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Antimalarial activity of *Toona ciliata* MJ Roem aqueous methanolic leaf extract and its antioxidant and phytochemical properties

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ABSTRACT

Background and aim: Malaria is a global health issue causing substantial morbidity and mortality. Screening of various traditionally important medicinal plants is a key source for the discovery of new antimalarials. We evaluated the antimalarial and antioxidant activities, and performed detailed phytochemical analyses of *Toona ciliata* MJ Roem aqueous methanolic leaf extract (*Tc*MLE).

Experimental procedures: In vitro antiplasmodial studies in *Plasmodium falciparum* (*Pf*) 3D7 and *Pf*Cam3.I^{R539T} strains were performed by [^{3}H]-hypoxanthine uptake assays. In vitro cytotoxicity in HeLa and HEK293T cell lines was evaluated using MTT assays. Hemolysis assay was performed using RBCs. Phytochemical analysis by GC-MS and in vitro antioxidant studies by DPPH and ABTS assays were performed. In vivo antimalarial studies in *Pb*-infected mice were carried out using Rane's test and Peters' 4-day test.

Results and conclusions: *Tc*MLE showed significant *in vitro* antioxidant activity and had phytochemicals reported for antimalarial activity. *In vitro* studies showed prominent antiplasmodial activity against *Pf*3D7 strain (IC$_{50}$ ~22 μg/ml) and *Pf*Cam3. I^{R539T}strain (IC$_{50}$ value ~43 μg/ml). *In vitro* cytotoxicity studies, *in vitro* hemolytic assays, and *in vivo* acute toxicity studies further suggested that *Tc*MLE is nontoxic. *In vivo* antimalarial studies using Rane's test showed a significant decrease in parasitemia by ~70% at 1200 mg/kg doses and delayed the mortality of mice by ~10–14 days. Peters' 4-day test also showed a similar pattern. The present study demonstrated the antimalarial potential of *Tc*MLE. These findings deliver a platform for further studies to identify the active components of *Tc*MLE and discover new antimalarials.

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Novelties

Although many plants from Meliaceae family possess diverse biological activities including the antimalarial properties, *Toona ciliata* has not been studied yet for its antimalarial activity. Several reports have described the medicinal values of *T. ciliata*. However, this study is the first report on its potential *in vitro* and *in vivo* antimalarial activities. The results suggest a prominent antiplasmodial activity against *P. falciparum* (*Pf*) 3D7 and artemisinin (ART)-resistant *Pf*Cam3.I^{R539T}strains. In the context of emerging resistance against ART and ART-based combination therapies (ACTs), our findings warrant further detailed investigations to identify new antimalarials.

List of abbreviations

ABTS	2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
ACTs	Artemisinin-based combination therapies
CPCSEA	The Committee for Control and Supervision of Experiments on Animals
CQ	Chloroquine
DMSO	Dimethyl sulfoxide
DPPH	2, 2-diphenyl-1-picrylhydrazyl
GC-MS	Gas chromatography-mass spectrometry
GCTM	Global Center for Traditional medicine
IBSD	Institute of Bioresources and Sustainable Development
MTT	4, 5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NIST	National Institute of Standard and Technology
OECD	The Organization of Economic Co-operation and Development
*Tc*MLE	*Toona ciliata* 70% aqueous methanolic leaf extract
*Tc*DLP	*Toona ciliata* dry leaf powder
pRBC	Parasitized red blood cell
PBS	Phosphate-buffered saline

1. Introduction

Plasmodium is a parasitic protozoan transmitted through infected female *Anopheles* mosquitoes causing malaria, a major public health problem. There were around 247 million malaria cases and 0.62 million deaths in 2021 across 85 endemic countries as reported in the World Malaria Report of 2022, WHO.[1] *P. falciparum* (*Pf*) and *P. vivax* are predominantly causing human malaria of which, *Pf* is mainly responsible for severe and fatal malaria.[2] The emergence of resistance against ACTs is a major concern for malaria eradication that demands the identification of new antimalarials. Plants and plant-based products have been used as traditional medicine for primary health care in rural areas. Over the years, studies have shown that medicinal plants having therapeutic properties are rich in bioactive secondary metabolites.[3] Secondary metabolites represent chemically, structurally, and functionally diverse pharmacophores. Plant secondary metabolites and their derivative drugs with diverse pharmacological activities are also considered to be effective and safe, thus providing a promising alternative to synthetic drugs.[4,5] Medicinal plants provide a credible source for identifying new antimalarials, and research on their chemical components could lead to the development of more effective antimalarial drugs.

Toona ciliata MJ Roem (common name - Toon tree, Red cedar, Indian mahogany, Moulmein cedar) is a forest tree belonging to the family Meliaceae that mainly grows in the tropical areas of Asia and Africa. It is cultivated for timber, agri-horticulture and medicinal uses.[6] Plants of this family have diverse biological activities including anti-malarial properties.[7,8] Some of the reported plant species of the Meliaceae family with potent antimalarial activities include *Azadirachta indica, Khaya sps, Melia azederach,* etc.[9–12] It has been used in traditional medicine to treat fever, diarrhea, ulcer, leprosy, menstrual disorders, etc (Table S1).[13–18] Several phytochemical investigations have been reported on *T. ciliata* MJ Roem possessing extensive biological potency like anti-bacterial,[19] anti-fungal,[20] analgesic,[21] anti-inflammatory,[22] antioxidant,[23] anti-diabetic,[24] anti-tumor,[25] antiulcer effect,[26] cytotoxic,[27] etc. It has also been traditionally used to cure fever, which is a major clinical symptom of malaria. Nevertheless, to the best of our knowledge, this is the first report on the antimalarial activity of *T. ciliata* methanolic leaf extract (*Tc*MLE).

To identify the potential uses of *T. ciliata*, we have evaluated the antiplasmodial and antimalarial activities of *Tc*MLE using *in vitro P. falciparum* cultures and *in vivo P. berghei*-infected mouse model. Further, we have examined the cytotoxic and antioxidant activities of *Tc*MLE and performed phytochemical screening. Our investigations bridge the gap between the traditional medicinal uses and scientific evaluation of *T. ciliata* leaf extract.

2. Methodology

2.1. Plant material collection and identification

Toona ciliata fresh leaves were collected from Lilong Chajing (latitude - N24°43′15.57552″; longitude − E93°56′26.65104″), Imphal, India. The plant material was identified and authenticated by the plant taxonomist of IBSD, Manipur. A herbarium specimen of the plant material (Voucher No.-IBSD/M − 278) was deposited in the IBSD herbarium.

