Research Article

In Vivo Antimalarial Activity of *Trichosanthes cucumerina* Against *Plasmodium berghei* NK65 in Mice

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ABSTRACT

Undoubtedly, malaria is a vector-borne infectious disease that is increasingly being given attention by many researchers in their efforts to find the best drugs for its treatment. Four groups of mice (6-8 weeks old, 20-25 gram body weight (g bw)) were inoculated with *Plasmodium berghei* NK65 intraperitoneally (i.p.) at 1.0×10^6 infected red blood cells (RBC) before being orally treated for the prophylactic and curative treatment regime with 0.2 mL of 100 mg/kg bw freeze-dried *T. cucumerina* aqueous extract. Parasitemia levels and inhibition rates were microscopically measured using Giemsa stained blood smear method. *Trichosanthes cucumerina* possessed strong antimalarial activities against *P. berghei* NK65 infection in mice. A significant correlation was successfully recorded between the survival time of the seven-day prophylactic treatment group (P7) with its ability to inhibit parasite growth as compared to the curative treatment groups. However, these values are still incomparable to the control group treated with the commercial drugs primaquine and chloroquine. In addition, blood biochemical toxicity analysis of ALT, AST, ALP, and STP showed that acute and sub-acute toxicity treatments of *T. cucumerina* did not cause liver injury and were non-toxic to the animals. Thus, this study significantly proves ($p \leq 0.05$, $n=6$) that *T. cucumerina* has antiparasitic properties that can be manipulated as an alternative antimalarial drug.

Key words: *Trichosanthes cucumerina*, snake gourd, antimalarial, *P. berghei* NK65

INTRODUCTION

Malaria can be considered one of the neglected vector-borne tropical diseases that has so far claimed millions of lives around the world (Zin *et al*., 2017). According to the Annual Report released by the Vector Borne Disease Control Unit, Ministry of Health Malaysia (MOH) 2019, a total of 3,372 cases of malaria have been recorded in Malaysia in 2018. Until today, the conventional treatment of malaria still entirely relies on synthetic chemical drugs that probably allow the development of antimalarial drug resistance. Realizing this scenario, many species of herbs and natural products have been used by the world's population as alternative drugs for remedies to treat many infectious diseases including malaria (Tajuddeen & Van Heerden, 2019).

Nowadays, however, the world's population tends to turn away and try their remedies towards natural based-alternative products to combat malaria. *Trichosanthes cucumerina* or commonly known as snake gourd or 'petola ular' in Malay is one of the naturally planted crops that is mostly cooked as a dish and eaten with rice in a daily meal, especially in South and Southeast Asian regions (Nadeeshani *et al*. 2016; Seshadri *et al*., 2020). Several earlier documents reported that *T. cucumerina* has been widely used since the ancestral era as a treatment for helminth, fungal and bacterial infections (Devi, 2017; Sirikantaramas *et al*., 2020). According to Aravindakshan and Thangavel (2020), *T. cucumerina* is rich in therapeutically active chemical constituents including flavonoids, carotenoids, and phenolic

acids. Due to this fact, this plant could be used as hepatoprotective, antidiabetic (Sassi *et al*., 2018; Kavitha 2020), gastroprotective, anti-inflammatory (Aravindakshan & Thangavel, 2020), and antioxidant (Seshadri *et al*., 2020)

By far, there are no studies documented the antimalarial effects of *T. cucumerina*. Therefore, using ICR strain mice as an *in vivo* model, this study aimed to evaluate the antimalarial activity of *T. cucumerina* aqueous extract. The assessment of liver enzymes and total protein levels in blood from the experimental animals was also conducted to determine the toxicity effect of the extract in this study.

MATERIALS AND METHODS

Preparation of *T. cucumerina* extract

About 2.2 kg of *T. cucumerina* or snake gourd was bought from a wet market at Jalan Pasar, Kuantan. The skin of this vegetable has been peeled before it is cut into small 2-3 cm cylindered sizes and washed with distilled water (dH_2O). The remaining 1.8 kg of *T. cucumerina* was blended with 2000 mL dH_2O and left overnight at 28 °C. Using filter paper, this mixture was filtered and repeated three times. For the freeze-drying process, all the filtered solutions are combined and distributed to eight containers. . Following the procedure by Tariq *et al*. (2018) with slight modification, a total of 250 g of the powder form of *T. cucumerina* aqueous extract was successfully obtained before it was stored in the refrigerator at 4 °C. To initiate the treatment, this powder is mixed with sdH_2O according to the targeted dosage and concentration.

