinic bond, but got significantly increased in its pivaloyl derivative (*PhY-4*),

Table 1 Anti-plasmodial activity of phytol and its derivatives against chloroquine sensitive *Plasmodium falciparum* NF-54 by measuring the parasite specific lactate dehydrogenase (pfLDH)

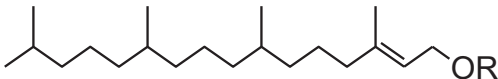
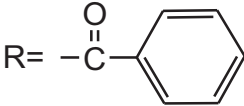
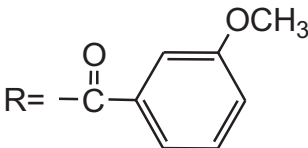
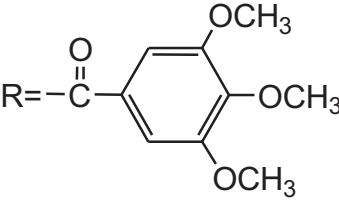
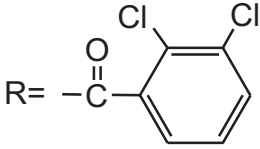
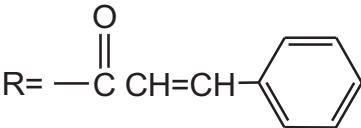
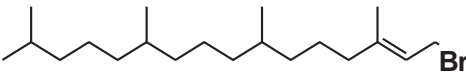
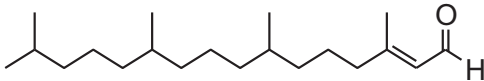
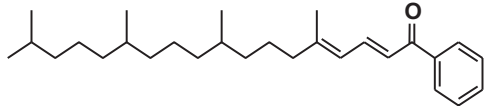
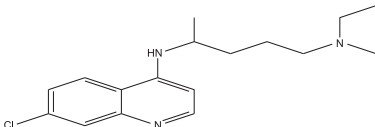
Codes	Structure	IC ₅₀ (μM) (mean ± SEM)
Phytol (PhY)		211.5 ± 0.93
PhY-1	R = H	263.9 ± 0.43
PhY-2	R = -COCH ₃	53.9 ± 0.23
PhY-3	R = -CO-CH ₂ CH ₂ (CH ₂) ₇ CH ₂ CH ₃	12.13 ± 0.31
PhY-4	R = -CO-CH ₂ CH ₂ (CH ₂) ₁₀ CH ₂ CH ₂ CH ₃	77.7 ± 0.61
PhY-5	R = -CO-C (CH ₃) ₃	>250
PhY-6	R = -CO-CH=CH CH ₃	>250
PhY-7		213.3 ± 0.34
PhY-8		18.8 ± 0.29
PhY-9		171.2 ± 0.26
PhY-10		177.8 ± 0.34
PhY-11		>250
		

Table 1 (continued)

Codes	Structure	IC ₅₀ (μM) (mean ± SEM)
PhY-12		109.3 ± 0.93
PhY-13		21.2 ± 0.29
CQ		0.042 ± 0.006

