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Original Article

Silybum marianum and *Trigonella foenum-graecum* Exhibit Moderate *In Vitro* Antimalarial Properties against *Plasmodium knowlesi*

Norsyafiqah Binti Ahmad Safri [1], *Fei Wen Cheong [2], Nur Zulaikha Zulkefli [2], Abolghasem Siyadatpanah [3], Roghayeh Norouzi [4], *Yee Ling Lau [2]

1. *Department of Biomedical Science, Faculty of Medicine, Universiti Malaya, 50603, Kuala Lumpur, Malaysia*
2. *Department of Parasitology, Faculty of Medicine, Universiti Malaya, 50603, Kuala Lumpur, Malaysia*
3. *Department of Microbiology, Faculty of Medicine, Infectious Diseases Research Center, Gonabad University of Medical Sciences, Gonabad, Iran*
4. *Department of Pathobiology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran*

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***Correspondence**
Email:
fwcheong18@um.edu.my,
lauyeeling@um.edu.my

Abstract

Background: Malaria remains a major global health burden, with increasing challenges posed by drug-resistant *Plasmodium* species. Another emerging threat is *Plasmodium knowlesi*, a zoonotic parasite responsible for rising human infections in Southeast Asia, particularly Malaysia. This study investigates the *in vitro* antimalarial activity of *Silybum marianum* (milk thistle) and *Trigonella foenum-graecum* (fenugreek) seed extracts against *P. knowlesi*, and their cytotoxicity against mammalian cells.

Methods: Using schizont maturation inhibition assay, *P. knowlesi* cultures were exposed to serial dilutions of both plant hydroethanolic extracts (0.20–100.00 µg/mL). Chloroquine was used as positive control. Cytotoxicity was evaluated on Vero cells using the MTT assay.

Results: Both extracts exhibited moderate antimalarial activity, with IC_{50} values of 16.12 ± 1.02 µg/mL (*S. marianum*) and 17.08 ± 1.26 µg/mL (*T. foenum-graecum*). Cytotoxicity testing showed that the extracts were non-toxic to mammalian cells, with CC_{50} values of 84.76 ± 15.12 µg/mL (*S. marianum*) and 72.75 ± 15.81 µg/mL (*T. foenum-graecum*). The selectivity indices were 5.26 and 4.26, respectively, indicating moderate selectivity towards the parasite over mammalian cells.

Conclusion: Both *S. marianum* and *T. foenum-graecum* demonstrate promising antimalarial potential against *P. knowlesi in vitro*, with acceptable cytotoxicity profiles. These findings support further investigation into their bioactive constituents and mechanism of action, offering preliminary evidence for their potential development as alternative antimalarial agents targeting zoonotic malaria.

Introduction

Malaria causes 282 million cases worldwide in 2024 (1). Four known species that cause malaria in humans as natural hosts, which are *Plasmodium falciparum, P. vivax, P. malariae, and P. ovale.* In addition, *Plasmodium knowlesi*, primarily found in macaques in Southeast Asia, has emerged as the fifth species causing malaria in humans via *Anopheles* mosquito bite (2,3). *P. knowlesi* has become the main species that con-

tributes to 1,927 human malaria cases in Malaysia in 2024 (1).

The emergence of drug-resistant strains towards multiple drugs including chloroquine and sulfadoxine-pyrimethamine in past decades posed a major barrier to malaria control and eradication (4,5). Furthermore, resistance of *Plasmodium* species towards the latest antimalarial drug, artemisinin, had firmly established in the Greater Mekong Subregion (6). Recently, partial artemisinin resistance has also been confirmed in the malaria endemic African region, poses a devastating risk in global malaria control (7,8). Therefore, development of new therapeutic compounds can no longer be delayed.

Historically, natural products have played a pivotal role in antimalarial development. Artemisinin was first isolated as a pure compound in 1972 from the plant *Artemisia annua* (9). Since then, studies to investigate antimalarial properties from other plant species remain robust. *Silybum marianum* (L.) Gaertn (milk thistle) and *Trigonella foenum-graecum* L. (fenugreek) are two medicinal plants with reported therapeutic properties. *S. marianum* is an annual herb native to southern Europe, Northern Africa and Asia Minor with reported hepatoprotective and antioxidant functions, ascribed mostly to its active component silymarin (10,11). Meanwhile, *T. foenum-graecum* is an annual herb currently cultivated globally for culinary and medicinal applications (12). It is well known for its anti-inflammatory, antibacterial and metabolic regulating properties (12,13).

