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RESEARCH ARTICLE

Vernonia amygdalina (Delile) exhibits *in vitro* anti-plasmodial activities against *Plasmodium knowlesi*

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ABSTRACT

Malaria is a global public health concern, where *Plasmodium knowlesi* contributes to most human malaria cases in Malaysia. The leaves of *Vernonia amygdalina* (Delile) were found to exhibit significant anti-plasmodial properties and are commonly used in sub-Saharan Africa countries for malaria treatment. Nonetheless, studies are lacking in the effect of this plant against *P. knowlesi* specifically. This study aimed to investigate the *in vitro* anti-plasmodial activity of crude extracts from *V. amygdalina* (Delile) against *P. knowlesi*. The fresh leaves of *V. amygdalina* were sequentially extracted in three solvents, dichloromethane (DCM), methanol (MeOH) and water (H_2O) to attain the crude extracts. Schizont maturation inhibition assays were performed using *in vitro P. knowlesi* culture (A1H1) to evaluate the anti-plasmodial activities. The *in vitro* cytotoxicity of the plant extracts was also evaluated with Vero cell line using MTT assay. Plant extracts in all solvents showed good to moderate anti-plasmodial activity against *P. knowlesi*, with concentration of drug required for 50% growth inhibition (IC_{50}) of 1.356 ± 0.23 µg/mL, 1.131 ± 0.16 µg/mL and 1.973 ± 0.30 µg/mL for DCM, MeOH and H_2O extracts, respectively. The concentration of drug that is cytotoxic to 50% cell populations (CC_{50}) for each extract is DCM = 48.61 ± 0.64 µg/mL, MeOH = 63.73 ± 0.78 µg/mL and H_2O = 86.91 ± 1.03 µg/mL, indicating the MeOH and H_2O extracts were likely non-toxic to the mammalian cells. In the present study, all crude extracts exhibited Selectivity Index (SI) values above 10, with MeOH extract having the highest selectivity towards malaria parasites. These findings suggest that the extracts of *V. amygdalina* leaves exhibit anti-plasmodial properties against *P. knowlesi* even in the crude form and may be a potential candidate for the development of new anti-plasmodial drug. This provides scientific evidence for usage of this plant in traditional medicine, and further studies are needed for identification and purification of the active metabolites.

Keywords: *Plasmodium knowlesi*; *Vernonia amygdalina* (Delile); anti-malarial; cytotoxicity; crude extracts.

INTRODUCTION

Malaria caused 263 million cases globally, leading to 597 000 deaths in the year 2023 (WHO, 2024) and is currently a significant health burden across the world. This disease is transmitted by female *Anopheles* mosquitoes to human during blood feeding. In Malaysia, *Plasmodium knowlesi* is the main causative agent of human malaria cases with 2879 cases and 14 deaths reported in 2023 (WHO, 2024). *P. knowlesi* was originally a simian malaria parasite but is recognised as the fifth human malaria species alongside *Plasmodium falciparum, Plasmodium malariae, Plasmodium ovale* and *Plasmodium vivax*. It is considered a zoonotic malaria species as the natural host for *P. knowlesi* are the long-tailed macaque (*Macaca fascicularis*), pig-tailed macaque (*Macaca nemestrina*), and banded-leaf monkey (*Presbytis melalophus*) (Coatney *et al*., 1971; Moyes *et al*., 2014). The uncontrollable passage of the parasites among the natural hosts has greatly impeded the government's aim for malaria eradication by the year 2020. This imposes a high risk for potential human malaria outbreaks via zoonotic transmission. On top of that, in the last few decades, the human *Plasmodium* species, particularly *P. falciparum* has shown to develop resistance to all the anti-plasmodial drug classes, including partial resistance to artemisinin, the last effective drug (Dorndorp *et al*., 2009). Resistance to artemisinin and its derivatives has resulted in failure of artemisinin-based combination therapies (ACT), which further threaten all major gains in malaria control as ACT has been the current backbone of malaria treatment and control worldwide (Thu *et al*., 2017; Dhorda *et al*., 2021). Hence, it is crucial to search for new anti-plasmodial agents with high efficacy and cost appropriate in order to combat the disease.

Compounds derived from medicinal plants have proven to be an excellent resource for anti-plasmodial agents. The use of herbal medicinal plants in the development of drugs and chemotherapeutics based on the acclaimed traditional use has gained interest. Two important anti-plasmodial drugs derived from plant sources namely quinine isolated from the bark of *Cinchona* species, and artemisinin, obtained from the Chinese herb *Artemisia annua* had gained interest since their first discovery (Tu *et al*., 1981; Phillipson & O'Neill, 1986). These findings indicate that natural compounds from plants play a

major role in drug discovery, highlighting the interest in investigating other plants that may possess pharmacological and medicinal properties. In this study, we focus on finding plants that can grow naturally in the wild in Malaysia with evidence of anti-plasmodial effects.