IC₅₀ concentration that inhibits 50% growth of parasites, $n = 3$

which consisted of five carbon branched chain. This increase in antiplasmodial activity with increase in carbon chain length was further observed in lauroyl (*PhY-2*) and palmitoyl (*PhY-3*) derivatives having twelve and sixteen carbon chains respectively. On comparing the antiplasmodial activities of lauroyl (*PhY-2*, IC₅₀ 53.9 ± 0.23 μM) and palmitoyl (*PhY-3*, IC₅₀ 12.13 ± 0.31 μM) derivatives, it was observed that *PhY-3* was four times more active than *PhY-2*, which may be considered due to four carbon increase in the chain length of *PhY-3* than of *PhY-2*. From the above SAR study, it may be concluded that acylation of the hydroxyl group in phytol by long chain (lipophilic) groups significantly increases antiplasmodial activity. We postulate that many of these ester derivatives will be working as prodrugs as these ester derivatives (eg. *PhY-3*) might have better cell penetration than the phytol and would be getting hydrolyzed back to phytol inside the cells. At the same time, it can't be ruled out that the activity is not due to phytol. Similarly, SAR could also be deduced among the acyl derivatives of phytol bearing substituted benzoyl groups. The antiplasmodial activity got reduced in the benzoyl (*PhY-6*, IC₅₀ > 250 μM), which got slightly improved in *m*-anisoyl (*PhY-7*, IC₅₀ 213.3 ± 0.34 μM) derivative. Further, it increased slightly in the dichloro substituted derivative (*PhY-9*; IC₅₀ 171.2 ± 0.26 μM), but it was drastically increased in the trimethoxy substituted derivative (*PhY-8*; IC₅₀ 18.8 ± 0.29 μM). On comparing the antiplasmodial activities of monomethoxy (*PhY-7*, IC₅₀ 213.3 ± 0.34 μM) and tri-methoxy (*PhY-8*, IC₅₀ 18.8 ± 0.29 μM) benzoyl derivatives, a ten-fold increase in the activity was observed due to the increase of two more methoxy groups in *PhY-9*. Hence, it may be concluded that,

substitution of hydroxyl group in phytol with benzoyl group however reduced the antiplasmodial activity, but substitution with tri-methoxy benzoyl derivative significantly increase the activity.

Finally, the other two phytol derivatives were also evaluated for their antiplasmodial activity. In comparison to phytol, a two-fold enhancement in the activity of phytol (*PhY-12*, IC₅₀ 109.3 ± 0.93 μM) was observed. The enhancement in activity may be due to the oxidation of aldehyde into the corresponding carboxylic acid inside the cells. The final benzoyl derivative *PhY-13* showed fourfold increase (IC₅₀ 21.2 ± 0.29 μM) in its activity, which may be accounted for its unhydrolysable keto group.

Cytotoxicity study

The in vitro cytotoxicity of *PhY-3*, a most active anti-malarial was carried out in the peritoneal macrophage cells isolated from the mice using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. No significant change in percent (%) dead cell population was observed ($P < 0.05$) at 10, 30 and 100 μg/mL concentration of the treatment when compared with un-treated cells, confirming that *PhY-3* is non-toxic (Supplementary Fig. 2).

In vivo study

Antimalarial activity in mice

The in vivo antimalarial model was employed for this study to validate the in vitro antiplasmodial potential of the most active *PhY-3*. Our results indicated that the intra-peritoneal

Table 2 Anti-malarial activity of *PhY-3* against *P. berghei* in Balb/c female infected mice

Groups	Dose (mg/kg)	Parasitaemia (%)	Suppression (%)	Mean survival time (Days)
Normal	–	NA	NA	NA
Vehicle control	–	34.05 ± 2.62	0.00	10.17
PhY-3	3	29.70 ± 0.85	12.77 ± 2.51	11.00
PhY-3	10	20.34 ± 2.24	40.26 ± 6.59	13.33
PhY-3	30	15.36 ± 0.79	54.89 ± 2.32	16.83
CQ + PhY-3	2.5 + 3	8.21 ± 1.32	75.88 ± 3.48	18.17
CQ	2.5	7.28 ± 0.88	78.62 ± 2.60	21

Data are expressed as mean ± SEM. $n = 6$

NA not applicable, CQ chloroquine

* $P < 0.05$; vehicle vs. treatment (ANOVA; Tukey's test)

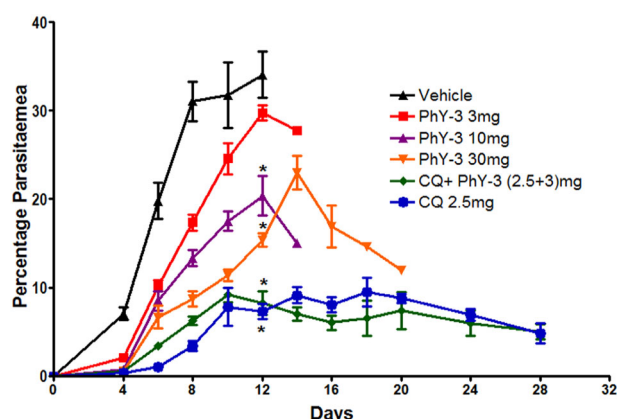


Fig. 1 Effect of *PhY-3* on percent parasitemia in *P. berghei*-induced malaria in mice. Data are expressed as mean ± SEM; * $P < 0.05$; vehicle vs. treatment (ANOVA; Tukey's test), $n = 6$