2.2. Preparation of dry leaf powder (TcDLP) and leaf extract (TcMLE) of Toona ciliata

T. ciliata leaves were air-dried and pulverized using a grinding machine to prepare *Tc*DLP. The fine powder obtained was stored in airtight container for further testing. For the preparation of *Tc*MLE, 150 g of *Tc*DLP was extracted in 1 L of 70% methanol in water for 72 h. To concentrate the filtrate, a rotary evaporator (Buchi Rotavapor, R-215) at 60 rpm and 45 °C was used. *Tc*MLE obtained was stored at 4 °C for further testing.

2.3. Extraction yield

The percentage yield of dried *Tc*MLE was calculated as follows: % Yield = (Weight of the crude dried leaf extract/Weight of the dry leaf powder) x 100.

2.4. Qualitative screening of phytochemicals

The qualitative phytochemical analysis was carried out based on the color reactions as mentioned with slight modifications.[28]

2.5. Estimation of total phenolic content

The Folin-Ciocalteu (FC) method was used to estimate the total phenolic content in *Tc*MLE as mentioned.[29] Briefly, 1 mg/ml of *Tc*MLE was mixed with FC reagent (1 ml), incubated for 5 min, and 4 ml Na_2CO_3 solution (20% w/v) was added. The reaction volume was doubled with distilled water and incubated in dark for 30 min. The absorbance of the sample was read at 765 nm. The percentage of total phenolic content was determined from the gallic acid calibration curve and represented as milligrams of gallic acid equivalent per gram of dried leaf extract (mg GAE)/g.

2.6. Estimation of total flavonoid content

The aluminum chloride method was used to estimate the total flavonoid content of *Tc*MLE as mentioned.[30] Briefly, 0.6 ml of varying concentrations of standard quercetin solution or *Tc*MLE and 0.6 ml of aluminum chloride (2%) were mixed, kept at RT for 1 h and the absorbance was read at 420 nm. The total flavonoid was determined using the quercetin calibration curve and represented

as milligrams of quercetin equivalent per gram of dried leaf sample (mg QE)/g.

2.7. Determination of antioxidant activity

2.7.1. DPPH assay

Free radical scavenging activity of *Tc*MLE was calculated by a calorimetry-based assay using DPPH radical as described.[30] In brief, 50 μL of standard (±) α-tocopherol or *Tc*MLE in various concentrations and 150 μL of DPPH solution (0.1 mM in methanol) were mixed. Thereafter, the reaction mixture was incubated for 30 min at RT in dark. The absorbance of the samples was measured at 517 nm. The antioxidant activity was represented as DPPH scavenging percentage and calculated as % DPPH scavenging activity = [(Control absorbance - Sample absorbance)/Control absorbance] x 100.

2.7.2. ABTS assay

The ABTS assay was performed as mentioned with slight modifications.[31] For the ABTS assay, ABTS stock solution (7 mM) was mixed with potassium persulfate (2.45 mM) to form the ABTS$^{\bullet+}$ solution. The solution was incubated for ~12–16 h at RT in the dark. Methanol was used to dilute the ABTS$^{\bullet+}$ solution to achieve an absorbance of 0.706 ± 0.01 units. The standard (±) α-tocopherol or *Tc*MLE (40 μL) was added to ABTS$^{\bullet+}$ solution (160 μL), incubated for 5–6 min, and the absorbance was read at 734 nm. The ABTS$^{\bullet+}$ scavenging percentage was determined as follows % ABTS$^{\bullet+}$ scavenging = [(AB-AA)/AB] x 100 wherein, AB denotes absorbance of ABTS$^{\bullet+}$ radical + methanol and AA denotes absorbance of ABTS$^{\bullet+}$ radical + *Tc*MLE or (±) α-tocopherol.

2.7.3. Reducing power assay

The reducing power of *Tc*MLE was determined as described.[32] Briefly, 100 μL of *Tc*MLE or (±) α-tocopherol (standard) was added to 250 μL of sodium phosphate buffer (0.2 M). Later, 250 μL of potassium ferricyanide (1%) was added and the mixture was incubated for 20 min at 50 °C. To this, 250 μL of trichloroacetic acid (10%) was added and centrifuged at 5000 g for 10 min. Finally, 250 μL of the supernatant was collected, mixed with 250 μL of water and 50 μL of ferric chloride (0.1% w/v). The absorbance of the reaction mixture was measured at 700 nm.

2.8. Chemical fingerprinting by GC-MS

The GC-MS analysis was performed with Thermo Scientific GC system Trace 1300 gas chromatograph using TG-5MS (5% diphenyl/9% dimethyl polysiloxane) GC column (30 m length × 0.25 mm ID × 0.25 μm film thickness) coupled with TSQ 8000 DUO mass spectrometry. An electron ionization system with an ionization energy of 70 eV was run in electron impact mode for quadruple GC-MS detection. A carrier gas of 99.99% helium was used, with an injection volume of 5 μL and a constant 1 ml/min flow rate. To calculate the relative percentage of each component, its average peak area was compared with the total area. To identify the peaks, the mass spectra were compared with the GC-MS Libraries (2017) from NIST.

2.9. In vitro antiplasmodial test

2.9.1. Pf culture and maintenance

In vitro Pf cultures (3D7 and Cam3.I^{R539T} strains) were maintained as mentioned[33] using O^{+ve} human RBCs from a healthy donor in RPMI-1640 medium supplemented with 50 mg/L hypoxanthine, 1% albumax II, and 50 μg/L gentamicin. The cultures were maintained at 5% hematocrit at 37°C, and the medium was

routinely changed and flushed with mixed gas (5% carbon dioxide, 5% oxygen, and 90% nitrogen). Giemsa-stained smears were prepared to monitor the parasite growth. To obtain ring-stage parasites, cultures were synchronized using 5% (w/v) D-sorbitol.

2.9.2. In vitro growth inhibition assay

The growth inhibition assay to determine the antiplasmodial activity of *Tc*MLE was done by measuring the [^{3}H]-hypoxanthine incorporation in the parasite nucleic acids.[34] Synchronized cultures of ring stage parasite (0.5–1.0% parasitemia) were treated with different concentrations of *Tc*MLE. After 3 h of treatment, [^{3}H]-hypoxanthine was added (5 μCi/ml) to the cultures and incubated at 37°C. The pRBC cultures (100 μL) were collected at various time intervals (12, 24, and 48 h), lysed in water followed by harvesting on glass fiber filters, and drying at 60 °C for 2–3 h. MicroBeta^2 Microplate Counter (PerkinElmer) was used to measure the radioactive counts by adding 150 μL of scintillation cocktail. Cultures were monitored for parasite number and morphology by preparing Giemsa-stained blood smears. Various concentrations of *Tc*MLE (1, 10, 25, 50, 75, and 100 μg/ml) in *in vitro Pf* cultures were achieved by diluting the stock solution (100 mg/ml). The solvent-treated culture (70% methanol in water) was used as a control. For positive control, 25 nM CQ-treated cultures of both the strains were used. IC$_{50}$ values were estimated using a non-linear regression fit of the dose-response curve.

