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ORIGINAL ARTICLE

In vivo Antimalarial Potential of *Tinospora crispa* Miers in Mice and Identification of the Bioactive Compound

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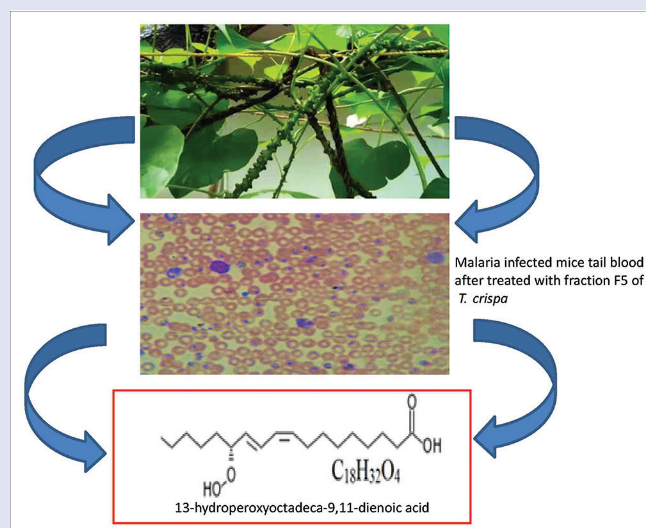
ABSTRACT

Background: Malaria is a serious disease that is causing huge toll on human health. The parasites causing this disease have developed resistance toward the current mainstream drugs. *Tinospora crispa* Miers is a well-known ethnopharmacological plant of Malaysia and has been traditionally used to treat malaria, fever, and other parasite-related illnesses. **Objective:** The present study investigated the potential of the crude methanol extract and antiparasitic fractions of *T. crispa* stem to exert antimalarial activity against *Plasmodium berghei*. **Materials and Methods:** The potent antiparasitic fractions of *T. crispa*, F4, and F5 were isolated in the previous study against *Toxoplasma gondii*. The same antiparasitic fractions along with crude methanol extract of different doses (10, 50, 100 mg/kg b. w.) were employed in the present study to determine the antimalarial activity against *P. berghei* ANKA strain. The survival curves of the treated mice were plotted by employing the log-rank (Mantel–Cox) test. The chemical composition of the most potent fraction F5 was determined spectrometrically using electrospray ionization-mass spectrometry (MS). **Results:** In a murine *P. berghei* model, fraction F5 displayed the highest parasitemia suppression effects compared to the crude methanol extract and other fraction. Subsequent chemical analysis by MS on fraction F5 has led to the tentative identification of 13-hydroperoxyoctadeca-9, 11-dienoic acid (13[*S*]-HPODE) compound. **Conclusion:** The crude methanol extract of *T. crispa* and its fraction F5 possess potent antimalarial activities, and the tentative discovery of the 13(*S*)-HPODE bioactive compound may serve as a precursor for developing a semisynthetic antiparasitic drug with enhanced efficacy and low toxicity.

Key words: 13-hydroperoxyoctadeca-9, 11-dienoic acid, antimalarial, parasitemia, *Plasmodium berghei*, *Tinospora crispa* Miers

SUMMARY

- Antiparasitic fraction F5 of *Tinospora crispa* Miers possess potent antimalarial activity, and the subsequent chemical analysis by mass spectrometry leads to the tentative discovery of 13-hydroperoxyoctadeca-9,11-dienoic acid compound from fraction F5.



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INTRODUCTION

Parasites cause fatal health complications in humans. To date, the most common parasite that trigger significant health impediment in human is *Plasmodium* species. Malaria is the most threatening parasitic disease in humans caused by *Plasmodium* parasites. According to World Malaria Report 2018 by the World Health Organization (WHO), an estimated 219 million cases of malaria occurred worldwide in 2017 and caused about 435,000 death cases. A total of about 92% of cases and 93% of deaths occurred in WHO African Region.[1] In 2017, children aged under 5 years had been largely affected by malaria and accounted for 61% (266,000) of all malaria deaths worldwide.[1] *Plasmodium* parasites are transmitted by *Anopheles* mosquitoes into a human host during a blood meal.[2] Severe malaria causes vital organ dysfunction and can be fatal without treatment. However, the increasing prevalence of *Plasmodium* parasites resistance to most of the available and affordable antimalarial drugs is a major concern in the treatment and control of malaria.[1] At present, to combat malarial infections, 1 or more classes of drugs are often given simultaneously. This is because no single drug has been discovered or manufactured to eradicate all forms of the parasite's life cycle. There are 4 major drug classes exploited to treat malaria synergistically, which include quinoline-related compounds, antifolates, artemisinin derivatives, and antimicrobials.[3] However, the enduring

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effectiveness of these regimens are often threatened by declining efficacy of artemisinin in southeast Asia.[4] As the prevention against malaria in regions where it is endemic, the regularly used antifolates have developed drug resistance, optimal prescription are imprecise, exorbitant especially for the nonimmune travelers and reduced tolerability in children.[5]