injection of *P. berghei* infected RBCs induced the peak of parasitemia on day 12 of post infection. Oral administration of *PhY-3* at 3, 10, and 30 mg/kg exhibited significant ($P < 0.05$) dose-dependent inhibition of parasitemia as compared to vehicle-treated infected control mice. The representative data of percentage of parasitemia and percentage of suppression of parasitemia are depicted in Table 2. Mortality rate improvement was observed in treated groups when compared to vehicle-treated infected control mice. The mean survival time of vehicle-treated control mice was 10.17 days whereas, *PhY-3* (3, 10 and 30 mg/kg) treated groups were 13.33, 16.83, and 18.17 days, respectively and chloroquine (2.5 mg/kg) and combination of *PhY-3* (3 mg/kg) plus chloroquine (2.5 mg/kg) was 21 and 18.17 days, respectively. The result of percent parasitemia and percent survival of mice are depicted in Figs. 1 and 2, respectively.

Effect of *PhY-3* on hemoglobin and blood glucose

The level of hemoglobin and glucose in blood were significantly decreased in vehicle-treated infected mice when compared with normal mice. The treatments with *PhY-3* and

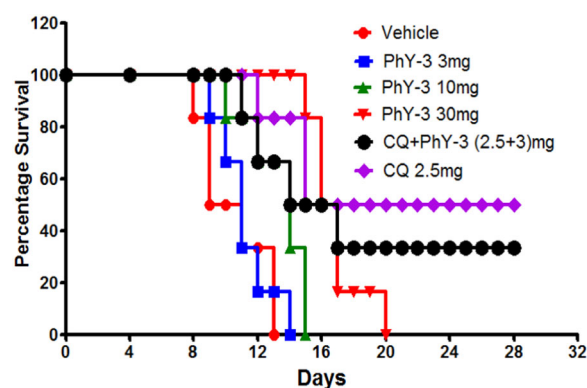


Fig. 2 Effect of *PhY-3* on percent survival in *P. berghei*-infected mice. $n = 6$

CQ were capable to restore the blood hemoglobin and glucose level towards normal (Fig. 3a, b).

Acute oral toxicity study in mice

The acute oral toxicity study of the most potent *PhY-3* showed that the administration of a single oral dose of 300 mg/kg body weight did not produce any toxic effects on the general behavior or appearance of mice as compared to the control group and all the mice survived during the whole experimental period. Similarly, no significant changes were recorded in body weight, serum biochemical (SGOT, SGPT, creatinine, cholesterol, and triglycerides,) as well as hematological parameters (RBCs and WBCs) of the treated group when compared to the control group (Table 3).

Discussion

A safe and effective treatment is essential to achieve significant and sustained reductions in malaria-related morbidity and mortality. The widely used antimalarial drugs in the market are plant-based quinine and artemisinin derivatives. The complexity of the *Plasmodium falciparum*

Fig. 3 Effect of *PhY-3* on hemoglobin and blood glucose level post infection of *P. berghei*-induced malaria in mice. **a** Blood glucose **b** hemoglobin. Data are expressed as mean \pm SEM; * $P < 0.05$; vehicle vs. treatment; # vehicle vs. normal; $n = 3$