Vernonia amygdalina (Delile) is a small perennial shrub from the family Asteraceae, which is native to African countries and widely distributed in Asia (Danladi *et al*., 2018; Abay *et al*., 2015). This herbal plant is also commonly found growing wild in Malaysia (Wong *et al*., 2013) and is locally known as "daun bismillah'' or bitter leaf (Zakaria *et al*., 2016). Multiple findings have shown that this plant has been widely used as a traditional medicinal practice for the treatment and prevention of malaria in native countries including Nigeria, Ethiopia, Cameroon, Rwanda and Democratic Republic of Congo (Challand & Willcox, 2009; Toyang & Verpoorte, 2013; Kadiri & Olawoye, 2016; Suleman *et al*., 2018). *V. amygdalina* has been found to be used as ethnomedicine in its native countries, where patients suffering from malaria attacks are advised to consume half a glass (approximately 250 ml) of the plant leaves soaked in hot water twice a day for four to seven consecutive days (Njan, 2012). Besides humans, studies by Koshimizu *et al*. (1994) and Huffman *et al*. (1993) observed that wild chimpanzees suffered from parasite-related illness chewed the pith and swallowed the bitter juice of *V. amygdalina*. Furthermore, the anti-plasmodial activity of this small shrub has been confirmed by numerous *in vitro* and *in vivo* studies with the ability to suppress *P. falciparum* and *P. berghei* (Madureira *et al*., 2002; Iwalokun, 2008; Mohd Abd Razak *et al*., 2014; Ajayi *et al*., 2017; Angupale *et al.,* 2024, Asianuba *et al*., 2024).

Most of the anti-plasmodial studies were carried out using *P. falciparum* and/or *P. berghei*. However, as Southeast Asian countries (especially Malaysia) are highly burdened by *P. knowlesi*, more efforts should be focused on this latest human malaria species. With the established *P. knowlesi in vitro* culture system, specifically the *P. knowlesi* A1H1 clone which can be cultured without using macaque erythrocytes (Moon *et al*., 2013), new therapeutic compounds could be easily assessed using growth inhibition assays. When it comes to drug susceptibility profiles, *P. knowlesi* differs from *P. falciparum*. For example, *P. knowlesi* is substantially less susceptible to sodium channel ATP4 inhibitors compared to *P. falciparum* (van Schalkwyk *et al*., 2019). Additionally, other human *Plasmodium* species, such as *P. vivax*, *P. ovale*, and *P. malariae*, demonstrated drug susceptibility profiles that were more closely resemble those of *P. knowlesi* than those of *P. falciparum* (van Schalkwyk *et al*., 2021). This indicates the importance of including *P. knowlesi* strains in the screening and development of new anti-plasmodial drugs, besides *P. falciparum*. To date, there is no report on the effect of *V. amygdalina* against *P. knowlesi* species. Hence, in the present study, we investigated the anti-plasmodial activity of crude extracts from *V. amygdalina* against *in vitro P. knowlesi* culture (A1H1). On the other hand, we also evaluated the *in vitro* cytotoxicity of the plant extracts towards mammalian cells.

MATERIALS & METHODS

Ethical approval

The usage and maintenance of *P. knowlesi* culture in this study were conducted with protocols and guidelines approved by the Medical Research Committee of the Ministry of Health, Malaysia (NMRR- 17-1718-35558) and Institutional Biosafety and Biosecurity Committee (IBBC) Universiti Malaya (UMIBBC/NOI/R/FOM/PARA-003/2019-04102021).

Plant collection and identification

Fresh whole *V. amygdalina* plants were collected from Klang, Selangor, Malaysia. Dr. Sugumaran Manickam of the Institute of Biological Sciences, Universiti Malaya, identified the plants. A voucher specimen (KLU 50183) was deposited at the herbarium of the Rimba Ilmu Botanic Garden, Institute of Biological Sciences, Faculty of Science, Universiti Malaya, Kuala Lumpur, Malaysia.

Plant extraction

Fresh plant leaves were collected and cleaned to remove any debris and were air-dried for two weeks under the shade at room temperature. They were then cut into small pieces and ground into coarse powder (1 kg) using an electric blender and stored in airtight plastic containers. The leaves were extracted using extraction procedures described by Mohd Abd Razak *et al*. (2014) with minor modifications. One kilogram of the grounded materials was defatted with 1 litre of hexane and were sequentially extracted with 1 litre of dichloromethane (DCM), methanol (MeOH), and sterile distilled water (H_2O) at room temperature for 24 hours. After the first DCM extraction, the residue was extracted again using 1 litre of fresh portion of DCM after the supernatant was collected using filter paper (Whatman No. 1, England). This step was repeated three times (3 x 1 litre). The combined DCM extracts were then concentrated by evaporation under reduced pressure using a rotary evaporator (Buchi Rotavapor R-200, Switzerland) at 40°C. The residue was further extracted using MeOH followed by sterile H_2O in similar procedures. Unlike DCM and MeOH extracts, the aqueous supernatant was freeze-dried using lyophiliser (Labconco FreeZone 6, USA) to obtain the aqueous crude extracts. All concentrated extracts were stored at -20°C in the dark until further use.