Past studies had reported the promising antimalarial properties of both plant extracts against *P. falciparum* (14,15). However, no studies have so far assessed their effectiveness against *P. knowlesi*, which has a relatively different drug susceptibility profile compared to *P. falciparum*. On top of that, other human *Plasmodium* species, such as *P. vivax, P. ovale*, and *P. malariae*, demonstrated drug susceptibility profiles that were more closely resembled those of *P. knowlesi* than those of *P. falciparum* (16). Moreover, *P. knowlesi* has a highly significant disease burden in Southeast Asian countries including Malaysia. Both plants used for this study are non-toxic, widely accessible, and traditionally used, hence, they are great candidates for drug discovery efforts.

We aimed to investigate the antimalarial properties of crude extracts from *S. marianum* and *T. foenum-graecum* against *P. knowlesi*, and their cytotoxicity against mammalian cells.

Material and Methods

Ethical clearance

Experiment procedures and ethics have been approved by Institutional Biosafety and Biosecurity Committee, Universiti Malaya (UMIBBC/NOI/R/FOM/PARA-003/2019-04102021) and National Medical Research Register, Ministry of Health Malaysia (NMRR-17-1718-35558) to maintain *P. knowlesi in vitro* culture A1H.1.

Plant collection and extraction

The plants, *S. marianum* and *T. foenum-graecum* were obtained from East Azerbaijan Province, Iran. The plant samples were delivered and deposited at the Herbarium of the Faculty of Pharmacy, Tabriz University of Medical Sciences, Iran (Herbarium number of *S. marianum*: TBZ-fph-514; *T. foenum-graecum*: TBZ-fph-2196). The seeds of the plants were ground into a powder using an electrical blender. Briefly, the hydroethanolic extract of *S. marianum* and *T. foenum-graecum* were prepared by maceration of 100 g of dried powder plant seeds in 70% ethanol for 3 d at room temperature. Then, the extracts were filtered through filter paper (Whatman Ltd., Buckinghamshire, UK). The alcohol in the sample was removed by vacuum rotary evaporator (Model VV 2000, Heidolph, Germany). After that, the extracts were dried in the incubator at 37 ℃ to obtain a dry powder extract for each plant. Both extracts were kept in -20 ℃ until further use.

Preparation of extract solutions

The concentrated crude hydroethanolic extracts of *S. marianum* and *T. foenum-graecum* were dissolved in dimethylsulfoxide (DMSO) to make stock solutions of 1 mg/mL and then serially diluted (2-fold dilution) in 70% ethanol with final concentrations from 0.20 µg/mL to 100.00 µg/mL (14,15). The reference drug used as standard control was chloroquine, prepared in absolute ethanol to make the stock solution of 515.86 mg/mL. Then, the chloroquine stock was serially diluted with 70% ethanol within the range of 1.60 pg/mL to 0.52 µg/mL (17). The 96-well flat-bottomed microtiter plates were pre-coated with 100 µL of the diluted suspensions of plant extracts and drug control for each well in each plate. For negative control, wells with 70% ethanol only were included. The pre-coated plates were air-dried overnight in the hood under sterile conditions.

P. knowlesi culture and maintenance

P. knowlesi A1H.1 was used in this study. This strain was derived from *P. knowlesi* H strain originated from Malaysia, after successful adaptation to long-term *in vitro* culture with human erythrocytes (18). The parasites were maintained in complete medium RPMI-1640 (Invitrogen, USA) supplemented with 10% (vol/vol) horse serum (Gibco, New Zealand), 0.05 g/L hypoxanthine, 2 g/L dextrose, 5 g/L AlbuMAX™ II, and 2.3 g/L sodium bicarbonate and 0.292 g/L L-glutamine. Then, the culture was incubated at 37 °C in a sealed flask gassed with a mixture of 5% O_2, 5% CO_2 and 90% N_2.

Tight synchronisation of P. knowlesi

Synchronisation of *P. knowlesi* was carried out using the Histodenz (Sigma, USA) density gradient method. Parasites at 50% haematocrit (2 mL) were layered over 5 mL of pre-warmed 55% Histodenz solution, followed by centrifugation at 2000 rpm for 15 min. After centrifugation, the brown interphase (containing schizonts) was collected, washed and returned to culture with fresh erythrocytes in the complete medium for 1 hour at 37 ℃ to allow schizont rupture, merozoite invasion into new erythrocytes and formation of new ring-stage parasites. The culture was then reapplied to another round of synchronisation. Next, the pellet, comprising newly invaded ring-stage parasites, was collected for experiment.