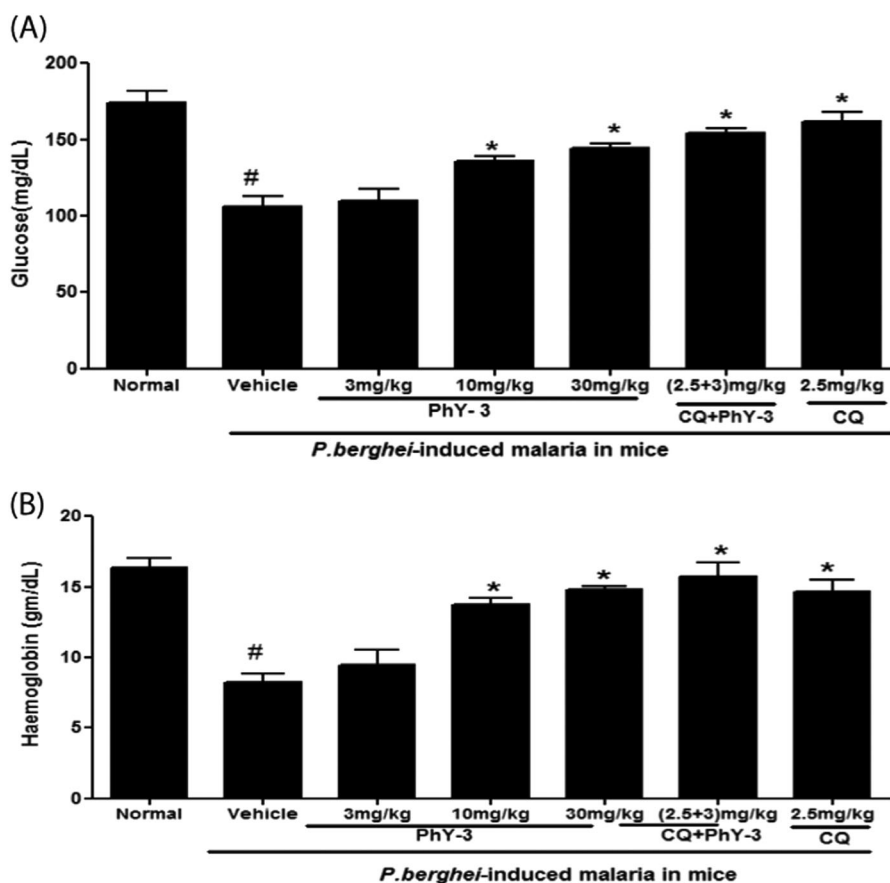


Table 3 Effect of *PhY-3* on acute oral toxicity at 300 mg/kg as a single oral dose in Balb/c female mice

Parameters	Basal value		7th day	
	Control	PhY-3	Control	PhY-3
Body weight (g)	28.5 \pm 0.31	27.84 \pm 0.34	28.2 \pm 0.47	28.06 \pm 0.67
SGOT (U/L)	24.15 \pm 3.36	25.27 \pm 2.78	22.64 \pm 1.98	23.31 \pm 2.73
SGPT (U/L)	19.57 \pm 1.33	23.52 \pm 1.92	20.53 \pm 2.04	21.98 \pm 2.12
Creatinine(mg/dL)	1.62 \pm 0.36	1.69 \pm 0.13	1.51 \pm 0.17	1.58 \pm 0.20
Cholesterol (mg/dL)	101.09 \pm 3.04	107.63 \pm 4.87	104.22 \pm 3.45	111.07 \pm 6.53
Triglycerides (mg/dL)	118.52 \pm 7.69	116.55 \pm 7.00	117.05 \pm 5.68	121.10 \pm 1.87
Haemoglobin (gm/dL)	10.52 \pm 0.29	11.19 \pm 0.75	11.17 \pm 0.68	10.93 \pm 0.78
RBC (million/mm ³)	6.12 \pm 0.27	6.19 \pm 0.46	6.47 \pm 0.35	6.23 \pm 0.29
WBC (thousands/mm ³)	7.82 \pm 0.37	7.79 \pm 0.56	7.44 \pm 0.34	7.5 \pm 0.40

Data are expressed as mean \pm SEM, $n = 4$

parasite leads to develop the progressive resistance to these frequently used drugs. World Health Organization (WHO) has recommended the use of combinations of drugs to replace the monotherapy in the treatment of malaria. Hence, as a part of our drug discovery program for antimalarial agents from plants; phytol, a very commonly occurring diterpene alcohol in the plant was investigated for antiplasmodial potential against the chloroquine-sensitive *Plasmodium falciparum* NF54 by measuring the parasite specific lactate dehydrogenase (pLDH) activity.