Thin-layer chromatography (TLC) profiling

TLC was done using aluminum sheets of silica gel F_{254} (Merck®) where aliquots of each crude extract were spotted onto a plate with volumetric micropipettes, along a virtual line situated 10 mm from the bottom edge of the plate (100 x 50 mm). The extract-loaded plates were developed in a developing chamber containing a mixture of solvents (ethyl acetate and petroleum ether) at 1:1 (v/v) solvent ratio. The developed plates were dried in a fume hood. The plates were visualised in a UV chamber at wavelengths 254 nm and then sprayed with vanillin-sulphuric acid as the chromogenic agent.

Preparation of extract solutions

The concentrated crude extracts of DCM, MeOH and sterile H_2O were solubilised in dimethylsulfoxide (DMSO) to make the stock solutions (1 mg/ml) and were kept at -20°C until further use. Prior to *in vitro* assays, the stock extracts were serially diluted (2-fold dilution) using 70% ethanol with final concentrations ranging from 0.19 µg/ml to 100 µg/ml (Omorgie *et al*., 2011; Mohd Abd Razak *et al*., 2014; Abay *et al*., 2015). Chloroquine was used as standard control drug, with final concentrations ranging from 1 ng/ml to 520 ng/ml (1.94 nM to 1 008.03 nM) (Fatih *et al*., 2013). To ensure the validity of the assay, the anti-plasmodial assay on the plant extracts was conducted concurrently with the standard anti-plasmodial drug. All crude extracts (stocks prepared in DMSO) and standard drug (stock prepared in distilled H_2O) were diluted using 70% ethanol and the 96-well flat bottom plates were pre-coated with 100 µl of the diluted suspensions in respective wells. The pre-coated wells were air-dried overnight in the hood under sterile condition. Wells with only 70% ethanol were included as the negative control in the assay.

P. knowlesi culture and maintenance

P. knowlesi culture used in this study, the A1H1 strain, is a well-established *in vitro* culture developed by Moon *et al*. (2013), with extensive genetic analyses performed on the strain. This strain has been successfully adapted for long-term *in vitro* culture using human erythrocytes, eliminating the need for macaque blood or animal facilities. A continuous culture of the *P. knowlesi* A1H1 strain was maintained with an initial parasitaemia of 1% at 2% haematocrit, following the method described by Moon *et al*. (2013). The parasites were grown in human A^+ erythrocytes and maintained in complete medium (pH 7.3) containing RPMI-1640 (Invitrogen,

USA) supplemented with 10% horse serum (Gibco, New Zealand), 2.3 g/L NaHCO3, 0.05 g/L hypoxanthine, 4 g/L glucose, 5.957 g/L HEPES, 0.292 g/L L-glutamine, and 5 g/L AlbuMAX II. The culture was incubated at 37°C in a humidified atmosphere of 90% N_2, 5% CO_2 and 5% O_2. Thin blood smears stained with 10% Giemsa solution were made daily to monitor the parasitaemia under a light microscope at 100x magnification.

Synchronisation of *P. knowlesi*

When the parasites in culture were majority in late stages, it was synchronised using Histodenz (Sigma Aldrich) as described by Moon *et al*. (2013) to obtain schizont stage parasites. Briefly, parasites were resuspended in 2 ml medium to achieve 50% haematocrit, and the suspension was layered over 5 ml pre-warmed 55% Histodenz solution before low brake centrifugation (10 minutes, 2000 rpm). Brown interphase containing schizonts was washed with incomplete media and returned to culture. The culture was allowed to grow for another 1-2 hours, followed by another round of synchronisation. This round, fraction containing early ring forms of parasites was collected.

***In vitro* anti-plasmodial assay using crude extracts**

Crude extracts in all three solvents were evaluated for their *in vitro* anti-plasmodial activity by schizont maturation inhibition assay, according to WHO method (*in vitro* micro test (Mark III)) (WHO, 2001). The synchronised ring form parasites (100 µl/well) were plated into the 96-well plates pre-coated with serially diluted extracts (abovementioned), adjusted to 1% parasitaemia at 2% haematocrit and were incubated at 37°C with 90% N_2, 5% CO_2 and 5% O_2. Cultures were harvested and thick blood smears were made from all wells when majority of the parasites in the negative control well (culture with only 70% ethanol) have developed into schizonts (containing ≥ 3 nuclei). The thick blood smears were stained for 30 min in 1.5% Giemsa solution (pH 7.2). The number of normal schizonts (containing ≥ 3 nuclei) out of a total of 200 parasites was counted. The number of schizonts in each well that contained extracts was compared to the negative control well. Each extract and drug control were performed in triplicates each round and the growth inhibition percentage were then calculated in comparison with the negative control using the formula below. Biological replicates were made, where experiments were repeated three times with parasites derived from three different batches of cultures.