2.10. Hemolysis assays

In vitro hemolytic effect of *Tc*MLE was examined as mentioned.[35] The fresh O^{+ve} human RBCs were centrifuged at 2000 g for 5 min, and the pellet was washed in PBS twice to prepare a 2% hematocrit solution. Different concentrations of *Tc*MLE (10 μL) were added to 190 μL of 2% hematocrit, followed by incubation for 3 h in a plate shaker (300 rpm) at RT. Thereafter, the samples were centrifuged at 2000 g for 5 min. The absorbance (A) of the supernatants was recorded at 415 nm. 70% methanol in water (solvent control), 0.4% Triton-X in PBS (positive control), and PBS (negative control) were used. The RBC viability percentage was determined as follows: [100 - (Absorbance of sample − Absorbance of PBS/Absorbance of Triton X-100) x 100].

2.11. Cell cultures and cytotoxicity assays

The *in vitro* cytotoxicity of *Tc*MLE was studied in HeLa and HEK 293 T (human embryonic kidney) cell lines. Cells were cultured in Dulbecco's modified medium as mentioned.[36] 10^4 cells were seeded for 24 h followed by treatment with various concentrations of *Tc*MLE (10–200 μg/ml) or 50 nM docetaxel (positive control) for 48 h at 37°C. Thereafter, 5 mg/ml of MTT (20 μL) was added and incubated for 3–4 h. Thereafter, DMSO (100 μL) was added and the sample absorbance (A) was read at 570 nm. The cell viability percentage was determined as follows: [Absorbance of treated sample/Absorbance of control sample] × 100.

2.12. Experimental animals

For animal studies, Balb/c male/female mice of 6–8 weeks of age were used. Mice were kept under a recommended environment (23 ± 4 °C, 30–70% humidity, diurnal cycle of 12 h light and 12 h dark) with *ad libitum* accessibility to drinking water and a pellet diet.

2.13. Acute toxicity test

Two groups of 6 mice were given a 5000 mg/kg dose of *Tc*DLP

and *Tc*MLE through an oral route after starving them for 2 h. Behavioral changes like anorexia, lacrimation, hypersalivation, tremors, reflexive response, diarrhea, seizure, and mortality were monitored as per the OECD guideline, 2008.[37]

2.14. In vivo antimalarial test

2.14.1. Parasite propagation and infection
Plasmodium berghei (*Pb* ANKA) strain was used in the present study. To initiate the blood-stage infections, $10^5/10^7$ pRBCs were injected intraperitoneally into the naïve mice.[38] Peripheral blood parasitemia was examined by Giemsa-stained blood smears prepared from tail vein blood.

2.14.2. In vivo Rane's curative test
The antimalarial potential of *Tc*DLP and *Tc*MLE were evaluated according to Rane's protocol.[39] Mice were intraperitoneally injected with 10^5 pRBCs on day 0 and divided randomly into 4 groups of six mice for each treatment. *Tc*DLP, *Tc*MLE, and CQ were prepared in the vehicle [10% Kolliphor® HS 15 (solutol) in 0.9% v/v saline]. Group 1 mice were administered with vehicle (control). Group 2 mice were administered with 400, 800, and 1200 mg/kg doses (subgroups) of *Tc*DLP. Group 3 mice were administered with 400, 800, and 1200 mg/kg doses (subgroups) of *Tc*MLE, and Group 4 mice were administered with 25 mg/kg dose of CQ (positive control). Mice were orally administered with the respective doses daily from day 3 post-infection and continued until day 7. Giemsa-stained blood smears were prepared to monitor parasitemia and the survival time was determined over 30 days. Body weight was recorded using a digital weighing machine and expressed as the percentage change in body weight of mice administered with *Tc*DLP or *Tc*MLE compared to the control (vehicle) group mice. The parasite growth inhibition was calculated as follows % Parasite growth inhibition = 100 × [(Average parasitemia of negative control group − Average parasitemia of treated group)/Average parasitemia of negative control group].

2.14.3. In vivo Peters' suppressive test
The antimalarial activity of *Tc*DLP and *Tc*MLE was evaluated by Peters' 4-day test as described.[40] Mice were intraperitoneally injected with 10^7 pRBCs and divided randomly into 4 groups of six mice for each treatment. Group 1 control mice were administered with the vehicle. Group 2 mice were administered with 800 and 1200 mg/kg doses (subgroups) of *Tc*DLP. Group 3 mice were administered with 800 and 1200 mg/kg doses (subgroups) of *Tc*MLE, and Group 4 positive control mice were administered with 25 mg/kg dose of CQ. The treatments with the respective doses were initiated 3 h post-infection orally and were continued daily for four consecutive days. On day 5, Giemsa-stained blood smears were prepared and the mice were observed for mortality until 30 days. The parasite growth inhibition was calculated as described in Rane's test.

2.15. Statistical analysis

The parasitemia percentage was determined as follows: % Parasitemia = (Number of pRBCs/Total number of RBCs) x 100. GraphPad Prism 9 was used to plot graphs and to determine statistical significance using an unpaired *t*-test, and log-rank Mantel-Cox test (n.s - not significant, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$). GraphPad Prism 9 was used for the nonlinear regression analysis and to calculate the R^2 values. Data were analyzed using Microsoft Excel 10 and represented as mean ± standard deviation (SD).

3. Results

3.1. Extraction yield and phytochemicals present in TcMLE

In this study, *T. ciliata* leaves extracted in 70% aqueous methanol showed a high extraction yield of 22.5% (based on dry weight). The qualitative phytochemical investigations of *Tc*MLE showed the presence of carbohydrates, reducing sugars, flavonoids, alkaloids, phenol, saponin, terpenoids, tannin, coumarin, proteins, and phytosterol as provided in Table 1. However, glycosides and anthroquinone were found to be absent and this could be because of their poor solubility in aqueous methanol.

3.2. Total phenolic and flavonoid contents of TcMLE

The phenolic content of *Tc*MLE was measured from the gallic acid calibration curve wherein, $y = 0.0053x + 0.3933$ (R^2 value = 0.9824). We found that *Tc*MLE had high phenolic content of 71.30 ± 3.32 mg GAE/g. The results for the total flavonoid content were derived from the quercetin calibration curve wherein, $y = 0.017x + 0.0507$ (R^2 value = 0.9845). *Tc*MLE had a total flavonoid content of 26.34 ± 1.07 mg QE/g (Table 2).

3.3. Antioxidant activity of TcMLE

*Tc*MLE showed a good DPPH radical scavenging potential having an IC_{50} value of 47.06 ± 1.33 μg/ml compared to α-tocopherol having an IC_{50} value of 24.59 ± 1.20 μg/ml. The antioxidant activity quantified by ABTS assay showed an IC_{50} value of 50.70 ± 2.1 μg/ml for *Tc*MLE while that of α-tocopherol was found to be

Table 1
Qualitative phytochemical screening of *Tc*MLE. The presence (+) and absence (−) of various phytochemicals are represented. All the tests were performed in triplicate with two different extract preparations.