In vitro antimalarial assay using crude extracts

The schizont maturation inhibition assay was used according to the WHO *in vitro* micro-test (Mark III) (19). One hundred microlitres of synchronised ring-stage parasites were plated in each well of 96-well plates pre-coated with plant crude extracts (as mentioned above). The culture parameters were adjusted to 1% parasitaemia at 1% haematocrit and were incubated at 37 ℃ in the incubator chamber with mixed gas. After incubation, the contents of the wells were harvested when >50% of the parasites in negative control well have developed into schizonts (containing ≥3 nuclei). The thick smears were made from each well and stained for 10 min in 10% Giemsa solution (pH 7.2). Then, the number of developed schizonts (containing ≥3 nuclei) out of a total of 200 parasites were counted. The number of schizonts in each well that contained extracts was compared to the negative control well. Both extracts and drug control were performed in triplicate wells each round. Moreover, three rounds of technical replications were performed to ensure reproducibility and reliability of the findings. Lastly, the parasite growth inhibition percentage for each dose extract was calculated in comparison with the negative control using the formula below:

Growth inhibition (%) =

$$\frac{\text{No. of} \geq 3 \text{ nuclei schizonts in negative control} - \text{No. of} \geq 3 \text{ nuclei schizonts in test well}}{\text{No. of} \geq 3 \text{ nuclei schizonts in negative control}} \times 100\%$$

Calculation of half-maximal inhibitory concentration (IC_{50}) concentration

The IC_{50}, defined as the extract concentration corresponding to 50% of parasite growth inhibition, was determined from the dose-response curves using the GraphPad Prism™ version 10.4 programme. Extracts with IC_{50} <5 µg/mL, 5-10 µg/mL and 11-25 µg/mL were classified as highly active, active and moderately active, respectively. Extracts with IC_{50} of 26-100 µg/mL were classified as having low activity and considered to be inactive extracts if the IC_{50} was > 100 µg/mL (20,21).

Cytotoxicity assessment with 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

Vero cell line (African green monkey kidney cells) was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 g/L sodium bicarbonate, and 100 U of Pen-Strep. The cytotoxicity was measured using MTT assay. 96-well flat bottom plates were pre-coated with the serially diluted of plant extracts (0.20 µg/ml to 100.00 µg/ml) as mentioned above. Vero cell suspensions (5×10^4 cells/well) were seeded into the pre-coated wells. Meanwhile, cell suspension with absolute methanol and cell suspension without test substance were used as positive control and negative control, respectively. The plates were incubated at 37 ℃ in 5% CO_2 incubator for 72 h. After incubation, each well was added with 10 µl of 2 mg/mL MTT solution, mixed gently and incubated for an additional 4 h at 37 ℃ in an incubator with 5% CO_2. The culture medium was removed after incubation and 100 µl of DMSO was added into each well to dissolve the MTT formazan product. The solution was then stirred for 15 min on a rotator shaker, and the absorbance of control and treated wells at 540 nm was measured using a microplate reader. The cytotoxic concentration 50% (CC_{50}) was calculated from a dose-response curve to find the cytotoxic concentration of each extract that resulted in a 50% reduction in cell viability, using the GraphPad Prism™ version 10.4 programme. All extracts were tested in triplicate wells each round, and a total of three rounds of experiments have been conducted. Following the background subtraction, the percentage of cytotoxicity was computed and compared to the negative control using the formula below:

$$\text{Cytotoxicity rate (\%)} = \frac{\text{Absorbance of negative control} - \text{Absorbance of test well}}{\text{Absorbance of negative control}} \times 100\%$$

According to the classification, an extract was classified as extremely toxic if its CC_{50} was less than 10 µg/mL, moderately toxic if it was between 11 and 30 µg/mL, slightly toxic if it was between 31 and 50 µg/mL, and non-toxic if it was greater than 50 µg/mL (22).