Growth inhibition rate (%)

$$= 100\% - \left[\frac{\text{No. of schizonts with} \geq 3 \text{ nuclei per 200 parasites in test well}}{\text{No. of schizonts with} \geq 3 \text{ nuclei per 200 parasites in negative control}} \text{ X } 100\%\right]$$

Calculation of IC_{50}

The IC_{50} (extract concentration at which parasite growth is inhibited by 50%) was determined from the dose-response curves using the GraphPad Prism™ version 9.0 programme. The anti-plasmodial activity of the plant extracts were then categorised as, IC_{50} < 0.1 µg/ml is considered to be very good, IC_{50} between 0.1 – 1.0 µg/ml is good, IC_{50} between 1.1 – 10.0 µg/ml is good to moderate, IC_{50} between 11.0 – 25.0 µg/ml is weak, IC_{50} between 26.0 – 50.0 µg/ml is very weak while IC_{50} > 50.0 µg/ml is considered inactive (Rosoanaivo *et al*., 2004).

Cytotoxicity assessment

Cytotoxicity of the plant extracts was assessed against Vero cell line (African green monkey kidney cells) cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 0.4% sodium bicarbonate (NaHCO3), 25 mM HEPES, 100U of PenStrep (100 U penicillin and 100 U streptomycin). The cytotoxicity was measured using 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. 96-well flat bottom plates were pre-coated with the serially diluted plant extracts (0.19 µg/ml to 100 µg/ml) as above mentioned. Cell suspensions (5 x 10^4 cells/well) were added into the pre-coated wells. Cell suspension without test substance and cell suspension with absolute methanol were used as negative control and positive control, respectively. The plates were incubated at 37°C in 5% CO_2 incubator for 72 hours. Each well was then added with 10 µl of MTT solutions (2 mg/ml) (in phosphate buffered saline), followed by four hours incubation at 37°C in an incubator with 5% CO_2. After removing the media, 100 µl of DMSO was added to dissolve the MTT formazan product. After the solution was shaked for 15 minutes on a rotator shaker, a microplate reader was used to measure the absorbance at 540 nm. Using the GraphPad Prism™ version 9.0 programme, the 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. All assays were performed in triplicates. Following the background subtraction, the percentage of cytotoxicity was computed and compared to the negative control using the following formula:

Cytotoxicity rate (%)

$$= 100\% - \left[\frac{\text{Absorbance of test well}}{\text{Absorbance of negative control well}} \text{ X } 100\%\right]$$

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 (García-Huertas *et al*., 2012).

Selectivity index

Selectivity index (SI) values were classified as follows: SI < 4 was considered not active, SI between 4–10 as somewhat active, and SI > 10 as active anti-malarial activity (Weniger *et al*., 2001). SI represents the ratio between cytotoxic and anti-plasmodial actions. SI value for each crude extract was determined using the following formula:

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

Data Analyses

Experiments were performed in triplicates and were repeated three times. The IC_{50} and CC_{50} were determined from dose-response curve using least-squares nonlinear regression in the GraphPad Prism™ version 9.0 programme. For multiple group comparisons of data, One-way ANOVA with Tukey's multiple comparison test (normally distributed) was performed; $p < 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSIONS

Extraction yields and TLC profiling

Three different solvents, DCM, MeOH and sterile H_2O were used to extract a wide range of the metabolites in *V. amygdalina* leaves. Each solvent has distinct properties that make it suitable for extracting different classes of compounds. For instance, DCM is ideal for non-polar compounds such as lipids and terpenoids; MeOH is a good solvent for a wide range of polar compounds such as alkaloids, flavonoids and phenolics; while water is a good solvent for water-soluble compounds like glycosides and certain phenolic acids. After sequential extraction of 1 kg of the coarse powder of *V. amygdalina* leaves using 100% DCM, MeOH, and sterile H_2O, the percentage yields of each extract were 2.00% w/w, 16.67% w/w, and 11.99% w/w, respectively. The solvent system for TLC profiling was done by using ethyl acetate and petroleum ether solvents at 1:1 (v/v) solvent ratio. The number of phytochemicals and their retention factor

(R_f) values of the extracts are presented in Table 1. The R_f values obtained from the different solvent extracts (DCM, MeOH, and H_2O) reveal a range of compound polarities present in the samples. In the DCM extract, higher R_f values (0.84, 0.80, and 0.74) suggest the presence of non-polar or weakly polar compounds, such as terpenoids or steroids, which tend to travel further on the TLC plate in non-polar solvent systems (Kumar *et al*., 2013). Conversely, the MeOH and H_2O extracts exhibited lower R_f values, especially in spots 1 to 4 (ranging from 0.17 to 0.38), indicating the presence of more polar phytochemicals, possibly flavonoids, phenolic compounds, or alkaloids (Kumar *et al*., 2013; Sureshkumar, 2021). The absence of higher R_f spots in the aqueous extract also supports this, as water tends to extract primarily polar constituents. This distribution of R_f values aligns with the general polarity trend of solvents used: DCM (non-polar) extracting non-polar compounds, MeOH (intermediate polarity) extracting both polar and non-polar compounds, and H_2O (polar) favouring polar phytochemicals (Kumar *et al*., 2013; Sureshkumar, 2021; Lee *et al*., 2024). These findings are consistent with those reported in prior studies, where similar R_f ranges were associated with specific phytochemical classes.