Sl.No.	Phytochemical detection tests	*Tc*MLE
1.	Carbohydrates	+
	a) Molisch's test	+
	b) Barfoed's test	
2.	Reducing sugars	+
	a) Fehling's test	+
	b) Benedict's test	
3.	Flavonoids	+
	a) Alkaline reagent test	+
	b) Shinoda's test	
4.	Alkaloids	+
	a) Mayer's test	+
	b) Wagner's test	
5.	Phenol	+
	a) Ferric chloride test	+
	b) Lead acetate test	
6.	Saponin	+
	a) Foam test	
7.	Terpenoid	+
	a) Salkowski's test	
8.	Tannin	+
	a) Braymer's test	
9.	Coumarin	+
	a) NaOH test	
10.	Proteins	+
	a) Xanthoproteic test	+
	b) Ninhydrin	
11.	Phytosterol	+
	a) Libermann-Burchard's test	
12.	Glycosides	−
	a) Modified Borntrager's test	−
	b) Legal's test	
13.	Anthraquinone	−
	a) Borntrager's test	

Table 2
Total phenolic and flavonoid contents of *Tc*MLE and its antioxidant activity. The data represent mean ± SD. The tests were performed in triplicate with two different extract preparations.

Extract/ Standard	Total phenolic content (mg GAE/ g)	Total flavonoid content (mg QE/ g)	DPPH assay IC$_{50}$ (µg/ ml)	ABTS assay IC$_{50}$ (µg/ ml)	Reducing power assay IC$_{50}$ (µg/ ml)
*Tc*MLE	71.30 ± 3.32	26.34 ± 1.07	47.06 ± 1.33	50.70 ± 2.1	51.54 ± 1.29
α-tocopherol	–	–	24.59 ± 1.20	28.16 ± 1.14	38.27 ± 1.25

28.16 ± 1.14 µg/ml. The ability of *Tc*MLE to reduce Fe^{3+} to Fe^{2+} was also evaluated by the reducing power assay and the results suggested that *Tc*MLE had good reducing power having an IC$_{50}$ value of 51.54 ± 1.29 µg/ml while the α-tocopherol was 38.27 ± 1.25 µg/ml. The data showed that *Tc*MLE had good antioxidant activity, comparable to that of α-tocopherol (Table 2).

3.4. GC-MS analysis of TcMLE

Phytochemical analysis of *Tc*MLE using GC-MS showed three prominent peaks along with five moderately prominent peaks. The phytocompounds of the respective peaks were identified based on their mass spectra from the NIST library. The most abundant phytocompounds found based on the peak areas were Hexadecanoic acid methyl ester (31.29%), followed by 9-Octadecenamide (10.74%) and Methyl 8,11,14-heptadecatrienoate (10.32%). Phytocompounds of moderate abundancy included Photocitral A (7.17%), 5,10-Pentadecadien-1-ol, (Z,Z)- (3.31%), 1,1,1,3,5,5,5-Heptamethyltrisil oxane (1.7%), 4-Nonene, 2,3,3-trimethyl-,(E)- (1.54%) and 1-Octylsilatrane (1.11%). The GC-MS chromatogram of *T. ciliata* methanolic leaf extract and the chemical structures of the eight major phytocompounds identified are shown in Fig. 1A and B, respectively. The entire set of phytocompounds present in *Tc*MLE is provided in Table S2.

3.5. Antiplasmodial activity of TcMLE

*Tc*MLE exhibited good antiplasmodial activity with dose-dependent parasite inhibition having IC$_{50}$ values of 22.07 ± 1.99 µg/ml and 42.68 ± 1.06 µg/ml against *Pf*3D7 and *Pf*Cam3.I^{R539T}strains, respectively (Fig. 2A and B). Microscopic analysis of the treated *Pf* cultures by Giemsa-stained thin blood smears further confirmed the dose-dependent growth inhibition pattern and the presence of dead and arrested parasites with pyknotic nuclei (Fig. 2C & D). CQ was used as a positive control for both *Pf*3D7 and *Pf*Cam3.I^{R539T}strains having IC$_{50}$ values of 8.29 ± 1.78 nM and 11.31 ± 1.02 nM, respectively. Growth inhibition and Giemsa-stained images of *Pf*3D7 and *Pf*Cam3.I^{R539T} cultures treated with CQ are shown in Fig. 2E and F, respectively.

3.6. Hemolysis and cytotoxicity effect of TcMLE

In vitro hemolytic assay results showed that *Tc*MLE did not possess hemolytic activity (Fig. 3A). Even at a higher concentration of 500 µg/ml, there was only <10% reduction in the viability of RBCs. *Tc*MLE exhibited low cytotoxicity (<20%) against HeLa and HEK293T cells at the highest tested concentration of 200 µg/ml (Fig. 3B).

3.7. Acute toxicity of TcDLP and TcMLE

The results obtained suggested no significant physical and behavioral changes in the mice. The treated mice did not show any mortality confirming the median lethal dose (LD$_{50}$) to be greater than 5000 mg/kg.

3.8. Antimalarial activity of TcDLP and TcMLE

In vivo antimalarial activity of *Tc*DLP and *Tc*MLE was evaluated against *Pb*-infected Balb/c mice by Rane's test, which determines their curative ability during infection, and Peters' 4-day suppressive test that evaluates their potential to suppress the parasite growth. In Rane's test, *Tc*DLP showed 54.76 ± 4.25%, 45.97 ± 3.34%, and 32.98 ± 2.93% decrease in parasite growth while *Tc*MLE showed 70.74 ± 3.53%, 51.13 ± 2.34% and 39.39 ± 3.29% decrease in parasite growth at 1200, 800 and 400 mg/kg doses, respectively (Table 3). Fig. 4A and B shows the survival curves of the *Pb*-infected mice treated with different doses of *Tc*DLP and *Tc*MLE in Rane's curative test respectively. A significant ~10–14 days delay in the mortality of mice treated with 800 and 1200 mg/kg doses of *Tc*DLP and *Tc*MLE compared to the control group (vehicle) was observed. There was also an increase in the body weight of mice in the treatment group in comparison to the control group mice during Rane's test (Fig. 4C). Giemsa-stained images of the blood smear prepared on day 7 post-infection from the tail vein blood of the infected mice treated with vehicle (control), 1200 mg/kg doses of *Tc*DLP and *Tc*MLE, and 25 mg/kg dose of CQ are provided in Fig. 4D. In Peters' test, *Tc*DLP showed 57.94 ± 4.25% and 43.56 ± 2.48% decrease in parasite growth whereas, *Tc*MLE showed 75.33 ± 5.77% and 56.71 ± 3.16% decrease in parasite growth at 1200 and 800 mg/kg doses, respectively (Table 4). The survival curves of the *Pb*-infected mice treated with different doses of *Tc*DLP and *Tc*MLE in Peters' suppressive test are provided in Fig. 4E and F. As observed in Rane's test, there was a significant ~10–14 days delay in the mortality of mice treated with 800 and 1200 mg/kg doses of *Tc*DLP and *Tc*MLE.

