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Antimalarial properties of *Artemisia vulgaris* L. ethanolic leaf extract in a *Plasmodium berghei* murine malaria model

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ABSTRACT

Background & objectives: Artemisinin isolated from *Artemisia annua* is the most potent antimalarial drug against chloroquine-resistant *Plasmodium falciparum* malaria. *Artemisia vulgaris,* an invasive weed, is the only *Artemisia* species available in Sri Lanka. A pilot study was undertaken to investigate the antiparasitic activity of an *A. vulgaris* ethanolic leaf extract (AVELE) in a *P. berghei* ANKA murine malaria model that elicits pathogenesis similar to falciparum malaria.

Methods: A 4-day suppressive and the curative assays determined the antiparasitic activity of AVELE using four doses (250, 500, 750 and 1000 mg/kg), Coartem® as the positive control and 5% ethanol as the negative control in male ICR mice infected with *P. berghei*.

Results: The 500, 750 and 1000 mg/kg doses of AVELE significantly ($p \leq 0.01$) inhibited parasitaemia by 79.3, 79.6 and 87.3% respectively, in the 4-day suppressive assay, but not in the curative assay. Chronic administration of the high dose of AVELE ruled out overt signs of toxicity and stress as well as hepatotoxicity, renotoxicity and haematotoxicity.

Interpretation & conclusion: The oral administration of a crude ethonolic leaf extract of *A. vulgaris* is non-toxic and possesses potent antimalarial properties in terms of antiparasitic activity.

Key words Antiplasmodial activity; *Artemisia vulgaris; Plasmodium berghei* ANKA; Sri Lanka; toxicity studies

INTRODUCTION

Resistance of *Plasmodium falciparum* to previous generations of therapeutics, such as chloroquine (CQ) and sulfadoxine-pyrimethamine (SP), became widespread in the 1970s and 1980s, undermining the malaria control efforts and reversing gains in child survival[1]. Artemisinin, a sesquiterpene lactone compound isolated from the plant *Artemisia annua*, is the most effective plant-based antimalarial used in the treatment of severe and multi-drug resistant malaria in the world at present. Artemisinin and its derivatives are considered keystone therapeutics for both rapid parasite clearance and quick fever resolution, in comparison to conventional antimalarials such as quinine[2]. The emergence of parasites resistant to artemisinin at the Thai-Cambodia border could seriously undermine the success of global malaria control efforts[3]. Thus, there is a need for improved use of existing drugs, as well as exploration of new antimalarial compounds where natural products could play a pivotal role in this persistent challenge. Accordingly, new artemisinin drugs and their derivatives warrant examination for potential antimalarial properties.

Plant species of the genus *Artemisia* (Family: Asteraceae) possess a plethora of bioactivities such as antihelminthic, antiseptic and is even widely used for its antiinflammatory properties[4–5]. Hitherto, rare attempts were reported on elucidating antimalarial properties of *A. vulgaris*, an invasive weed, growing on nitrogenous soils, found in waste dumps and on roadsides. It is the only reported *Artemisia* species in Sri Lanka. A previous preliminary study by our group substantiated moderate antiparasitic activity of *A. vulgaris* leaf extract when administered orally, against *Plasmodium yoelii* rodent malaria parasites[6].

Thus, we undertook a pilot study to investigate antimalarial properties of an *A. vulgaris* ethanolic leaf extract (AVELE) in the *P. berghei* ANKA (lethal strain) murine malaria model that elicits pathogenesis similar to falciparum malaria[7]. *In vivo* antiparasitic activity and toxicity of AVELE were investigated using standard methodologies.

MATERIAL & METHODS

Ethical clearance

Ethical clearance for this research study was obtained from the Ethics Review Committee of the Faculty of Medicine, University of Colombo, Sri Lanka (Ref. No. EC-10-132).