***In vitro* anti-plasmodial activity**

Extracts that exhibit an IC_{50} value less than 10.0 µg/ml are considered to have potential anti-plasmodial activity. The *in vitro* anti-plasmodial activities of leaf extracts from *V. amygdalina* against *P. knowlesi* are shown in Table 2. Of the three crude extracts tested, MeOH extract of *V. amygdalina* has the lowest IC_{50} (1.131 ± 0.16 µg/mL) against *P. knowlesi* A1H1 among the three extracts. Meanwhile, DCM extract and H_2O extract of *V. amygdalina* have IC_{50} values of 1.356 ± 0.23 µg/mL and 1.973 ± 0.30 µg/mL, respectively. Overall, all three crude extracts showed good to moderate anti-plasmodial activity against *P. knowlesi* A1H1, in which MeOH showed most promising anti-plasmodial activity followed by DCM extract and H_2O extract. The plant extracts were subjected to the schizont maturation inhibition assay in parallel with chloroquine. In this study, the IC_{50} of chloroquine is 28.631 ± 0.93 nM. Hence the findings of the study were considered valid, with the IC_{50} of chloroquine for *P. knowlesi* reported to be 11 – 100 nM from the previous studies (Ekland & Fidock, 2008; Fatih *et al*., 2013; Barber *et al*., 2017).

Microscopic examination of Giemsa-stained slides for DCM extract showed no developed schizonts with ≥ 3 nuclei from 25 µg/ml onwards (25, 50, 100 µg/ml). In contrast, the MeOH extract showed total absence at 100 µg/ml (Figure 1). The H_2O extract, however, did not exhibit total inhibition; even at 100 µg/ml, it still contained 10.01% schizonts with ≥ 3 nuclei. These findings suggest that the active constituents in the extracts may be cytotoxic to *P. knowlesi* A1H1 trophozoites, preventing their development into the schizont stage. While the anti-plasmodial activity of *V. amygdalina* has been reported in several studies, none focused on *P. knowlesi*. When compared to negative control, significant inhibition of schizont maturation was observed with *V. amygdalina* DCM crude extract for all concentrations, with $p = 0.0002$ for 0.19 µg/ml, and $p < 0.0001$ from 0.39 µg/ml to 100 µg/ml. Similar findings were found for MeOH extract, with $p < 0.0001$ for all concentrations (0.19 µg/ml to 100 µg/ml). For H_2O extract, significant growth inhibition was found from 0.78 µg/ml to 100 µg/ml ($p < 0.0001$), with no significant inhibition was observed at 0.19 µg/ml ($p = 0.0847$) and 0.39 µg/ml ($p = 0.0769$) (Figure 2). We then compared parasite maturation inhibition across DCM, MeOH, and H_2O extracts at each concentration. No significant difference was observed between DCM and MeOH in most of the concentrations (except 25 µg/ml), whereas these two compounds were found to have significant difference in inhibition rate compared to H_2O extract in most of the concentrations (Figure 3).

Table 1. Retention factor (R_f) values and number of spots at wavelength UV254 nm of *V. amygdalina* crude extracts

No. of spot	DCM extract	MeOH extract	H_2O extract
1	0.46	0.17	0.17
2	0.54	0.24	0.21
3	0.60	0.27	0.30
4	0.68	0.35	0.38
5	0.74	0.56	0.54
6	0.80	0.66	–
7	0.84	0.71	–

Table 2. *In vitro* anti-plasmodial activity of extracts and standard drugs against *P. knowlesi*

Concentration of Extracts (µg/ml)	Crude Extracts		
	DCM (% inhibition)	MeOH (% inhibition)	H_2O (% inhibition)
100	100 ± 0	100 ± 0	89.99 ± 1.86
50	100 ± 0	95.58 ± 3.03	83.83 ± 2.65
25	100 ± 0	89.15 ± 0.45	78.56 ± 2.62
12.5	77.82 ± 4.63	84.00 ± 2.09	71.27 ± 6.82
6.25	72.78 ± 3.93	77.11 ± 3.81	64.19 ± 5.97
3.12	67.40 ± 5.91	67.78 ± 1.65	55.80 ± 1.82
1.56	59.38 ± 5.93	57.00 ± 2.14	41.66 ± 1.90
0.78	36.98 ± 11.38	41.53 ± 6.83	24.28 ± 6.12
0.39	33.14 ± 8.73	35.03 ± 7.34	9.71 ± 2.19
0.19	17.10 ± 1.26	26.09 ± 8.91	9.55 ± 1.74
IC_{50} (µg/ml)	1.356 ± 0.23	1.131 ± 0.16	1.973 ± 0.30

Data are presented as the mean ± standard deviation (SD) of three independent experiments performed in triplicate assays.