Experimental animal

All animals (male, 6-8 weeks old, 20-25 g bw) in this study were performed according to standard procedures and guidelines by IACUC-IIUM with code number IIUM/IACUC Approval/2019 (6) (27). All groups of mice (*n*=6 per group) were placed in stainless steel cages and put at room temperature. They were all treated with daily meal *ad-libitum* at 12-12 h both with and without light periods.

Parasite inoculum

The rodent malarial parasite used in this study is the *P. berghei* NK65 strain. To secure this parasite, all tested mice were intraperitoneally (i.p.) injected with an inoculum containing 1.0×10^6 *P. berghei* parasitized RBC (obtained from donor mice) in 0.1 mL of Alsever's solution. Then, a serial dilution was performed on parasitized RBCs using Alsever's solution, before each mouse was intraperitoneally (i.p.) injected with a lethal dose of 0.1 mL blood solution containing 1.0×10^6 *P. berghei* parasitized RBC on Day-0 (D0).

Treatment regimen

Daily, all four tested mice groups were force-fed with 0.2 mL of 100 mg/kg body weight (bw) of sdH_2O-*T. cucumerina* extract. Under the prophylactic regimen, the mice in PT7 and PT3 groups are treated from D7 and D3 pre-infection respectively, while under the curative regimen, the mice in CT3 and CT5 groups are treated from D3 and D5 post-infection respectively. There are two positive control groups designated in this study. PPC (preventive positive control) group was given 0.1 mL 15 mg/kg bw of primaquine and the CPC (curative positive control) group was given 0.1 mL 10 mg/kg bw of chloroquine, both on D3 pre-infection and post-infection respectively. These two groups were treated with a single dose only. A negative control group labeled with SNC which was daily given with 0.1 mL 0.9% normal saline (NS) from D7 pre-infection was also formed in this study.

Microscopy observation and parameter measurement

By applying the Giemsa blood-stained smear, a thorough observation of parasitized RBCs was performed under a light microscope (Zeiss Primo Star, New York Microscope Co., New York, NY, USA). The four-day suppression test (4DST) method was applied to measure parasitemia density (%) and inhibition rate (%) of all groups of mice where the percentage of inhibition rate exceeding 65% was considered to have significant *in vivo* antimalarial activity. The survival period of mice was measured on a daily observation basis from the day of infection (D0), where the group of mice that recorded the longest survival period was counted as having received the best treatment regimen (Baba *et al*., 2015).

Biochemical toxicity assessment

Two phases of toxicity testing were established in this study, namely acute toxicity (daily treatment for 7 days) and sub-acute toxicity (daily treatment for 28 days). Each of these phases, it is divided into two treatments, namely treatment without infection and treatment within 2 h after infection. All four groups of mice in this toxicity test were labeled as AEA (acute toxicity without infection), AEB (acute toxicity within 2 h post-infection), SAA (sub-acute toxicity without infection), and SAB (sub-acute toxicity within 2 h post-infection). Two control toxicity groups were initiated in this study and labeled as CNA (normal mice without infection and treatment) and CLB (mice administered with a lethal dose of blood solution containing 1.0×10^6 *P. berghei* parasitized RBC. At the end of this assessment, the mice's blood was taken from cardiac puncture, and the level of all blood enzymes; alanine aminotransferase

(ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and serum total protein (STP) was recorded to be compared with the normal range. All these enzymes were chosen as it normally became the indicator for any toxicity in the liver and kidney that might occur after treatment with newly tested materials (Baba *et al*., 2015).

Statistical analysis

The Shapiro-Wilk test has been applied to assess significance analysis. All the mice groups were normally distributed. The values obtained for antimalarial screening and biochemical toxicity tests were documented as mean ± standard deviation (SD). When the *p*-value is equal to or less than 0.05, the significance of the test is declared.