Medicinal plants are capable treatments of malaria. Quinine and artemisinin are the two examples of antimalarial drugs isolated from medicinal plants. However, over the years, the nonconformance in the use of antimalarial drugs has led to resistance. Therefore, the need for new antimalarial drugs from herbal medicines or botanical sources is indispensable. *Tinospora crispa* Miers, a member of Menispermaceae family, is a climbing plant widely distributed in tropical and subtropical parts of Asia such as Indonesia, Malaysia, Thailand, India, China, and Vietnam.[6] It is alternatively known by its Malay name as "patawali." The stem decoction of the plant is claimed to possess antimalarial and antihelmintic properties while decoction of the whole plant is used as an antidiabetic remedy among the Malay and Indonesian communities.[7-11] Stem extract of *T. crispa* is proven for well accepted pharmacological activities such as antipyretic, antidiabetic, anti-inflammatory, and antimalarial properties in Thai traditional medicine.[12] In traditional Chinese medicine, stem extract of *T. crispa* is commonly used to treat septicemia, fever, scabies, and other tropical ulcer-related disorders.[13]

Previous studies had reported that *T. crispa* possesses potential cytotoxicity, antioxidant, and antiparasitic activities. Rahman discovered the methanol extract of *T. crispa* injected intraperitoneally at a dose of 5 mg/kg in mice infected with *Plasmodium berghei* ANKA strain and had extended the life of the mice for 1 day compared to control mice.[14] The *in vitro* study of methanol extract of *T. crispa* was shown to have more than 90% of parasitemia inhibition at the concentration of 0.03 μg/mL using FCR-3 strain (chloroquine resistant) of *Plasmodium falciparum*.[15] Researchers have also proved that the crude ethanol extract of *T. crispa* stem has *in vivo* antimalarial effect in a dose-dependent manner against *Plasmodium yoelii*.[16]

Even though *T. crispa* crude extracts have been researched earlier for their antimalarial activities, there have been no reports on the fractionation of the crude plant extract and identification of bioactive chemical compounds against the malaria parasite, *P. berghei* using animal model. Earlier, we have reported the bioactivity-guided fractionation of *T. crispa* against *Toxoplasma gondii*, where fractions F4 and F5 had showed the most prominent *in vitro* antiparasitic activities. Knowing that *T. gondii*, *Plasmodium* sp., and many of their apicomplexan families undergo similar developmental process in the cells of their hosts, which includes genome replication, cell division, and the assembly of new invasive stages, the selected fractions of F4 and F5 from *in vitro* model were employed in the present study to investigate the antimalarial potentials against *Plasmodium berghei* in animal model. The present study is also assumed the first to report the tentative identification of chemical compound accountable for antimalarial activity from this ethnomedicinal plant.

MATERIALS AND METHODS

Chemicals and solvents

Analytical grade solvents, high-performance liquid chromatography (HPLC) grade solvents, acetic acid (98%), and silica gel 60 (0.063–0.200 mm) were from Merck (Darmstadt, Germany). DMSO, Tween 20, phosphate-buffered saline (PBS), and Leishman stain were obtained from Sigma-Aldrich (Germany). Reference drug chloroquine was from Sigma-Aldrich (Germany).

Plant collection and authentication

The fresh stems of *T. crispa* Miers were obtained from Kepala Batas, Pulau Pinang, Malaysia. The stems of *T. crispa* Miers was authenticated by Mr. Shunmugam, the botanist of the School of Biological Sciences, Universiti Sains Malaysia, where voucher specimen (No. 11262) was deposited in the Herbarium Unit of the school.

Extraction of plant material

The stems of *T. crispa* Miers were cleaned and air-dried under sun shade for 6 days. The dried stem parts were cut and ground to mesh size No.40. Powdered stems (300 g) were macerated exhaustively with methanol by ratio of 10 g of ground plant material in 100 ml of methanol at room temperature for 2 days. Fresh solvent was used for subsequent maceration after each extraction, and the process was repeated three times. The extracts were filtered, pooled, and concentrated under reduced pressure at 40°C in a rotary evaporator (Rotavapor® R-200, Buchi, Switzerland) until dryness to yield a sticky dark brown crude residue which was then freeze-dried (FreeZone®, MO) to afford 24.36 g of dried extract. The crude extract was stored at 4°C until use.

Fractionation of the crude methanol extract of *Tinospora crispa* Miers by column chromatography

An amount of 600 g of silica gel 60 (0.063–0.200 mm, Merck) was premixed with hexane and then packed into a sintered glass column measuring 60 cm long and 6.5 cm in diameter. An amount of 50 g of silica gel (70-230 mesh) preabsorbed with 10 g of *T. crispa* Miers crude extract was loaded into the column. The column was then equilibrated and fractionated with hexane and eluted subsequently with various gradient mixtures (2 L each) of hexane and acetone (95:5, 90:10, 85:15; 80:20, 75:25, 70:30, 65:35, 60:40, 50:50, 40:60, 30:70, 20:80, and 10:90 v/v) and pure acetone, respectively. A total of 1530 fractions (20 mL each) were collected. The eluted fractions were analyzed by thin-layer chromatography, and identical fractions with similar retention factor (R_f) values were pooled into eight fractions denoted as F1-F8. The R_f value is defined as the ratio between the migration distance of a solute and the migration distance of the solvent front. The pooled fractions (F1-F8) were freeze dried and stored at 4°C before further analysis. In the previous study, fractions F4 and F5 of *T. crispa* showed the most prominent antiparasitic activities against *T. gondii*, where their inhibitory activities were comparable to positive control clindamycin.[17] Therefore, in the subsequent *in vivo* antimalarial study, we had only employed the well-proved antiparasitic fractions, F4 and F5 of *T. crispa*.