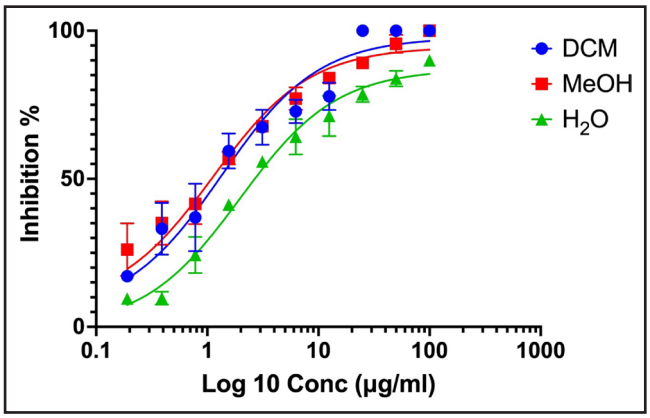

Figure 1. The parasite growth inhibition rate versus the concentrations of the *V. amygdalina* extracts in all three solvents. DCM and MeOH extracts exhibited 100% growth inhibition at 25 µg/ml and 100 µg/ml, respectively. H_2O extract exhibited 89.99% growth inhibition at the highest concentration (100 µg/ml). DCM, dichloromethane, MeOH, methanol, H_2O, water extract.

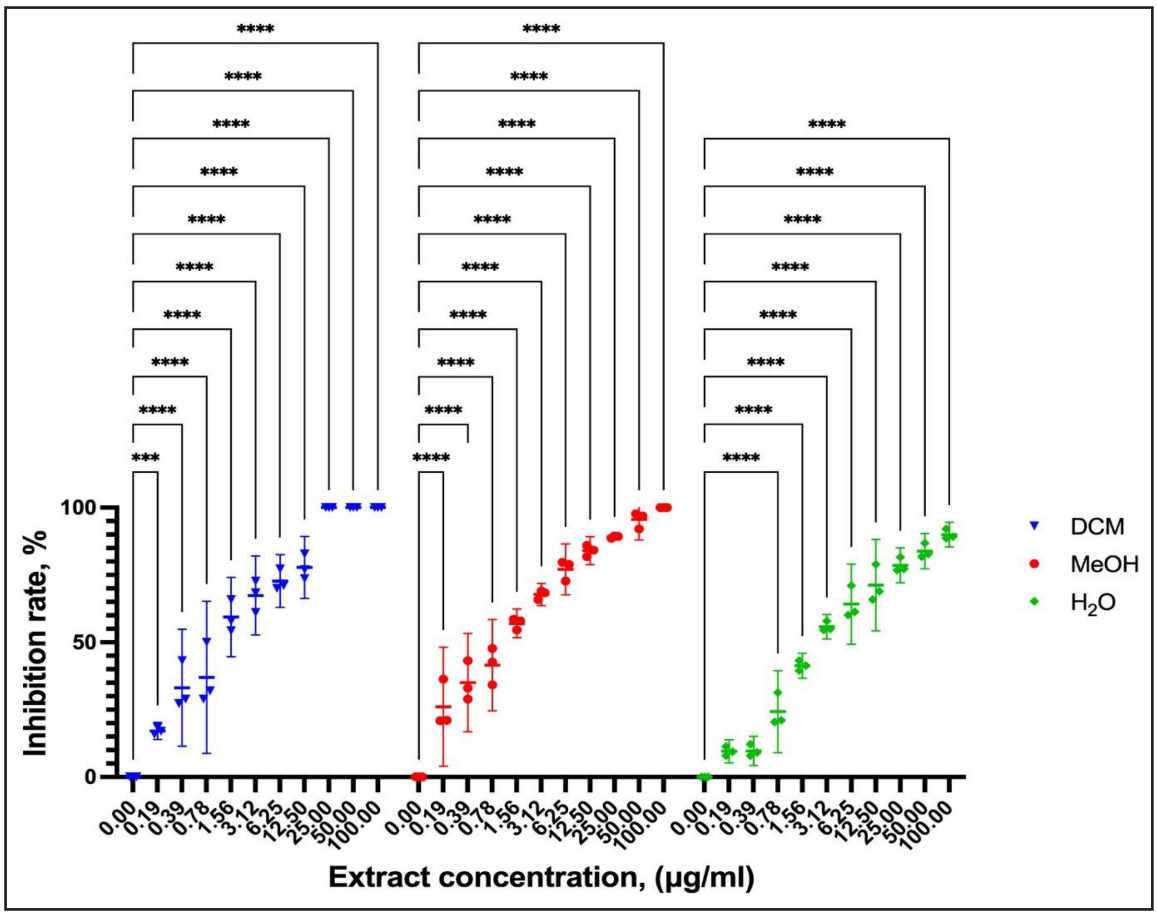

Figure 2. Comparison of schizont maturation inhibition rate at each concentration of *V. amygdalina* crude extracts in all three solvents to negative control. Significant inhibition of schizont maturation was observed in DCM and MeOH crude extracts for all concentrations (0.19 to 100 µg/ml), while significant growth inhibition was found from 0.78 µg/ml to 100 µg/ml for H_2O extract. The assays were performed in three independent experiments; error bars represent means with 95% CI; DCM, dichloromethane, MeOH, methanol, H_2O, water extract, *** $p < 0.001$, **** $p < 0.0001$.