RESULTS AND DISCUSSION

Parasitemia density, inhibition rate, and survival time of the mice

There was a significant difference recorded between prophylactic treatment groups, PT7 and PT3 where PT7 recorded the lowest parasitemia density (1.29 ± 2.19 %) among all groups except for those two positive control (PPC and CPC) groups (Table 1). No difference in the improvement of parasitemia density pattern recorded for the two groups in curative regimes was discovered. For all experimental groups, daily treatment of *T. cucumerina* extract still gave lower parasitemia values compared to the mice in the SNC group. Since the values of inhibition rates for the CT3, CT5, and SNC groups were lower than 65%, only the PT7 and PT3 groups provided the best values of antimalarial activity in this study. Some chemical constituents present in *T. cucumerina* are cucurbitacin B, cucurbitacin E, isocucurbitacin B, 23,24-dihydroisocucurbitacin B, 23,24-dihydrocucurbitacin E, sterols 2 β-sitosterol stigmasterol (Badejo *et al*., 2016). The phytochemical analysis of *T. cucumerina* revealed the presence of alkaloids, flavonoids, phenolics, tannins, and terpenoids. In addition, the phytochemical screening of the ethanolic extract of seeds showed the presence of triterpenoids and sterols (Azeez & Morakinyo, 2004). Trichosanthes species plays an important role in the Ayurvedic and Siddha system of medicine due to their various medicinal values anti as HIV, cardioprotective, anti-ulcer, antidiabetic, hepatoprotective, anti-inflammatory, and larvicidal effects (Rekha, 2015). The nutritional composition of *T. cucumerina* plants includes proteins, fat, fiber, carbohydrates, minerals, and vitamins A and E in significant quantities (Ojiako & Igwe, 2008). The results obtained are in line with previous documentation which states that the percentage value of parasitemia is directly parallel to the percentage value of inhibition rate, whereby the lower the value of parasitemia, the higher the value of inhibition rate (Baba *et al*., 2015). This study may obtain more accurate and concrete results if several other factors are taken into account such as the value of inhibition rate recorded according to the specific stage of *Plasmodium* spp. life cycle (Zin *et al*., 2017). In addition to the proliferation factors and progression of the erythrocytic cycle in *Plasmodium*-infected hosts that are still debated (Pasini & Kocken, 2021; Henry *et al*., 2022), some researchers and scientists believe that the level of immunity and its development in malaria-infected hosts in fighting the disease may influence the results of antimalarial activity of the tested material (Huang *et al*., 2021). All these factors were not considered in this study. As far as our extended searching, the presented study is the first attempt to evaluate the biological property of *T. cucumerina* against *P. berghei* using animal models.

In line with the significant percentage of inhibition rate, mice of the PT7 group recorded the longest survival time (122.42 ± 0.09 days) as compared to the other three treatment groups. Thus, there was a significant difference (*p*≤0.05, *n*=6) in the survival period of mice in this group as compared to the others except for the two positive control groups, PPC and CPC. Due to the perfect inhibition rate at 100%, the mice in PPC and CPC groups survived more than a year. Previous *in vivo* and *ex vivo* studies found that the survival time of *Plasmodium*-infected hosts would be longer and extended at higher inhibition rate values (Baba *et al*., 2015; Zin *et al*., 2017; Gebrehiwot, *et al*., 2021). The survival time for the PT3 group was also prolonged and evidenced a significant difference compared to the two curative regime groups CT3 and CT5. This proves that malarial-infected hosts consuming prophylactic treatment could potentially expand their lifespan. Prophylactically in daily life, primaquine should be taken on the third day before entering any endemic regions of malaria (Chu & White, 2021). Similarly preventively, the 4DST method is used as the signs and symptoms of malaria practically will appear within between the third or fourth day after infection and subsequently treated with chloroquine as a first-line drug against malaria (Kweyamba *et al*., 2019). All these elements are applied in this study.

Biochemical toxicity assessment

All ALT, AST, ALP, and STP values recorded by all four toxicity mice groups and two control groups for the blood biochemical toxicity assessment were situated in the normal range (Table 2). All values for ALT, AST, ALP, and STP recorded for four groups of treatment (AA, AB, SA, & SB) were recorded within the normal level as documented in the circulation issues by the Research Animal

Table 1. Parasitemia density (%), inhibition rate (%), and survival time (day) of the mice group treated with 0.2 mL of 100 mg/kg bw of sdH2O-*T. cucumerina*

Regimen	Group	Parasitemia density (%) ± SD	Inhibition rate (%) ± SD	Survival time (day) ± SD
Preventive	PT7	1.29 ± 2.19 [s]	81.44 ± 1.06 [s]	122.42 ± 0.09 [s]
	PT3	5.60 ± 0.96	67.07 ± 3.77	60.11 ± 4.20 [s]
Curative	CT3	7.44 ± 2.25	32.72 ± 0.65	32.09 ± 2.52
	CT5	8.83 ± 4.03	20.13 ± 2.22	18.78 ± 1.78
Control	PPC	0	100	>360
	CPC	0	100	>360
	SNC	10.07 ± 0.15	0	13.38 ± 2.22

Notes: (s) value indicated the significantly different ($p≤0.05$, n=6). Prophylactic Treatment on Day 7 (PT7), Prophylactic Treatment on Day 3 (PT3), Curative Treatment on day 3 (CT3), Curative Treatment on Day-5 (PT5), Primaquine Positive Control (PPC), Chloroquine Positive Control (CPC) and Normal Saline Negative Control (SNC), standard deviation (SD).