4. Discussion

Traditional medicine comprised of indigenous health practices, knowledge and beliefs has remained effective for numerous generations to treat infectious and chronic diseases. According to GCTM (WHO), several countries use different forms of traditional medicine that include herbal medicines, Ayurveda, Siddha, Unani and other practices, acupuncture, yoga, etc. Over 40% of pharmaceutical formulations are also based on natural products and traditional medicine. Hence, in the context of the importance of traditional medicine in ameliorating public health care, there is an immense need to generate evidence-based data to strengthen the practices of traditional medicine for sustainable development.[15] Medicinal plants serve as a fundamental component of various traditional formulations and are rich in phytochemicals. Meliaceae family is known for its anti-inflammatory, antioxidant, antidiabetic, antipyretic, antiseptic, antiulcer, anticancer, antibacterial, antifungal, and antiprotozoal properties in traditional medicine. For this study, we chose *Tc*MLE and examined its antimalarial activity. While there are many reports describing the medicinal values of *T. ciliata*, its antimalarial activity has not been studied yet. Methanol is the commonly used solvent for the extraction of polyphenolic compounds from plant materials due to its high polarity. The methanolic leaf extracts of *T. ciliata* used in the present study showed high total phenolic and flavonoid contents. Several studies have shown that *T. ciliata* extracts are enriched
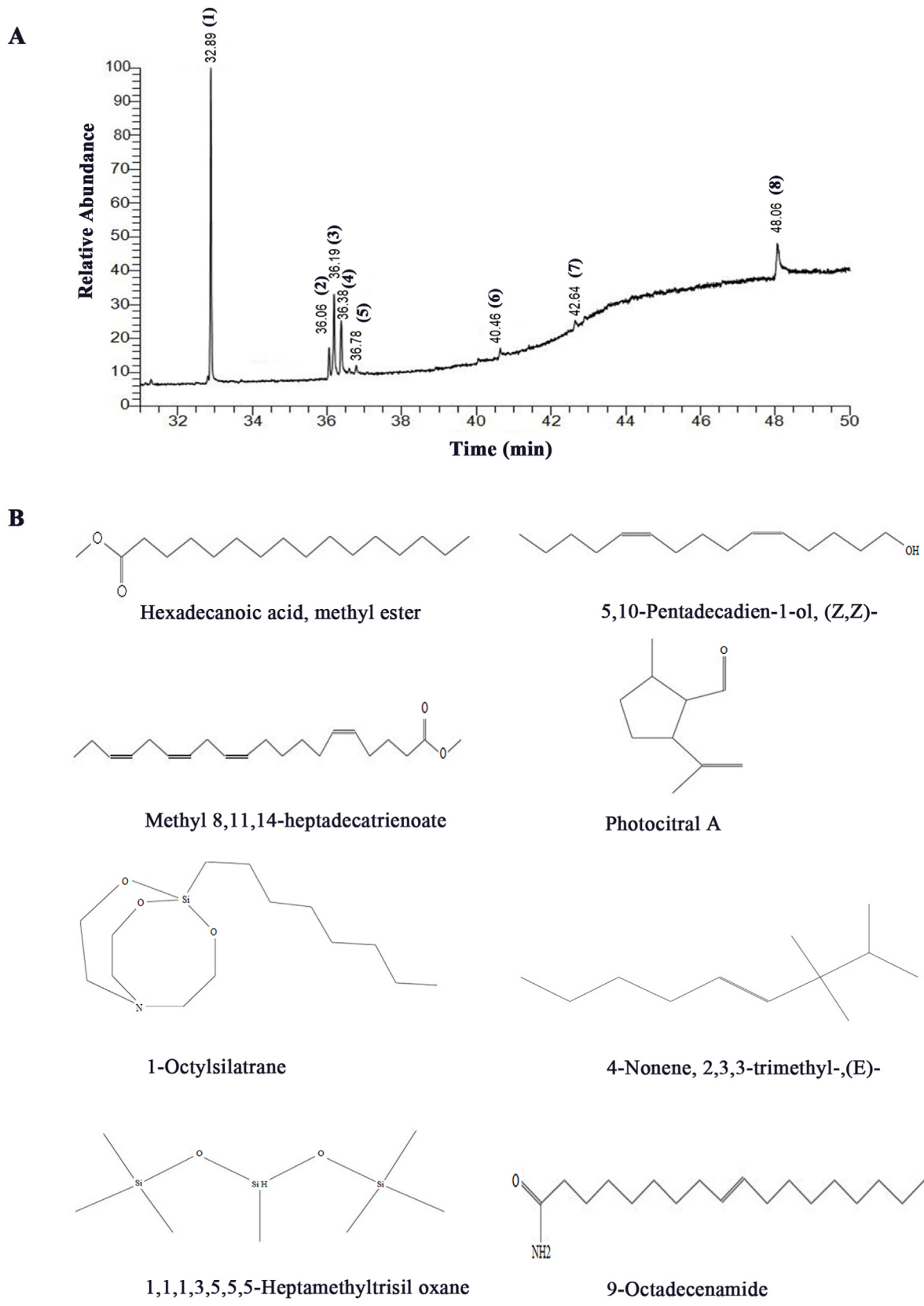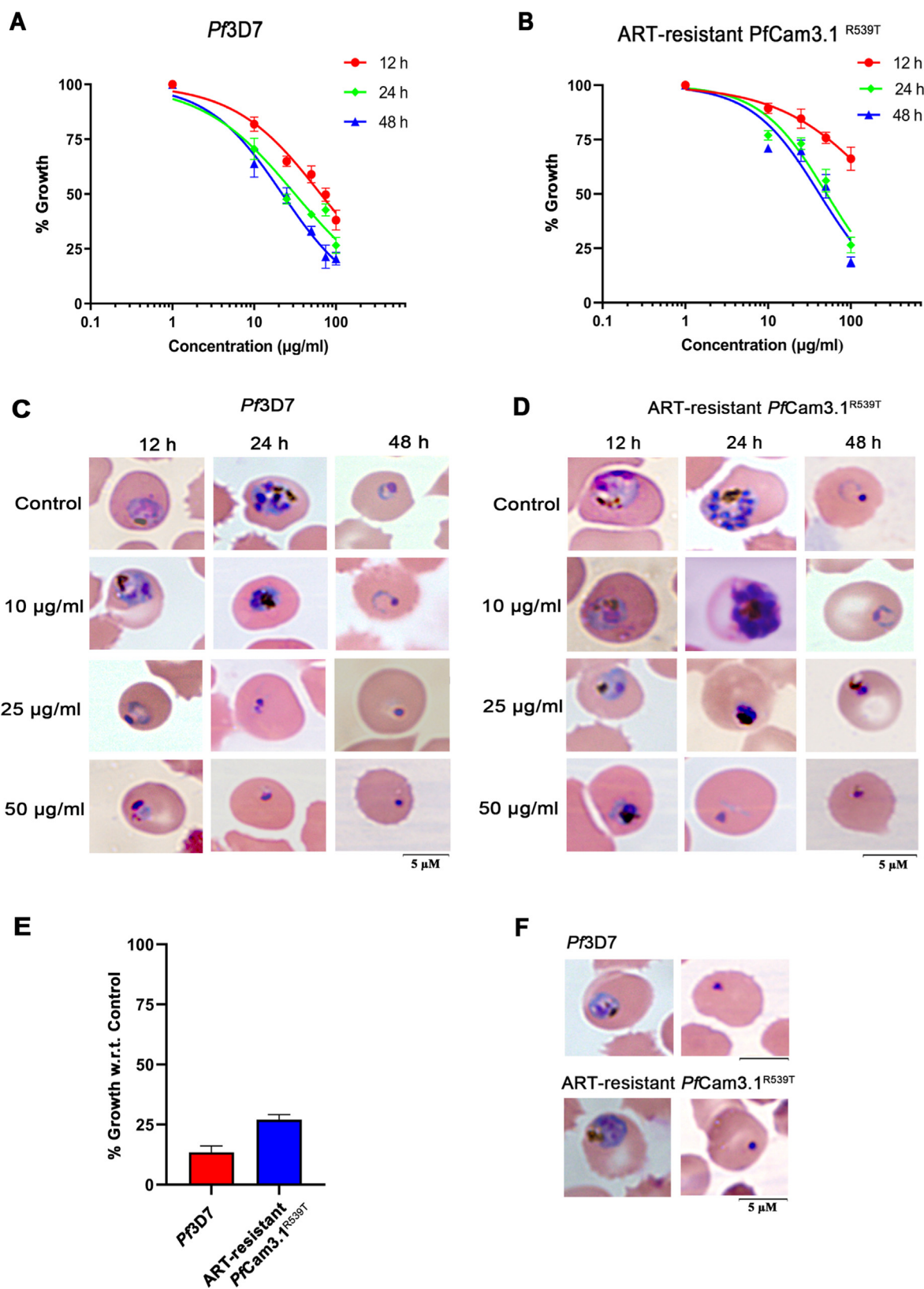
Fig. 1. GC-MS analysis of *Tc*MLE. (A) GC-MS chromatogram of *Tc*MLE. **(B)** Chemical structure of the eight prominent phytocompounds identified.