In vivo antimalarial activity

Experimental animal

Healthy male ICR mice initially weighing between 17 and 20 g were obtained from Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. They were housed at 28–30°C and fed on standard chow diet and tap water *ad libitum* during experiment. The animals were kept in cages covered with mosquito net to avoid any malaria infection transmission during the experiment. All the animals' procedures conducted have been critically reviewed and approved by the Animal Care and Use Committee (ACUC) of Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (ACUC approval number: UPM/FPSK/PADS/BR-UUH/00399). At the end of experiments, mice were euthanized by CO_2 exposure. All sections of this report adhere to the ARRIVE Guidelines for reporting animal research.[18]

Parasite and infection procedures

P. berghei ANKA strain (chloroquine sensitive) was used to initiate malaria infection in ICR (Institute of Cancer Research) mice. The infection procedures were based on the method described by Basir.[19]

Parasitemia measurement

Parasitemia was measured using method provided by Basir with slight modification.[19] Parasitemia was monitored daily with a digital microscope (Nikon Eclipse 80i, US) at 1000 times magnification using standard immersion oil. The Leishman-positive cells in five different fields with an estimation of 200 erythrocytes per field were counted manually using manual tagging option from Image Pro Plus (version 5.1) software. The calculated positive Leishman cells observed from the blood smear represented the parasitemia level in the animals. Parasite counts were expressed as percentage of red blood cells and were calculated as below:

$$\text{Percentage parasitaemia}\ (\%) = \frac{\text{Number of Leishman positive bodies}}{\text{Total number of red blood cells}} \times 100\%$$

In vivo antimalarial assessment

A total of 60 male ICR mice were employed in this study. Animals were randomized into six groups of ten individuals. Animals were randomized into treatment groups by picking numbers out of a hat. A timeline of the study is shown in Figure 1. The mice were inoculated intraperitoneally with 0.2 mL of 2×10^7 PRBCs while the control group received uninfected RBCs in an equivalent volume and dilution. The mice were inoculated intraperitoneally using sterile 1 mL syringe with 25-gauge needle. The day of inoculation was regarded as day 0. Twenty-four hours after the parasite inoculation, each group of mice was orally fed with test extract for ten consecutive days beginning at day 1 to day 10 postinoculation. The groups and treatment received were summarized as follows:

- Group 1: Control mice receiving vehicle, 10% Tween 20 in PBS (0.2 mL/animal, oral feed)
- Group 2: Malaria-infected mice receiving vehicle, 10% Tween 20 in PBS (0.2 mL/animal oral feed)
- Group 3: Malaria-infected mice receiving chloroquine (20 mg/kg animal weight, 0.2 mL/animal, oral feed)
- Group 4: Malaria-infected mice receiving low-dose extract (10 mg/kg animal weight, 0.2 mL/animal, oral feed)
- Group 5: Malaria-infected mice receiving medium-dose extract (50 mg/kg animal weight, 0.2 mL/animal, oral feed)

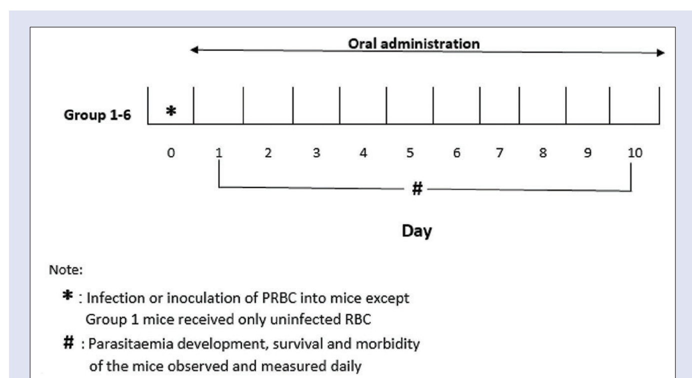

Figure 1: Experimental design and timeline of the study

- Group 6: Malaria-infected mice receiving high-dose extract (100 mg/kg body weight, 0.2 mL/animal, oral feed).

The entire *in vivo* antimalarial assessment was repeated with the two most potent fractions (F4 and F5) determined from the *in vitro* antitoxoplasmosis assay from previous study.[17] Parasitemia development in these mice was also observed and measured daily. Every parasitemia counts within a single experiment were executed by the same investigator. The percentage parasitemia suppression for each of the treatment was calculated according to the formula below:

$$\text{Percentage suppression}\ (\%) = \frac{\text{Parasitaemia of negative control mice} - \text{parasitaemia of treated malaria mice}}{\text{Parasitaemia of negative control mice}} \times 100\%$$

The daily survival and morbidity of the mice in each group of treatment were monitored for 10-day postinfection. The survival data recorded the number of mice that endured throughout the treatment period. Mice found moribund during the study were sacrificed (euthanized by CO_2 exposure) and recorded as dead. The percentage survival was calculated as below:

$$\text{Percentage survival}\ (\%) = \frac{\text{Number of survived mice}}{\text{Total experimental mice}} \times 100\%$$