Identification of potential drugs targeting malaria parasites are aimed at the blood stages of the parasites (Singhal et al. 2011) phytol, a parent molecule showed moderate activity (IC_{50} 211.5 \pm 0.93 μ M). On the basis of SAR strategy which generally provides exciting results with high bioactivity and low toxicity (Upadhyay et al. 2014). We, further, phytol was chemically converted into thirteen derivatives, which were also evaluated for their antiplasmodial potential.

To test the effect of phytol and its derivatives on malaria parasites, we performed parasite specific lactate

dehydrogenase (*p*/LDH) assay in the chloroquine-sensitive *P. falciparum* NF54. All the derivatives were active, but the *PhY-3* (IC_{50} $12.13 \pm 0.31 \mu M$) followed by *PhY-8* (IC_{50} $18.8 \pm 0.29 \mu M$) and *PhY-13* ($21.2 \pm 0.29 \mu M$) were significantly active against the *P. falciparum*. The *P. falciparum* lactate dehydrogenase enzyme (PfLDH) has been considered as a potential molecular target for antimalarials due to this parasite's dependence on glycolysis for energy production. Because the LDH enzymes found in *P. vivax*, *P. malariae*, and *P. ovale* exhibit around 90% identity to PfLDH, it would be desirable to have new anti-pLDH drugs, particularly ones that are effective against *P. falciparum*, the most virulent species of human malaria (Penna-Coutinho et al. 2011). Among all this most active derivative was *PhY-3*. To confirm the safety profile, in vitro cytotoxicity of the most active *PhY-3* in primary macrophages isolated from mice revealed that it was non-toxic even at the higher concentration. To substantiate the physiological function of *PhY-3*, we further evaluated its therapeutic efficacy and safety profile in mice. In vivo efficacy study in infected mice indicated that the oral treatment with *PhY-3* (3, 10, and 30 mg/kg), showed antimalarial activity by reducing the parasitemia in a dose-dependent manner when compared to vehicle treated infected control mice. Further, treatment was able to improve the mean survival time of *P. berghei* infected mice. There are several reports on the antimalarial activity of plant-derived molecules and its derivatives against the blood stages of the parasites by reducing the parasitemia and improve the mean survival time (Mohanty et al. 2015; Saxena et al. 2016). Hyperparasitemia, hypoglycemia, cerebral malaria, severe anemia, respiratory distress are the clinical manifestations of severe malaria (Perkins et al. 2011) and it has been correlated with mortality in many parts of the world especially in children and pregnant women (Schantz-Dunn and Nour 2009). Infection and destruction of red blood cells is central to the malaria-causing plasmodium parasite's reproduction and survival. Since red blood cells consist of hemoglobin, an oxygen transport protein, their destruction causes severe anemia (Boeuf et al. 2012). Malaria parasites also dependent on glucose as a nutrient source. As Plasmodium has no capacity to store energy in the form of glycogen they rely entirely on an exogenous supply of glucose and the parasites use a large portion of this energy both to break apart hemoglobin proteins within the cells (Ogetii et al. 2010). The oral treatment of *PhY-3* to the *P. berghei* infected mice also attributed the improvement of blood glucose and hemoglobin level when compared with vehicle-treated infected mice. *PhY-3* treatment kills the malaria parasite, so loss of blood glucose and hemoglobin due to malaria infection in mice was improved in *PhY-3* treated groups.

In this study, we have attempted to find out any synergistic interaction standard antimalarial drug chloroquine and

PhY-3. Chloroquine (CQ) is a cost effective antimalarial drug with a relatively good safety profile. However, CQ is no longer used alone to treat patients with *P. falciparum* due to the emergence and spread of CQ-resistant (Aguiar et al. 2014). The results on combination study of CQ and *PhY-3* showed no synergism. In vitro cytotoxicity in primary macrophage cells and in vivo acute oral toxicity study in mice revealed that the *PhY-3* was non-toxic even at the higher concentration. There are several reports concluding that plant-derived molecules are safe at higher concentration (Mohanty et al. 2015; Saxena et al. 2016).

Conclusion

Phytol and its derivatives showing antiplasmodial activity. The phytol derivative *PhY-3* possesses significant antimalarial potential in both in vitro and in vivo bioassay and also non toxic even at the higher dose to the mice.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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