Fig. 2. Growth curves of *Pf*3D7 and ART-resistant *Pf*Cam3.I[R539T] strains treated with *Tc*MLE. (A) Dose-dependent growth inhibition of *Pf*3D7 cultures. (B) Dose-dependent growth inhibition of ART-resistant *Pf*Cam3.I[R539T] cultures. The data (mean ± SD) represent three independent experiments. (C) Giemsa-stained images of *Pf*3D7 parasites treated with *Tc*MLE. (D) Giemsa-stained images of ART-resistant *Pf*Cam3.I[R539T] parasites treated with *Tc*MLE. (E) Growth analyses of *Pf*3D7 and ART-resistant *Pf*Cam3.I[R539T] cultures treated with 25 nM CQ for positive control. The data (mean ± SD) represent the growth inhibition at 24 h from three independent experiments. (F) Giemsa-stained images of *Pf*3D7 and ART-resistant *Pf*Cam3.I[R539T] cultures treated with 25 nM CQ. All images were captured with an inverted Olympus IX83 microscope using 100x objective. Scale bar = 5 μM.
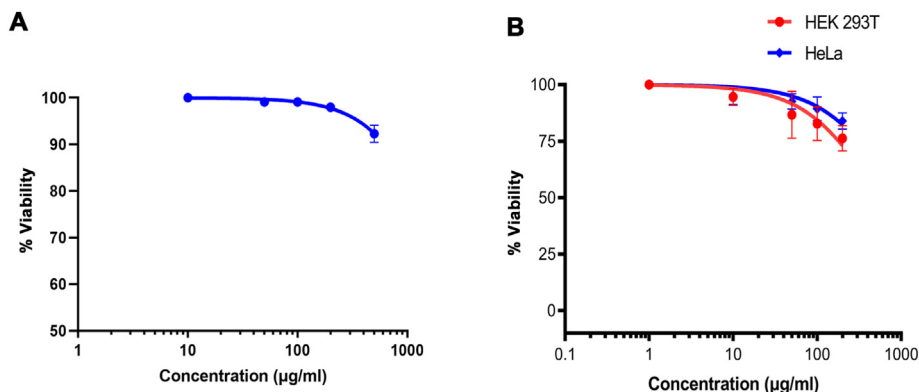
Fig. 3. *In vitro* hemolytic and cytotoxic effects of *Tc*MLE. (**A**) Percentage viability of human O^{+ve} RBCs treated with *Tc*MLE. (**B**) Percentage viability of HeLa cells and HEK 293 T treated with *Tc*MLE. The data (mean ± SD) represent three independent experiments.

Table 3
Percentage parasitemia and mean survival of *Pb*-infected mice administered orally with *Tc*DLP and *Tc*MLE in Rane's test. The data are expressed as mean ± SD that represent 6 mice for each treatment from two independent experiments. NA-not applicable [***$P < 0.001$, n.s - not significant, log-rank (Mantel-Cox) test].

Groups	Doses (mg/kg/day)	% Parasitemia				% Suppression w.r.t. Vehicle control (Day 7)	Mean survival day
		Day 4	Day 5	Day 6	Day 7		
Vehicle (Control)	NA	1.96 ± 0.74	6.96 ± 1.16	15.03 ± 1.3	22.48 ± 0.57	NA	11.83 ± 2.11
*Tc*DLP	400	1.20 ± 0.51	5.99 ± 0.85	8.51 ± 1.24	15.05 ± 0.46	32.98 ± 2.93	14.17 ± 2.1^{ns}
	800	1.08 ± 0.46	3.15 ± 0.93	7.34 ± 1.1	12.15 ± 0.82	45.97 ± 3.34	19.17 ± 2.2 ***
	1200	1.04 ± 0.51	3.13 ± 0.72	6.97 ± 0.79	10.16 ± 0.90	54.76 ± 4.25	22.33 ± 1.1***
*Tc*MLE	400	1.1 ± 0.41	4.08 ± 1.05	8.23 ± 1.11	13.61 ± 0.52	39.39 ± 3.29	13.67 ± 1.49 ^{ns}
	800	1.55 ± 0.82	4.62 ± 1.03	6.64 ± 1.13	10.98 ± 0.47	51.13 ± 2.34	20.33 ± 2.36***
	1200	1.49 ± 0.66	3.56 ± 0.93	5.63 ± 1.39	6.58 ± 0.85	70.74 ± 3.53	25.17 ± 1.57***
CQ	25	0.94 ± 0.36	1.47 ± 0.62	0	0	100	NA