Preparative liquid chromatography analysis

The most potent fraction F5 which has demonstrated better survival rate as determined from the *in vivo* antimalarial study, was chosen for LC analysis. The preparative LC analysis was carried out at room temperature using Shimadzu preparative LC LC-20AP equipped with a communication bus module CBM-20A, an auto sampler SIL-10AP, a photodiode array detector (DAD), and a fraction collector FRC-10A. Separation was carried out on a reversed phase column (Thermo Scientific Hypersil Gold prep C 18, 250 mm × 21.2 mm, 5 µm). The mobile phase system was composed of 40% (v/v) acetonitrile (ACN) in water (solvent A) and 80% (v/v) of ACN in water (solvent B). Both solvent A and B were attuned to pH 3 with acetic acid. The solvent gradient elution was programmed as follows with total analysis time of 40 min: 0–30 min, linear gradient 10%–60% B, 30–35 min, isocratic 100% B; and 35–40 min, isocratic 10% B. The flow rate was at 21.0 mL/min. The sample injection volume and column temperature were set at 500 µL (10 mg/mL) and 30°C, respectively. The DAD acquisition was implemented at 260 nm. The purity of the peak collected from fraction F5 with retention time of 12.128 min was more than 99%, as verified by HPLC-photodiode array.

Electrospray ionization-mass spectrometry analysis

Chemical characterization of the peak (retention time = 12.128 min) from fraction F5 was carried out by direct inlet flow using quadrupole ion trap mass spectrometer equipped with an Electrospray Ionization (ESI) ion source, in the negative ion mode. Direct infusion was performed using a syringe pump of 500 µL volume at a flow rate of 10 µL/min. The following conditions were used: spray voltage 4.70 kV; capillary temperature 275°C; sheath gas flow 15 arbitrary units (AU); auxiliary gas flow 21 AU; and sweep gas flow 0 AU. For mass spectrometry (MS) analysis, mass spectrometer was scanned from 10 to 1000 m/z.

Statistical analysis

Statistical analysis was carried out using the GraphPad Prism version 5.0 software. All experimental values shown are of mean ± standard deviation. One-way analysis of variance was performed to analyze the difference among at least three independent groups. The level of significant difference was set at $P < 0.05$ or $P < 0.001$. Dunnett's test was employed to compare the effect of crude extract or fractions treatment to the positive-control chloroquine. Besides, the survival curves of the extract or fractions treated mice were plotted and compared to the positive control chloroquine by employing the log-rank (Mantel–Cox) test.

RESULTS

Suppression levels and survival of the malarial mice

The parasitemia level in the malarial mice was represented by the percentage of Leishman positive cells observed on microscopic examination. Figure 2a-e revealed thin films of blood obtained from tail blood of malarial-infected mice under various treatments. Parasitized erythrocytes were shown stained in blue and were utilized to determine the percentage parasitemia level on day 6 postinoculation. Table 1 illustrated the parasitemia levels of distinct groups of malaria-infected mice under different treatment conditions from day 1 until day 10 postinoculation. Parasitemia suppression exhibited by different treatments that were given to the mice from day 2 until day 6 postinoculation was tabulated in Table 2. Control mice group showed Leishman-positive bodies level of 1.70% on all days and served as the baseline indicator for the parasites clearance level. The groups of mice that received 100 mg/kg treatments of crude *T. crispa*, fraction F4, and fraction F5 (50 and 100 mg/kg), displayed lower parasitemia level on day 6 postinoculation. The parasitemia level for crude *T. crispa* (100 mg/kg) was 41.05% ± 0.41%, meanwhile the parasitemia level of fraction F4 (100 mg/kg) was recorded at 42.38% ± 0.33% on the day 6. The same treatments exhibited parasite inhibitory effects of 42.85% ± 0.58% (crude extract) and 41.01% ± 0.46% (fraction F4) on the day 6. Interestingly, fraction F5 (100 mg/kg) had achieved the lowest parasitemia level (32.82% ± 0.17%) with suppression level of 54.32% ± 0.24% on day 6. The highest dosage treatment of fraction F4 and fraction F5 exerted maximum suppressive activity of 61.29% ± 0.28% on day 4 and 58.73% ± 0.36% on day 5, respectively. However, the suppression effect of those two treatments declined afterward and the parasitemia level increased until all the mice were dead on day 10 postinoculation [Table 1]. The survival percentage of mice in the crude extract treatment groups (10, 50, 100 mg/kg) dropped to 40%, 60%, and 60%, respectively, on day 6 [Figure 3a]. For the fraction F4 treatment group, all the mice from the three different dosage treatment groups survived from day 1 until day 6 postinoculation. As for the fraction F5, all the ten mice in the 50 mg/kg treatment group were viable from day 1 until day 8 postinoculation. None of the mice in all the three groups survived until day 10 postinoculation. The survival curve [Figure 3] of mice treated with 3 different doses of crude *T. crispa* extract, fraction