Selectivity index (SI)

SI represents the ratio between cytotoxic and antimalarial actions. SI values were classified as not active if SI < 4, SI between 4-10 as somewhat active and SI > 10 as active antimalarial activity (highly selective towards malaria parasites but not the tested mammalian cells) (23). SI value for each crude extract was determined using the formula:

$$\text{SI} = \frac{CC_{50} \text{ of Vero cell lines}}{IC_{50} \text{ of } \textit{Plasmodium knowlesi}}$$

Statistical analyses

The IC_{50} and CC_{50} were determined from dose-response curve using least-squares nonlinear regression in the GraphPad Prism™ ver. 10.4 programme. For multiple group data comparisons, One-way ANOVA with Dunnett's multiple comparison test (normally distributed) was performed; $P<0.05$ was considered statistically significant.

Results

In vitro antimalarial activity

Table 1 shows the parasite growth inhibition for different concentrations of *S. marianum* and *T. foenum-graecum* extracts. Marked inhibition was seen at 25.00 µg/mL and above, with 93.57 ± 6.94% inhibition rate at 100.00 µg/mL for *S. marianum* extract. A similar pattern was seen for *T. foenum-graecum* with a slightly higher inhibition rate at 100.00 µg/mL, which was 94.53 ± 4.65%.

S. marianum and *T. foenum-graecum* extracts have the IC_{50} values of 16.12 ± 1.02 µg/mL and 17.08 ± 1.26 µg/mL, respectively. Meanwhile, chloroquine, which is the positive control drug, has IC_{50} value of 341.90 ± 9.12 pg/ml, which is within the IC_{50} reference range for chloroquine. The microscopic examination of Giemsa-stained slides at hour 24 showed less schizonts inhibition at the lowest concentration (0.20 µg/mL) for both extracts. Then, the percentage of inhibition gradually increased starting from 0.39 µg/mL onwards. There were huge differences in schizonts inhibition between 12.50 µg/mL and 25.00 µg/mL for both extracts (Fig. 1).

Table 1: *In vitro* antimalarial activity of plant extracts against *P. knowlesi*

Concentration of Extracts (µg/mL)	Parasite Growth Inhibition (%)	
	Silybum marianum	***Trigonella foenum-graecum***
100.00	93.57 ± 6.94	94.53 ± 4.65
50.00	90.43 ± 10.54	91.01 ± 5.31
25.00	82.63 ± 9.08	77.54 ± 4.64
12.50	31.28 ± 4.36	30.11 ± 4.82
6.25	19.36 ± 11.86	20.73 ± 1.55
3.13	15.03 ± 8.28	14.31 ± 1.79
1.56	12.07 ± 7.02	11.65 ± 1.50
0.78	11.94 ± 5.56	9.48 ± 2.26
0.39	10.46 ± 3.39	5.89 ± 1.80
0.20	6.63 ± 5.00	3.73 ± 2.11
IC_{50} (µg/mL)	16.12 ± 1.02	17.08 ± 1.26

Data are presented as the mean ± standard deviation (SD) of three independent experiments performed in triplicate wells. The assay for standard antimalarial drug (chloroquine) was conducted in parallel with the crude plant extracts

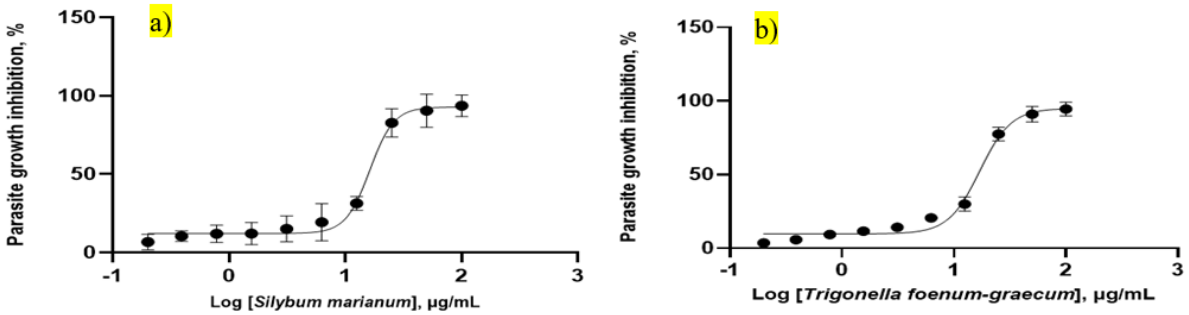

Fig. 1: Dose-dependent inhibition of schizont maturation by (a) *S. marianum* and (b) *T. foenum-graecum* plant extracts. The data was presented in mean ± SD for each of the plant extract log concentrations