with plant metabolites like terpenoids, cedrelone, limonoids, polyynes etc with diverse pharmacological properties.[24] In particular, limonoids (triterpenoids) are abundantly present in Meliaceae plants including *T. ciliata*. The bioactive limonoids isolated from the extracts of bark, stem leaves and twigs of *T. ciliata* are shown to have anti-inflammatory activity by inhibiting the NLRP3 (nucleotide-binding domain (NOD)-like receptor protein 3) inflammasome required for the production of pro-inflammatory cytokines like IL-1β and IL-18.[41–43] Several limonoids isolated from the crude bark and seed extract of *Khaya grandifoliola* of Meliaceae family have shown potent antiplasmodial activity with IC$_{50}$ values between 1.25 and 9.63 μg/ml.[11] It is worth mentioning that the traditional antimalarial drug like chloroquine also possesses anti-inflammatory property. Further, phytochemicals isolated from plants represent a structurally diverse group that includes terpenoids, steroids, quinolines and its derivatives etc. with promising antimalarial activity.[44] Hemisynthetic alkaloids affect the vital processes like DNA replication and protein translation by targeting various proteins at different stages of *Plasmodium* life cycle thereby, affecting proliferation and transmission.[45] Bergenin, a naturally occurring polyphenol derivative obtained from plant shows potent antimalarial activity by inhibiting the heme polymerization pathway of malaria parasite that leads to reduction in hemozoin formation which is a key regulator of disease pathogenesis.[46] Pentacyclic terpenoids like boswellic acids show potent anti-inflammatory activity by targeting the NF-κB transcription factor and show antimalarial activity by inhibiting the heme detoxification pathway. These in turn lead to increase in the reactive oxygen species (ROS) and nitric oxide (NO) causing parasite death.[47,48] Fatty acids and its derivatives like hexadecanoic acid from the plant extracts also show potent antimalarial activity by inhibiting the type II fatty acid biosynthesis pathway of malarial parasite.[49] Terpenes cause growth arrest by

inhibition of isoprenoid biosynthetic pathway in malaria parasite affecting the tRNA function and post-translational modification.[50] Our results suggested that *Tc*MLE had various bioactive metabolites such as alkaloids, flavonoids, glycosides, saponin, phenols, terpenoids, tannins, steroids and coumarins. GC-MS analysis of *T. ciliata* methanolic leaf extract indicated the presence of fatty acid derivatives such as Hexadecanoic acid, methyl ester, 9-Octadecenamide and Methyl 8,11,14-heptadecatrienoate, and monocyclic terpenoid such as photocitral A that are known to have antimalarial effects.[51] As *Tc*MLE is enriched with terpenoids and fatty acid derivatives, the inhibition of heme detoxification and fatty acid biosynthetic pathways could be its plausible mode of action, although further detailed studies are necessary to confirm this. *Tc*MLE showed significant antioxidant activity and reducing power comparable to that of α-tocopherol. These findings were in agreement with the earlier reports on *T. ciliata* leaf and fruit extracts prepared using various solvents.[52] It is known that saponin present in plant extracts can cause hemolysis.[53] Although we could detect saponin in our extract preparations, there was no significant hemolysis even at concentrations higher than the IC$_{50}$ values exhibiting *in vitro* antiplasmodial activity. This could be because of the presence of limited quantities of saponin in *Tc*MLE. Similarly, *Tc*MLE showed less *in vitro* cytotoxicity. This was further verified by performing acute toxicity studies wherein, no sign of mortality and physical or behavioral changes were observed in mice treated with 5000 mg/kg of *Tc*DLP and *Tc*MLE. Further, previous reports on the ethanolic extract of *T. ciliata* did not show any toxicity.[27] All these results highlighted the suitability of *Tc*DLP and *Tc*MLE for therapeutic purposes.