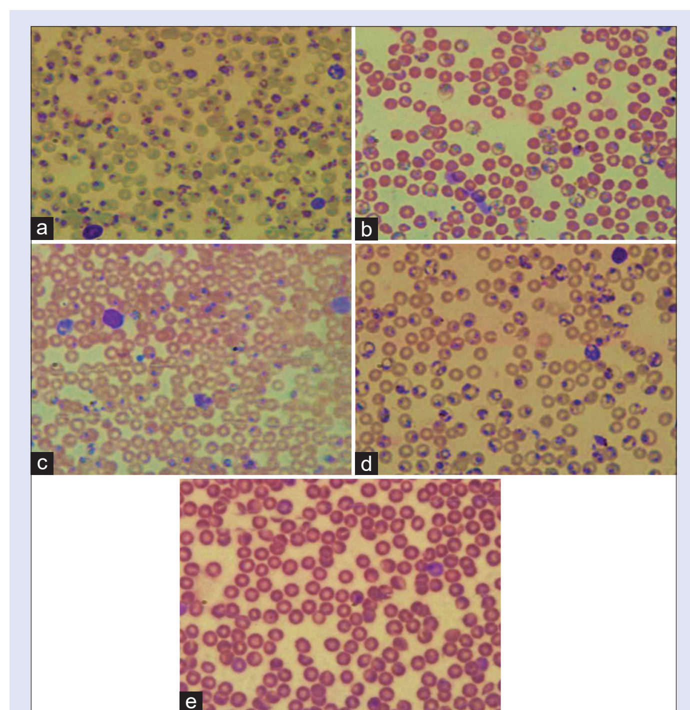

Figure 2: Leishman's stained films of malaria-infected mice tail blood on day 6 postinoculation observed under ×1000 with oil immersion field. (a) Treatment with crude *Tinospora crispa* Miers, 100 mg/kg animal weight; (b) treatment with F4, 100 mg/kg animal weight; (c) treatment with F5, 100 mg/kg animal weight; (d) untreated control; (e) treatment with chloroquine. The blue stains inside the red blood cell represented parasite infected-red blood cell to determine the percentage parasitaemia level

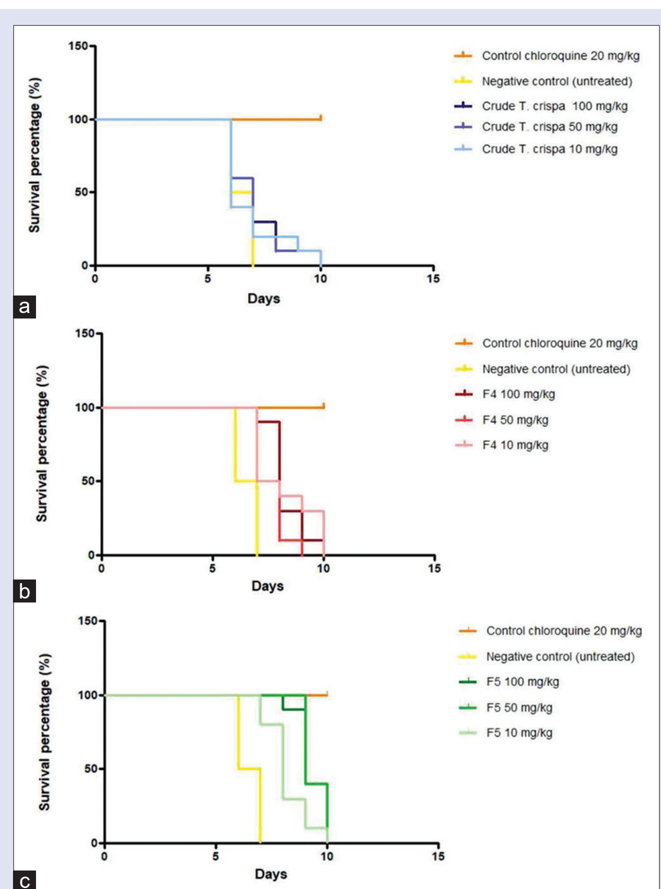

Figure 3: Survival curves of *Plasmodium berghei*-infected mice treated with different doses of plant extract and fractions including untreated and chloroquine-treated malarial-infected mice. (a) Treatments of crude methanol stem extract of *Tinospora crispa* Miers; (b) treatments of fraction F4; (c) treatments of fraction F5. The survival curves were significantly different at $P < 0.001$ compared to chloroquine-treated group

Table 1: Daily mean percentage of parasitemia level of distinct groups of *Plasmodium berghei*-infected mice