Collection and authentication of plant material

Leaves of *A. vulgaris* (English – Common wormwood or mugwort; Sinhala – *Walkolondu;* Tamil – *Mâcipattiri*) were collected from the road side in Nuwara Eliya, Sri Lanka (altitude: 1868 m, 6.9667° N, 80.7667° E). The specimen was authenticated by the Herbal Technology Division, Industrial Technology Institute, Sri Lanka, and a voucher specimen was deposited in the museum of the Department of Zoology, Faculty of Science, University of Colombo, Sri Lanka (GY 01/2010).

Preparation of the plant extract

Leaves of *A. vulgaris* were washed with tap water; air dried under the shade for 21 days and powdered using a mechanical grinder. Ground leaves (weight: 575 g, moisture: 13.15%) were soaked in 2.7 L of an organic solvent mixture consisting of diethyl ether, methanol and petroleum ether at a 1:1:1 ratio (i.e. in 900 ml of each component) at room temperature (~28° C) for 48 h. The resulting dark green solution was filtered using a Buckner funnel under suction filtration. The leaves were subsequently soaked in the organic solvent mixture for a second time for 48 h. The extracted solution was then evaporated at reduced pressure using a rotavapour to obtain the crude extract in the form of a paste[8]. This crude extract was suspended in 5% ethanol to obtain the required dosages. Since specific human dosages were not available for this plant extract (as this plant is not used for antimalarial treatment), dosages were determined using the human dose for the artemisinin-based combination therapy (ACT) drug, Coartem®. Taking the metabolic rate of mice into consideration, the test animals were orally treated with the *A. vulgaris* ethanolic leaf extract (AVELE) at doses of 250, 500, 750 and 1000 mg/kg of body weight, that represented low, human equivalent, moderately high and high doses, respectively.

Experimental animals

Healthy adult male ICR (Institute of Cancer Research) mice weighing 25–30 g, purchased from the Medical Research Institute, Colombo, were used in this study. All animals were housed in plastic cages in the animal house, Department of Zoology, University of Colombo under standard conditions (temperature 28–31°C, photoperiod: approximately 12 h natural light per day and relative humidity: 50–55%). The animals were fed with food pellets (Master Feed Ltd., Colombo, Sri Lanka) and drinking water *ad libitum*. Except at the time of experimental procedure, the animals were handled only during cage cleaning.

Phytochemical analysis of AVELE

The AVELE was subjected to phytochemical screening by using qualitative chemical tests performed according to the standard protocols[9].

TLC analysis of the plant extract

AVELE (3 mg) was dissolved in 1.5 ml of 1:1:1 ratio of methanol : diethyl ether : petroleum ether mixture concentrated to 0.5 ml and spotted on a TLC plate. The mobile phase solvent system used was methanol: ethyl acetate : dichloro methane : hexane in a 0.25 : 1.25 : 1.5 : 2 ratio, respectively. The retention factor (R_f) values were determined by visualizing bands at 254 and 366 nm by using UV light. Furthermore, vanillin sulphate was sprayed on to the TLC plate and heated up to 110°C for 5 min for color development[10].

Parasite isolates

Plasmodium berghei ANKA parasite line maintained through serial passage of blood in mice was used to assess *in vivo* antimalarial activity of AVELE. This parasite strain was kindly provided by the Queensland Institute of Medical Research, Australia.

Parasitic inoculation

Desired amount of blood diluted in RPMI 1640 medium to obtain 10^7 parasitized red blood cells in 0.5 ml of suspension was injected intraperitoneally (IP) into adult male ICR mice using a 26 gauge needle.

Antiparasitic activity of the test plant extract

The 4-day suppressive assay: In vivo antiparasitic activity of four doses of AVELE (250, 500, 750, 1000 mg/kg body weight) was assessed using the 4-day suppressive assay that evaluates antiparasitic activity on an early infection[11]. Coartem® (artemisinin-based combination therapy drug, Novartis Pharmaceuticals) was used as the positive control at 450 mg/kg body weight while 5% ethanol served as the negative control. Six male ICR mice were randomly allocated for each dose.