For *S. marianum* extract, no statistically significant differences were seen at concentrations ranging from 0.20 µg/mL to 3.13 µg/mL (adjusted $P>0.05$). Significant inhibitory effects were observed at 6.25 µg/mL ($P=0.0267$), 12.50 µg/mL ($P=0.0003$), 25.00 µg/mL ($P<0.0001$), 50.00 µg/mL ($P<0.0001$), and 100.00 µg/mL ($P<0.0001$). Meanwhile, *T. foenum-graecum* exhibited statistically significant differences in antimalarial activity to the untreated control at lower concentration compared to *S. marianum*, starting at concentrations of 0.78 µg/mL ($P=0.0125$), followed by 1.56 µg/mL ($P=0.0018$), 3.13 µg/mL ($P=0.0002$) and 6.25 µg/mL and above ($P<0.0001$). Conversely, lower concentrations (0.20 and 0.39 µg/mL) did not exhibit a significant difference ($P = 0.6741$ and 0.2074, respectively) compared to the control. The inhibition increased with concentration showing a dose-dependent effect, with the most significant effects reported at the higher concentrations of 25.00 µg/mL and above (Fig. 2).

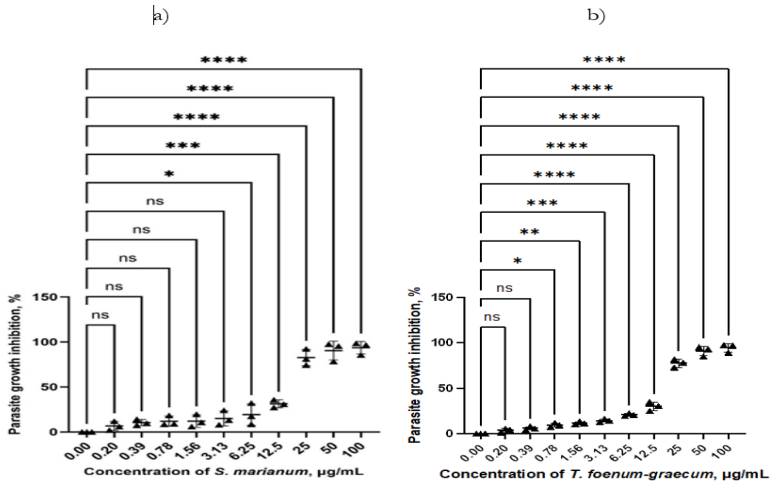

Fig. 2: Effect of different concentrations of (a) *S. marianum* and (b) *T. foenum-graecum* on parasite growth inhibition of *P. knowlesi* compared to the control. The data was presented in mean ± SD for each of the plant extract concentrations. ns: not significant. Significant differences to the parasite control: *($P<0.05$); ** ($P<0.01$); *** ($P<0.001$); **** ($P<0.0001$)

In vitro cytotoxicity activity

The CC_{50} of both plant extracts on Vero cells were determined using dose-response curve and the results are shown in Table 2. In this study, the CC_{50} for hydroethanolic extract of *S. marianum* and *T. foenum-graecum* were 84.76 ± 15.12 µg/mL and 72.75 ± 15.81 µg/mL, respectively.

Selectivity index (SI)

The value of the SI was calculated to assess the safety and therapeutic potential of a compound. In this study, both hydroethanolic extracts of *S. marianum* and *T. foenum-graecum* exhibited values below 10, with SI values 5.26 and 4.26 (Table 2).

Table 2: The antimalarial and cytotoxicity of hydroethanolic extracts of *S. marianum* and *T. foenum-graecum* seeds

Plant extracts	*P. knowlesi* A1H.1 IC_{50} (µg/mL)	Vero cells CC_{50} (µg/mL)	Selectivity index (SI)
S. marianum	16.12 ± 1.02	84.76 ± 15.12	5.26
T. foenum-graecum	17.08 ± 1.26	72.75 ± 15.81	4.26

Discussion

In vitro antimalarial activity

S. marianum hydroethanolic extract demonstrated moderate antimalarial activity with dose-dependent inhibition of parasite growth. This observation is consistent with previous findings against *P. falciparum*, where silymarin exhibited antimalarial activity against *P. falciparum* (NF-54 strain) with IC_{50} value of 14 ± 0.33 µM (similar to 6.75 µg/mL) with high SI (>100) (15). Silymarin is a complex of flavonolignans extracted from the seeds of *S. marianum*, where silymarin demonstrated antimalarial action by inhibiting both heme-polymerisation and degradation. Excess heme leads to redox imbalance and eventually apoptotic cell death. Besides, the presence of silymarin-heme complex triggers the destabilisation of membrane, inducing high oxidative stress causing redox imbalance, leading to lipid peroxidation and damaging food vacuole membrane (24). On the other hand, *S. marianum* fruit was also found to exhibit antimalarial activity with IC_{50} value of 2 µg/mL against *P. falciparum* NF-54 strain with a good SI of 27.3 (25).