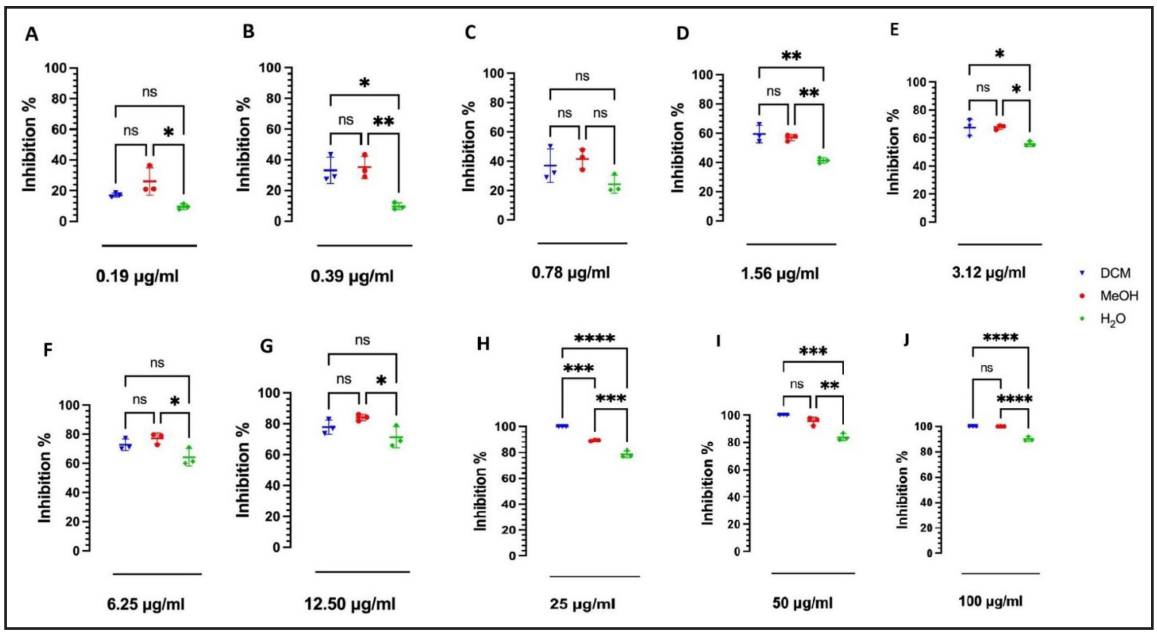

Figure 3. Comparison of schizont maturation inhibition rate of *V. amygdalina* crude extracts in all three solvents at different concentrations. No significant difference was observed between DCM and MeOH in most of the concentrations (except 25 µg/ml), while H_2O extract was found to have significant differences in inhibition rate compared these two compounds (DCM and MeOH) in most of the concentrations. (A) 0.19 µg/ml, (B) 0.39 µg/ml, (C) 0.78 µg/ml, (D) 1.56 µg/ml, (E) 3.12 µg/ml, (F) 6.25 µg/ml, (G) 12.50 µg/ml, (H) 25 µg/ml, (I) 50 µg/ml, and (J) 100 µg/ml. DCM; dichloromethane, MeOH; methanol, H_2O; water extract, ns; non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

In one local study by Mohd Abd Razak *et al*. (2014) found that *V. amygdalina* leaf extracts exhibit promising moderate anti-plasmodial activity (IC_{50} < 10 µg/ml) in *in vitro P. falciparum* K1 culture (IC_{50} of artemisinin 0.51 ng/ml) with no toxic effect to bovine kidney cell line (SI ≥ 10), which is in agreement with our results where the methanolic extract showed moderate activity with IC_{50} = 1.131 µg/ml. Moreover, the study by Mohd Abd Razak *et al*. (2014) also specifically showed that the methanol extract of the plant had higher anti-plasmodial activity with EC50 (50% Effective Concentration) = 1.09 µg/ml, as compared to the aqueous extract with EC_{50} = 8.23 µg/ml, which were in line with our findings. Other studies also showed that extracting plants using a semi-polar solvent similar to methanol such as ethyl alcohol possessed a higher anti-plasmodial effect as compared to water (Omeregie *et al*., 2011; Zemicheal & Mekonnen, 2018). Other studies also revealed that *V. amygdalina* leaf extracts in ethanol, petroleum ether, methylene chloride, and methanol were found to elicit IC_{50} values of less than 10 µg/ml against *P. falciparum* strains *in vitro* (Madureira, 2002; Tona *et al*., 2004; Zofou *et al*., 2011). According to previous research, the plant's ability to inhibit malaria may stem from the existence of its active ingredients from the terpernoids and sesquiterpene lactones class which includes hydroxyvernodalin, vernolepin, vernolin, and vernolide (Phillipson *et al*., 1993; Tona *et al*., 2004; Ugbogu *et al*., 2021; Edo *et al*., 2023). As most terpenoids and sesquiterpene lactones have poor solubility in water, this could possibly be the reason of lower anti-plasmodial activity in the aqueous extract compared to methanol extract. Nonetheless, there is also study reported that the aqueous extract of *V. amygdalina* leaves possessed anti-plasmodial activities on *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae* (Odeh & Usman, 2017). Water soluble compounds including tannins and saponins were detected in *V. amydalina* (Degu *et al.,* 2024), and small amount of sesquiterpene lactones could also be extracted using aqueous solvent, which possibly contributing to the anti-plasmodial activities in the aqueous extract.