A detailed analysis using *in vitro* cultures of *Pf*3D7 and ART-resistant *Pf*Cam3.I^{R539T} strains suggested the *in vitro* antiplasmodial activity of *Tc*MLE. The antiplasmodial activity could be
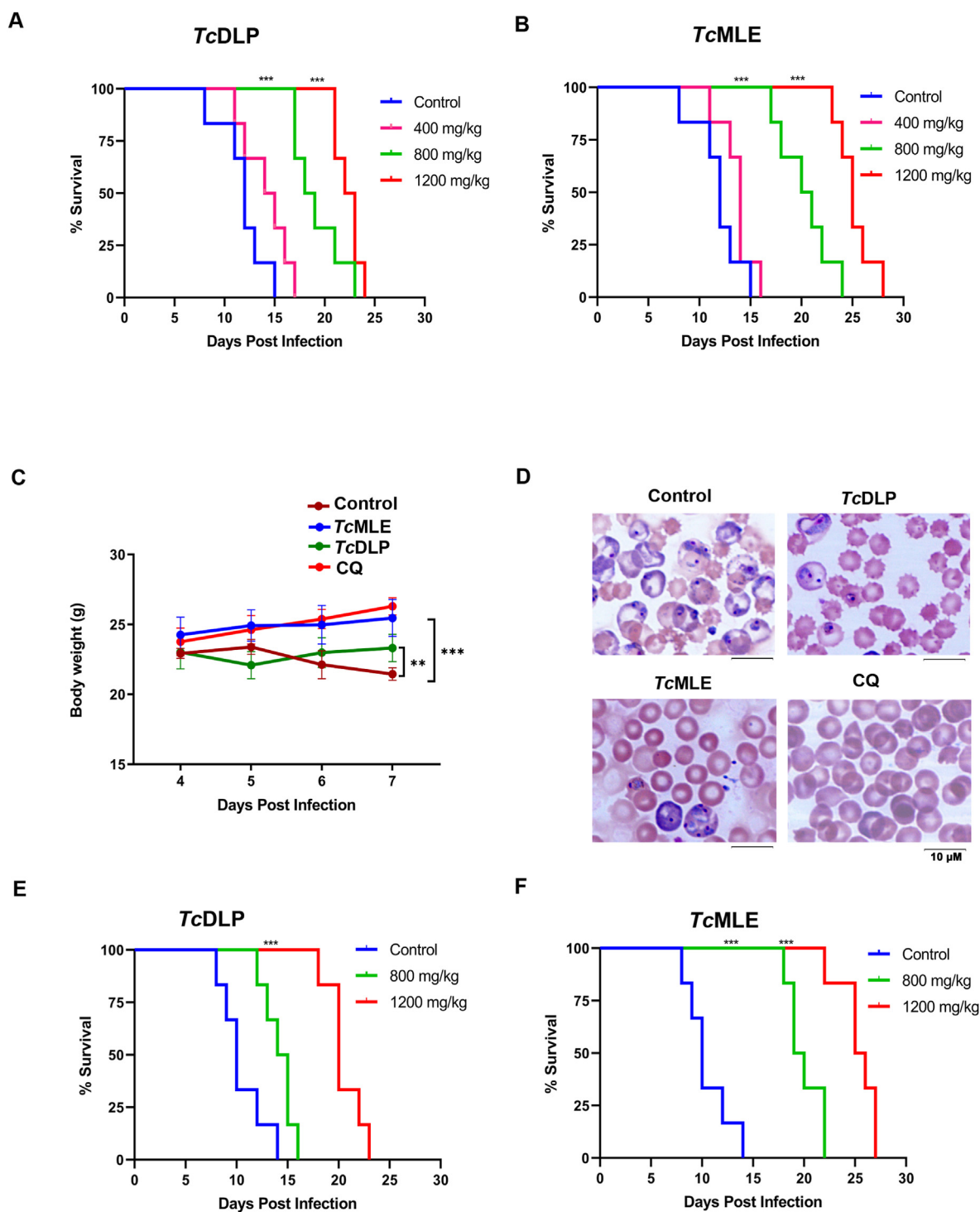
Fig. 4. ***In vivo* antimalarial activity of *Tc*DLP and *Tc*MLE in Rane's test and Peters' 4-day test.** Survival curves of *Pb*-infected mice administered with **(A)** *Tc*DLP and **(B)** *Tc*MLE in Rane's test. **(C)** Body weight analyses of *Pb*-infected mice administered with 1200 mg/kg doses of *Tc*DLP and *Tc*MLE in Rane's test. The data represent six mice for each treatment from two independent experiments (**P < 0.01, ***P < 0.001, unpaired *t*-test; two-sided). **(D)** Giemsa-stained images of the blood smear prepared from *Pb*-infected mice on day 7 in Rane's test. All images were captured with an inverted Olympus IX83 microscope using 60x objective. Scale bar = 10 μM. Survival curves of *Pb*-infected mice administered with **(E)** *Tc*DLP and **(F)** *Tc*MLE in Peters' test. All Survival curve data represent six mice for each treatment from two independent experiments. [***P < 0.001, log-rank (Mantel-Cox) test].

readily detected by prominent growth arrest in the treated asexual stage parasites as early as 24 h of treatment. There was a clear dose-dependent inhibition with IC_{50} values in the range of ~20–40 μg/ml. Importantly, *Tc*MLE was effective against ART-resistant *Pf*Ca-m3.I^{R539T} strain. It is worthwhile to mention that the emergence of ART resistance leading to delayed parasite clearance is a major

setback in malaria control, treatment, and elimination. Further, our results suggested the high phenolic and flavonoid contents of *Tc*MLE and its antioxidant activity. Several reports have indicated that the extracts from medicinal plants rich in bioflavonoids and antioxidants could elicit potential antiplasmodial activity although the molecular mechanisms are yet to be illustrated.[54–56] GC-MS

Table 4
Percentage parasitemia and mean survival of *Pb*-infected mice administered orally with *Tc*DLP and *Tc*MLE in Peters' 4-day suppressive test. The data are expressed as mean ± SD that represent 6 mice for each treatment from two independent experiments. NA-not applicable [*P < 0.05, ***P < 0.001, log-rank (Mantel-Cox) test].

Groups	Doses (mg/kg/day)	%Parasitemia (Day 4)	% Suppression w.r.t. Vehicle control (Day 4)	Mean survival day
Vehicle (Control)	NA	14.76 ± 0.58	NA	10.5 ± 1.98
*Tc*DLP	800	8.3 ± 0.54	43.56 ± 2.48	14.17 ± 1.34*
	1200	6.2 ± 0.81	57.94 ± 4.25	20.5 ± 1.61 ***
*Tc*MLE	800	6.29 ± 0.76	56.71 ± 3.16	20 ± 1.53***
	1200	3.62 ± 0.74	75.33 ± 5.77	25.33 ± 1.70***
CQ	25	0	100	NA

analysis of *Tc*MLE indicated the presence of fatty acid derivatives such as Hexadecanoic acid, methyl ester, 9-Octadecenamide, and Methyl 8,11,14-heptadecatrienoate, and monocyclic terpenoid such as photocitral A. There are also reports on the antimalarial effects of fatty acids and terpenoids.[57] All these findings together with the safety data obtained from the toxicity studies prompted us to evaluate the *in vivo* antimalarial activity of *Tc*MLE and *Tc*DLP. Our findings suggested that oral administration of *Tc*DLP and *Tc*MLE could effectively lead to 50–75% growth inhibition at a dose range of 800–1200 mg/kg. In particular, the treated *Pb*-infected mice showed a prolonged survival by ~10–14 days. For the respective doses that were tested, *in vivo* antimalarial activity of *Tc*MLE was higher than *Tc*DLP. This in turn suggested that the methanolic extraction of *Tc*DLP could lead to the enrichment of active phytochemicals in *Tc*MLE. Similar enrichment procedures would also be of help to enhance the potency of traditional preparations. A 1200 mg/kg dose of *Tc*MLE in mouse translates to a human equivalent dose of 97.2 mg/kg.[58,59] Many studies have suggested that active components isolated from the crude fractions of different plant extracts display enhanced *in vitro* antiplasmodial and *in vivo* antimalarial activity.[60,61] Taken into consideration of the crude methanolic leaf extract used in this study, the isolation of active components will significantly reduce the effective dosage and increase the antiplasmodial efficiency. Altogether, our results emphasized the antimalarial potential of *Tc*MLE.

5. Conclusion

*Tc*MLE showed a prominent *in vitro* antiplasmodial activity against *Pf*3D7 and ART-resistant *Pf*Cam3.I^{R539T}strains and a promising *in vivo* antimalarial activity against *Pb*. This is the first report on the antimalarial activity of *T. ciliata* leaf extract to the best of our knowledge. Our findings justify the ethnopharmacological potential of *T. ciliata* and deliver a platform for further fractionation studies to identify the active components of *Tc*MLE and explore their potential as new antimalarials.

Ethics approval statement

The mice were inbred in the animal house facility at the Institute of Life Science, Bhubaneshwar, as suggested by CPCSEA (Registration No. 76/GO/ReRcBi/S/99/CPCSEA) and the study protocol was approved by Institutional Animal Ethics Committee (ILS/IAEC-239-AH/21).

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2023.05.004.

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