Treatment groups	Daily mean percentage of parasitemia level									
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
Crude *T. crispa* low dose	5.73±0.09	11.01±0.14[a,b]	22.68±0.23[a,b]	38.32±0.44[a,b]	48.88±0.67[a,b]	52.96±1.19[a,b]	56.58±0.95[a,b]	66.39±0.70[a,b]	74.83±0.00[a,b]	-
Crude *T. crispa* medium dose	5.57±0.12	10.53±0.14[a,b]	18.37±0.18[a,b]	27.38±0.35[a,b]	39.92±0.30[a,b]	47.22±0.42[a,b]	51.99±0.66[a,b]	59.62±0.00[a,b]	68.57±0.00[a,b]	-
Crude *T. crispa* high dose	5.46±0.09	10.43±0.08[a,b]	17.19±0.13[a,b]	23.89±0.20[a,b]	34.06±0.46[a,b]	41.05±0.41[a,b]	51.05±0.44[a,b]	59.14±0.00[a,b]	69.24±0.00[a,b]	-
F4 low dose	4.21±0.07	8.94±0.09[a,b]	18.91±0.16[a,b]	24.24±0.39[a,b]	41.23±0.32[a,b]	56.66±0.28[a,b]	60.99±0.28[a,b]	66.11±0.61[a,b]	72.23±0.26[a,b]	-
F4 medium dose	4.11±0.11	8.96±0.12[a,b]	17.45±0.20[a,b]	20.02±0.16[a,b]	35.93±0.21[a,b]	54.06±0.49[a,b]	61.89±0.21[a,b]	70.31±0.00[a,b]	-	-
F4 high dose	4.23±0.11	9.13±0.09[a,b]	12.47±0.16[a,b]	16.89±0.12[a,b]	28.66±0.21[a,b]	42.38±0.33[a,b]	58.71±0.59[a,b]	62.97±0.71[a,b]	69.72±0.00[a,b]	-
F5 low dose	4.20±0.05	8.86±0.09[a,b]	18.57±0.16[a,b]	31.60±0.22[a,b]	36.39±0.35[a,b]	48.95±0.40[a,b]	58.02±0.30[a,b]	64.63±0.59[a,b]	71.15±0.00[a,b]	-
F5 medium dose	4.22±0.06	8.88±0.07[a,b]	17.87±0.14[a,b]	24.57±0.19[a,b]	31.84±0.19[a,b]	38.71±0.18[a,b]	49.55±0.25[a,b]	58.76±0.25[a,b]	65.44±0.43[a,b]	-
F5 high dose	4.24±0.09	8.98±0.11[a,b]	13.49±0.16[a,b]	19.79±0.24[a,b]	26.17±0.22[a,b]	32.82±0.17[a,b]	44.58±0.24[a,b]	57.19±0.62[a,b]	62.36±0.47[a,b]	-
Chloroquine	4.22±0.04	4.57±0.05	4.04±0.03	3.22±0.03	2.52±0.02	2.03±0.02	1.70±0.02	1.65±0.02	1.60±0.02	1.62±0.03
Control untreated	4.28±0.05	9.43±0.11	20.69±0.27	43.62±0.77	63.41±0.88	71.83±0.31	-	-	-	-
Control uninfected (baseline)	1.67±0.03	1.70±0.01	1.74±0.02	1.71±0.02	1.74±0.02	1.71±0.03	1.72±0.05	1.71±0.02	1.75±0.03	1.73±0.03

[a]*P*<0.05, [b]*P*<0.001. The values were presented as mean±SD (*n*=10). -: Means all the mice were dead; SD: Standard deviation; *T. crispa: Tinospora crispa*

Table 2: Daily mean percentage of parasitemia suppression of distinct groups of *Plasmodium berghei*-infected mice

Treatment groups	Daily mean percentage of parasitemia suppression level (%)				
	Day 2	Day 3	Day 4	Day 5	Day 6
Crude extract low dose	4.38±1.19[a,b]	−2.63±1.06*	12.17±1.01[a,b]	22.91±1.06[a,b]	26.28±1.66[a,b]
Crude extract medium dose	8.52±1.19[a,b]	16.85±0.83[a,b]	37.23±0.80[a,b]	37.04±0.48[a,b]	34.27±0.59[a,b]
Crude extract high dose	9.38±0.70[a,b]	22.21±0.59[a,b]	45.25±0.46[a,b]	46.28±0.72[a,b]	42.85±0.58[a,b]
F4 low dose	22.32±0.78[a,b]	14.40±0.71[a,b]	44.44±0.91[a,b]	34.98±0.51[a,b]	21.13±0.37[a,b]
F4 medium dose	22.15±1.06[a,b]	21.04±0.92[a,b]	54.11±0.37[a,b]	43.34±0.32[a,b]	24.75±0.69[a,b]
F4 high dose	20.71±0.77[a,b]	43.58±0.74[a,b]	61.29±0.28[a,b]	54.80±0.33[a,b]	41.01±0.46[a,b]
F5 low dose	23.06±0.75[a,b]	15.95±0.74[a,b]	27.56±0.50[a,b]	37.88±0.56[a,b]	31.86±0.55[a,b]
F5 medium dose	22.86±0.63[a,b]	19.13±0.65[a,b]	43.68±0.43[a,b]	49.78±0.31[a,b]	46.12±0.25[a,b]
F5 high dose	21.97±0.93[a,b]	38.93±0.74[a,b]	54.64±0.56[a,b]	58.73±0.36[a,b]	54.32±0.24[a,b]
Chloroquine	60.26±0.42	81.70±0.15	92.63±0.07	96.02±0.03	97.17±0.03

*The negative value of the mean was the results of some mice in the crude *T. crispa* low-dose treatment group developed parasitemia level higher compared to the negative control group. [a]*P*<0.05, [b]*P*<0.001 compared to chloroquine. The values were presented as mean±SD (*n*=10). SD: Standard deviation; *T. crispa*: *Tinospora crispa*

F4, and F5 were significantly different ($P < 0.001$) compared to the positive-control chloroquine-treated group.