Cytotoxicity activity to mammalian cell line

The crude plant extracts from *V. amygdalina* leaves were subjected to MTT cytotoxicity assay and tested against Vero cell lines. The MTT test was based on the reduction of the tetrazolium salt by mitochondrial enzymes, which is possible only in live cells. The CC_{50} of the extracts on Vero cells were determined and the results are listed in Table 3. In this study, the cytotoxicity value for DCM, MeOH and H_2O extracts were 48.61 ± 0.64 µg/ml, 63.73 ± 0.78 µg/ml and 86.91 ± 1.03 µg/ml respectively. The DCM extract of *V. amygdalina* was slightly toxic against Vero cell lines with CC_{50} below 50 µg/mL. In contrast, the MeOH and H_2O extracts were potentially non-toxic to the mammalian cells as the CC_{50} value was above 50 µg/mL, with MeOH extract demonstrating a relatively higher anti-plasmodial effect as compared to H_2O extract. The cytotoxicity trends observed in this study align with those of a local study by Mohd Abd Razak *et al*. (2014), which tested crude extracts of *V. amygdalina* leaves against Madin-Darby Bovine Kidney (MDBK) cell lines. In that study, the DCM extract exhibited the highest EC_{50} at 157.69 µg/ml, followed by the MeOH extract with an EC_{50} of >300 µg/ml and the H_2O extract with an $EC_{50}$of > 600 µg/ml (Mohd Abd Razak *et al*., 2014). Other research involving *V. amygdalina* extracts against Vero cells found that the ethanolic extract had an IC_{50} of 60.33 µg/ml (Omoregie *et al*., 2011), similar to the MeOH data from this study due to comparable solvent polarity. Conversely, the aqueous extract demonstrated low toxicity to Vero cells, with an IC_{50} of 414 µg/ml (Omoregie *et al*., 2011). Additionally, Samutrtai *et al*. (2024) reported IC_{50} values for the ethyl acetate extract of *V. amygdalina* leaves of 4.043 µg/ml in Vero cells and 0.0767 µg/ml in human HeLa cells. Current cytotoxic data indicate that the crude extracts of *V. amygdalina* leaves vary depending on the extraction solvent used and the specific cell lines tested.

Selectivity index

To assess the safety of a compound, the value of the SI was calculated as the fractional ratio between the CC_{50} for Vero cells and IC_{50} of *P. knowlesi*. In the present study, all crude extracts exhibited SI values above 10, with SI values of 35.84, 56.34 and 44.04 for DCM, MeOH, and H_2O extracts respectively (Table 3). A hit compound must exhibit a SI value of at least 10 (a ten-fold higher activity against parasites than against a mammalian cell line) in accordance with the consensus on anti-plasmodial drugs development in order to be taken into consideration for further development (Pink *et al*., 2005). MeOH extract has the highest selectivity followed by H_2O and DCM extract. MeOH extract was shown to be non-toxic to mammalian cell line and exhibits the highest anti-plasmodial activities among the three compounds. Hence, MeOH extract was proposed to be chosen for the future studies in purifying and identifying the pure compounds and active metabolites.

Table 3. The anti-plasmodial and cytotoxicity activities of DCM, MeOH and H_2O extracts of *V. amygdalina* leaves

Crude Extracts	IC_{50} (µg/ml) *P. knowlesi* A1H1	CC_{50} (µg/ml) Vero cells	Selectivity Index (SI)
DCM	1.356 ± 0.23	48.61 ± 0.64	35.84
MeOH	1.131 ± 0.16	63.73 ± 0.78	56.34
H_2O	1.973 ± 0.30	86.91 ± 1.03	44.04

DCM, dichloromethane, MeOH, methanol, H_2O, water extracts. Data are presented as the mean ± standard deviation (SD) of three independent experiments performed in triplicate assays.

CONCLUSION

Overall, the findings from this present study in evaluating the *in vitro* anti-*Plasmodium* activity of crude extracts from *V. amygdalina* against *P. knowlesi* observed a similar trend with previous studies using *P. falciparum*, strongly supporting its usage as anti-plasmodial across the human *Plasmodium* spp. This study is the first to investigate the anti-plasmodial activity of crude extracts from *V. amygdalina* plant obtained from a local source in Malaysia against *P. knowlesi in vitro* culture. The MeOH extract showed promising anti-plasmodial activity with non-toxic properties against mammalian cells, supported by the highest selectivity value, followed by H_2O extract and DCM extract. The results confirmed that this plant possesses *in vitro* anti-plasmodial potential and justified its use as ethnomedicine in the native countries. However, *in vivo* or *ex vivo* studies on this medicinal plant are necessary and should seek to determine the toxicity of the active constituents, and its pharmacokinetic properties. In addition, future work is desirable for extracts that exhibit anti-plasmodial properties to undergo phytochemical analysis to isolate and identify the pure compounds, and the anti-plasmodial activities of each pure compounds against *P. knowlesi* should also be evaluated.

Conflict of Interests

The authors declare that they have no conflict of interest.

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