Table 2. Level of ALT, AST, ALP, and STP of the mice group treated with 0.2 mL 100 mg/kg bw of sdH$_2$O-*T. cucumerina* extract as compared with the normal range value

Regime	Group	ALT (IU/L) ± SD	AST (IU/L) ± SD	ALP (IU/L) ± SD	STP (g/dL) ± SD
Acute exposure	AEA	53.33 ± 1.22	126.10 ± 2.38	82.16 ± 1.10	7.76 ± 2.06
	AEB	74.49 ± 2.04	177.78 ± 3.01	77.42 ± 3.03	9.01 ± 1.45
Sub-acute exposure	SAA	60.01 ± 4.34	165.02 ± 2.23	65.19 ± 4.40	8.34 ± 2.96
	SAB	57.53 ± 2.39	159.64 ± 1.99	71.70 ± 0.99	7.22 ± 4.00
Control	CNA	64.50 ± 0.97	123.19 ± 2.07	80.58 ± 3.32	6.95 ± 2.22
	CLB	59.09 ± 3.77	170.54 ± 3.22	69.02 ± 2.02	7.70 ± 3.11
Indicator	NR	40 – 93	92 – 206	54 – 115	5.8 – 9.5

Notes: The indicator for normal range (NR) was documented by Research Animal Resources, University of Minnesota, USA. Abbreviations: bw: body weight, ALT: alanine aminotransferase, AST: aspartate aminotransferase, ALP: alkaline phosphatase, STP: serum total protein, IU/L: international unit per er liter, g/dL: gram per deciliter, AEA: Acute toxicity without infection, AEB: Acute toxicity within 2 h post-infection, SAA: Sub-acute toxicity without infection, SAB: subacute toxicity within 2 h post-infection, CNA: Normal mice without infection and treatment, CLB: mice administered with a lethal dose of blood solution containing $1.0 × 10^6$ *P. berghei* parasitized RBC, NR: Negative control mice treated with normal saline, SD: Standard deviation.

Resources, University of Minnesota, USA. With a dose and concentration of 0.2 mL of 100 mg/kg bw of sdH$_2$O-*T. cucumerina* given to all mice in this study, no effect of undesired toxicity on all types of blood enzymes and serum total protein as per guidelines issued by The Department of Animal Resources Research, University of Minnesota (MN, USA). As the increased ALT, AST, and ALP and decreased STP levels in blood serum may indicate the toxicity effects of tested plan material in the Plasmodial-infected host, this study exhibited there are no toxicity effects of *T. cucumerina* in the malarial-animal models with the respected dosage and concentration. Devi (2017) and Kavitha (2020) in their review article have reported that *T. cucumerina* did not leave any undesired toxicity effects in anti-inflammatory and anticancer studies. Antimicrobial studies on the crude extract of this plant on *Staphylococcus aureus* and *Bacillus cereus* also did not leave any toxicity effects on rats (Reddy *et al*. 2010). According to Chinwe *et al*. (2020), practically, the level of blood enzymes is always associated with the severity of infectious disease, type of cancer, and immune-mediated diseases. Megabiaw *et al*. (2022) highlighted that in clinical practice, ALT and AST levels are the two gold standard indicators for liver failure and damage. The Department of Animal Resources Research, University of Minnesota also circulated that all the values of blood enzymes and serum total protein can be varied and reach beyond the normal levels depending on the weight, gender, age, and type of animal model. In connection to this fact, often, blood enzymes will be oversecreted whenever the animals go through the aging maturity process (Lim *et al*., 2018). In the infected host *Plasmodium*, the life cycle of this protozoa allows the occurrence of toxicity in the liver when the schizont stage hibernates in the liver parenchymal cells (Zin *et al.*, 2017). In this case, the action of bioactive compounds in *T. cucumerina* in inhibiting the development of *P. berghei* at the stage of the exo-erythrocytic cycle on the schizont is expected to have a toxic effect on the liver at the non-optimal dosages and concentrations. This hypothesis is supported by a study from Pasini and Kocken (2021) who concluded that bioactive compounds that accumulate in the liver at non-

therapeutic quantities can influence and change the inhibition rate of *P. vivax* and *P. ovale.*

CONCLUSION
The antimalarial properties of *T. cucumerina* demonstrated that the earlier the daily prophylactic consumption of *T. cucumerina*, the greater the results can be obtained in inhibiting the development of the malarial agent. More in-depth studies are needed to gain a more precise understanding in terms of the mechanism of action of *T. cucumerina* against this parasite and in terms of how manipulation of dosage and concentration of *T. cucumerina* can be applied to both animals and humans to obtain a better effect on the prophylactic treatment against malaria.

ETHICAL STATEMENT
This study was approved by an ethical committee from the Institutional Animal Care and Use Committee (IACUC-IIUM) with the code IIUM/ IACUC Approval/2019 (6) (27).

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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