Chemical compound identification

Figure 4 shows the HPLC chromatogram of fraction F5. The peak showed high purity (99%) and was subjected to MS analysis. In the ESI-MS-negative ion mode, this compound formed deprotonated molecule ([M-H]⁻) at m/z 311.1690 [Figure 5]. The fragmented ions occurred at m/z 293.1534 obtained following the loss of one water molecule [M-H_2O] from the parent ion. The mass spectrum of this compound correlates to 13-Hydroperoxyoctadeca-9,11-dienoic acid (13[*S*]-HPODE) molecule with molecular formula of $C_{18}H_{32}O_4$. The possible fragmentation pattern of the compound found in fraction F5 is given in Figure 6.

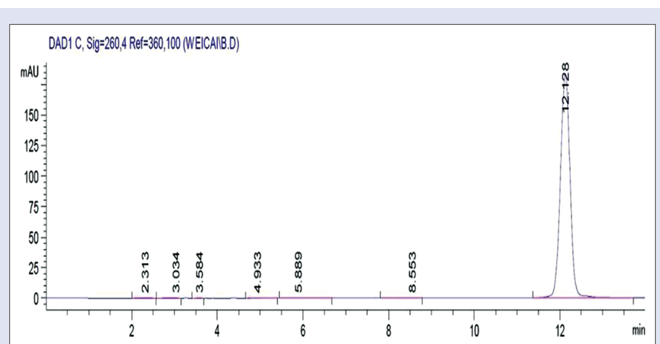

Figure 4: High-performance liquid chromatography chromatogram of compound in fraction F5

DISCUSSION

To date, malaria chemotherapy is the most general and widely used strategy in its control. However, the widespread development of resistance in malaria parasites against existing clinical antimalarial drugs such as chloroquine and even quinine has prompted a search for alternative treatments. The major ethnobotanical use of *T. crispa* has been in the treatments of malaria among Asians for decades. This had provoked an insightful study to investigate the *in vivo* antimalarial potential of crude methanol stem extract of *T. crispa* and its fractions against *P. berghei*. Earlier, the author and his coworkers had reported the cytotoxic activities of the crude methanol extract and fractions of *T. crispa* Miers.[17] The published data supported the finding of crude methanol extract of *T. crispa* and its fractions (F1-F8) being nontoxic to the mammalian cells. The selectivity index values reported were 19.13 (crude extract), 24.54 (fraction F4), and 28.37 (fraction F5), respectively.[17] Therefore, the plant extract as well as its fractions were established safe toward the experimental animal in the current study.

In this work, the antimalarial properties of the plant extract and fractions were determined using an *in vivo* mice model. Although the pharmacokinetics of drugs differ between humans and mice, Landau and Gautret have acknowledged that all mammalian *Plasmodium* species have comparable life cycles and are equally sensitive toward

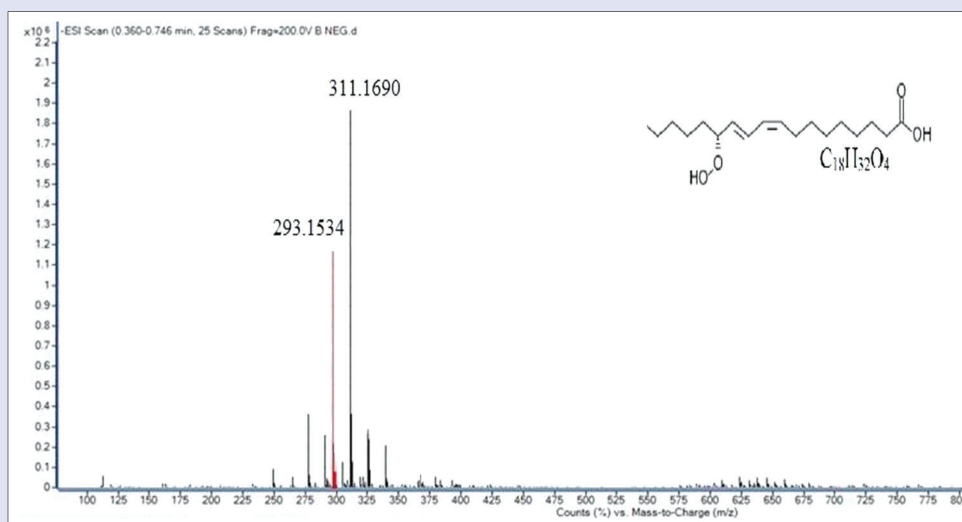

Figure 5: Mass spectrum and molecular structure of the compound in fraction F5

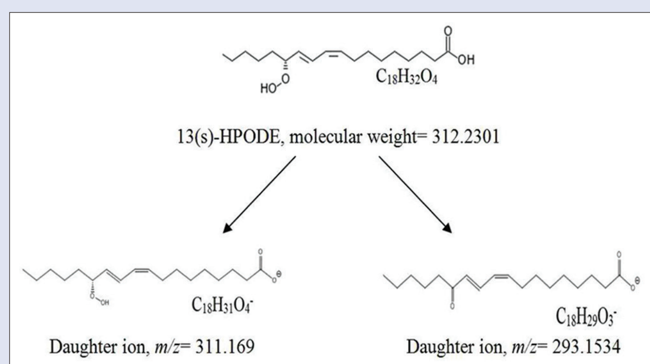

Figure 6: Fragmentation pattern of compound 13-hydroperoxyoctadeca-9, 11-dienoic acid as detected from mass spectrometry analysis

the antimalarial drugs used.[20] According to Stevenson, rodent plasmodia-species-infected-mouse strains have always been adopted as model in investigating possible new chemotherapeutic agents and their antimalarial related interventions.[21] Therefore, the rationale of using a rodent model to test for antimalarial drug efficacy is widely established and is considered substantially reliable, reproducible, and affordable. However, further study for confirmation using humanized SCID model (infected with *P. falciparum*) is indeed required.