On the other hand, *T. foenum-graecum* extract also demonstrated moderate antimalarial activity, and dose-dependent inhibition of parasite growth. Significant inhibitory effects were found starting at 0.78 µg/mL, implying a more sensitive antimalarial action at lower concentration than *S. marianum*. *T. foenum-graecum* is postulated to possess antimalarial activity due to its high phytochemical content, especially flavonoids, saponins, and alkaloids such as trigonelline, already shown antibacterial and antiparasitic properties (26,27). The 50% hydroethanolic extract possessed profound *in vitro* antimalarial activity with IC_{50} value of 8.75 ± 0.35 µg/mL and 10.25 ± 0.35 µg/mL against chloroquine-sensitive and resistant *P. falciparum* isolates, respectively, which agrees with our findings in the present study (14).

Nonetheless, the IC_{50} values of both plants used in the present study were higher compared to the previous studies. These could be due to different *Plasmodium* species being investigated, since *P. falciparum* and *P. knowlesi* have different drug susceptibility profiles due to their biology and genetic makeup. Besides, hydroethanolic solvent (70% ethanol) was used in the present study compared to the other studies that used methanol, ethanol and ethyl acetate. Each solvent differs in polarity and extraction efficiency, influencing the phytochemical profile and concentration of bioactive compounds in the final extract. Moreover, comparing different parts of the plants also could contribute to the differences, as each part of the plants could contain different bioactive compounds. Previous studies that used different parts of plants compared to the use

of seeds in this study may have resulted in varying levels of antimalarial activity.

Cytotoxicity of plant extracts against mammalian cell line

The hydroethanolic extract of *S. marianum* was not toxic against Vero cells. This finding is aligned with previous study which silymarin demonstrated high selectivity value (> 100), indicating the compound is not toxic to the tested mammalian cells (15). Besides, the *S. marianum* ethyl acetate extract is exhibiting selective cytotoxicity, with significantly higher tolerance (higher IC_{50}) in normal cells (baby hamster kidney cells) compared to cancerous cell lines HeLa and HepG2 (28).

On the other hand, extract of *T. foenum-graecum* was also not toxic against Vero cells. The finding is in concordance with several previous studies. Al-Oqail et al. investigated the *in vitro* cytotoxic activity of seed oil of fenugreek, and among the used cell lines, HEp-2 cells showed the highest reduction in cell viability, followed by MCF-7, WISH and least in Vero cells, with only 14% cytotoxicity at 1000 µg/mL (29). Similarly, Al-Timimi observed no cytotoxic effect of at high concentrations of ethanol fenugreek seed extract (400-1000 µg/mL) towards Vero cells (30). The minimum inhibition concentration of 70% ethanol fenugreek seed extract towards Vero cells was reported to be 10 mg/mL, indicating that concentrations below this value are relatively non-toxic to normal cells (31).

Despite the CC_{50} values are favorable, the SI value for both plant extracts falling below the threshold of 10, which is generally considered as acceptable therapeutic SI values (32). Although the extracts exhibit a moderate level of antimalarial activity; they have a narrow safety margin in relation to cytotoxicity. To increase the therapeutic index, purification or fractionation may be required to separate the most bioactive yet less mammalian cell-toxic compounds.

Conclusion

This study is the first to investigate the antimalarial properties of crude extracts from *S. marianum* and *T. foenum-graecum* seeds against *P. knowlesi*. Both the plant extracts exhibit moderate antimalarial activities, non-toxic to mammalian cells, with moderate selectivity towards *P. knowlesi* and Vero cells. The results pave the way to further evaluate their potentials as antimalarials. In the present study, only crude extracts were tested and the specific phytochemicals responsible for the antimalarial activity remain unidentified. Hence, future research should focus on isolating and identifying the pure chemicals from crude extracts of these two plants to determine the metabolites' profile and to identify the active compounds which exert antimalarial activities yet highly selective towards malaria parasites at the same time.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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