It is very conceivable that the conventional microscopic examination of Leishman-stained thin blood smears is an established and accurate procedure for peripheral detection and identification of malarial parasites.[22] In the present work, the crude extract as well as the fractions (F4 and F5) had significantly decreased the parasitemia level in suppressive, prophylactic, and in a dose-dependent manner. These declines showed promising schizontocidal effect of *T. crispa* plant extract on the plasmodial parasite growth and maturation. Of note, the study revealed that at the end of day 6, all the mice in the untreated control group were dead. This finding was in accordance with the *in vivo* study by Zakaria where the investigation had observed maximum parasitemia percentage of *P. berghei* infection around day 6 due to the rapid propagation of the parasites in the mice blood circulation.[23] The untreated malarial-infected mice which were vehicle treated (10% Tween 20 in PBS, 0.2 mL) were observed to increase continuously. This showed that there was no inhibitory effect by the vehicle on the malaria parasites as all the mice were dead within seven days due to heavy parasites load. The parasitemia propagation level is likely influenced by several factors such as body immunity, interactions among the parasites, and available RBCs as the source for parasitic invasion.[24]

To further complement the results of parasitemia percentage, the parasitemia suppression percentage was determined. The suppression percentage disclosed the effectiveness of the positive control (chloroquine) or plant treatments (crude extract and fractions) in inhibiting the parasites' propagation. It is worth mentioning that the percentage of parasitemia suppression by various treatment groups was only assessed from day 2 until day 6 because all the mice on day 1 were never exposed to any treatment when the blood was withdrawn as well as the entire mice in the negative control group died at the end of day 6. Thus, the parasitemia suppression was unable to be ascertained from day 7 onward. As shown in Table 2, *P. berghei* ANKA strain used in this experiment was chloroquine-sensitive strain. Hence, the antimalarial drug displayed excellent parasites' suppression level from day 2 to day 6. This result contrasts with parasite inhibiting activity by the crude methanol *T. crispa* Miers extract, fraction F4, and fraction F5, respectively, which recorded lower parasitemia suppression percentages. It is interesting to note that Niljan *et al*. found that the crude methanol *T. crispa* stem extract confirmed percentage inhibition of 35% and 50% in the *P. berghei*-infected mice receiving treatment doses of 100 mg/kg and 200 mg/kg, respectively, on day 4 postinoculation.[25] In another study, 2.5 mg/mL of the methanol extract of *T. crispa* stem was able to completely inhibit *P. falciparum* parasites after 72 h incubation.[14] These findings attested *T. crispa* crude extract which contains potential phytocompounds that may act as potential antimalarial remedy.

From the survival curves obtained, the survival times of the mice receiving treatments of crude *T. crispa* extract, fraction F4, and F5 were significantly prolonged compared to the untreated malarial mice. In addition, it was evident that by increasing the treatment dosages of extract or fractions, it would lengthen the survival of the malarial mice. However, mice treated with 50 mg/kg and 100 mg/kg of fraction F5 showed better survival rates between day 7 and day 9 postinoculation compared to mice treated with crude methanol *T. crispa* extract and fraction F4 during the study. This finding suggested that the potential chemical constituent in fraction F5 may be beneficial in eradicating malaria, and therefore, fraction F5 was subjected to chemical identification study. The compound 13(S)-HPODE

was subsequently identified in fraction F5 using MS. Literature shows that 13(S)-HPODE is produced by the oxidation of linoleic acid by lipoxygenase-1 in many plants including soybean, flaxseed, apples, and tea leaves.[26,27] 13(*S*)-HPODE is a precursor for several important plant metabolites such as plant hormone jasmonic acid.[28] Jasmonic acid and its various metabolites are responsible in regulating plant responses to abiotic and biotic stresses, as well as plant growth and development.[29] In mammalian tissues, 13(S)-HPODE is generally reduced to 13(S)-HODE, a compound which exhibits many biological activities.[30] To the best of our knowledge, the present study would be the first to investigate the bioactive compound responsible for the antimalarial activity from *T. crispa* stem extract. The chemical compound 13(*S*)-HPODE has been isolated and identified tentatively for the first time from this plant which is responsible for antimalarial activity.

CONCLUSION

In conclusion, the findings from this study seemed to validate the traditional use of *T. crispa* Miers to treat malaria. Fraction F5 which had exerted parasite inhibitory effect on *P. berghei* was found to comprise 13(*S*)-HPODE chemical compound. Further, NMR studies, mechanism of action, and structural modification of bioactive compound 13(S)-HPODE may lead to the development of novel antimalarial therapeutic agent in the future. In addition, unidentified compounds from other fractions of *T. crispa* are also vital to be further investigated for their potential *in vitro* and *in vivo* antimalarial activities against *Plasmodium* sp.

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Conflicts of interest

There are no conflicts of interest.

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