The mice were inoculated IP with 10^7 infected RBC (diluted with RPMI) on Day 0. Administration of the extracts, positive and negative control took place daily from D0 through D3. On Days 4, 5 and 6, thin blood smears were prepared from tail bleed of mice, stained with Giemsa and the degree of parasitaemia was calculated. Percentage inhibition of parasitaemia was also calculated using the following formula:

$$\% \text{ Inhibition} = \frac{\text{Parasitaemia of negative control} - \text{Parasitaemia with AVELE}}{\text{Parasitaemia of negative control}} \times 100$$

Mice used for the 4-day suppressive assay were observed daily for 8 consecutive days (D0–D8) and their parasitaemia was monitored. The animals were treated orally with Coartem® when the parasitaemia reached 50%.

Curative assay

The curative assay evaluates the antiparasitic activity on an established infection[12]. Three groups (n=6) of male ICR mice were injected IP with 10^7 infected RBC on D0. Seventy-two hours following inoculation (on D3), blood smears were prepared by tail bleed after which the test group was treated with AVELE (1000 mg/kg body weight dose). Coartem® at 450 mg/kg body weight was used as the positive control, while the third group was treated with 5% ethanol as the negative control. Preparation of blood smears and treatment with the plant extract and the control were carried out for two more days, on Days 4 and 5, to evaluate the curative action of the plant extract. Parasitaemia levels were determined for each group.

Evaluation of chronic toxicity

Two groups of adult ICR male mice (n=6/group) were used. One group was treated with AVELE at 1000 mg/kg body weight while the second group received 5% ethanol as the control, daily (0900–1000 hrs) for 30 consecutive days. Mice were closely observed on each day of treatment from Day 1 post-treatment for overt signs of toxicity (salivation, diarrhoea, yellowing of fur, loss of fur, postural abnormalities and behavioral change), stress (fur erection and exophthalmos), and aversive behavior (biting and scratching behavior, licking of tail, paw and penis, intense grooming behavior, and vocalization)[6]. Each animal, after fasting for about 12 h, was weighed at weekly intervals using an electronic balance (MP 6000, Chyo Balance Corporation, Tokyo, Japan).

The food intake of the animals was assessed fortnightly by placing individual mice with 50 g of food pellets for 24 h. The food intake was calculated by deducting the weight of the left over pellets by the total given and expressed as mg/100 g body weight. The water intake of animals was also assessed fortnightly by placing individual mice with 100 ml of water for 24 h. The water intake was calculated by deducing the remaining volume of water in the bottle by the total given, and expressed as ml/100 g body weight.

Evaluation of the effect of AVELE on mouse hematological and serum parameters was determined on Day 31 post-treatment. Tail bleed (0.3–0.5 ml) was collected into vials containing heparin under mild ether anesthesia using aseptic precautions. The white blood cell (WBC), red blood cell (RBC) and WBC differential (DC) counts of fresh blood was determined[13]. Serum parameters (AST, ALT, urea and creatinine levels) were determined by using Randox kits (Randox Laboratories Ltd., Co., Antrium, U.K.) using a spectrophotometer (Jasco V560, Jasco Corporation, Tokyo, Japan)[14]. On Day 31 post-treatment, mice used for this toxicity study were sacrificed, and their vital organs (liver, spleen, lungs, kidneys and heart) were immediately excised, blotted dry and weight recorded.

Statistical analysis

Data, expressed as mean ± SEM, were analysed using the Minitab 15 statistical package for Windows. Statistical analysis was performed by using the Mann-Whitney U-test. Linear regression analysis was performed to assess dose-dependencies. IC_{50} values were also calculated[15]. Significance was set at $p \leq 0.05$.

RESULTS

Phytochemical analysis

Preliminary phytochemical analysis revealed that the AVELE contained alkaloids and coumarins, while thin layer chromatography (TLC) evidenced a group that contained higher alcohols, phenols, steroids and essential oils.

Antimalarial activity of AVELE in P. berghei murine model

Effect of AVELE on early infection: Parasitaemia measurements obtained from the 4-day suppressive antimalarial assay and percent inhibition of parasitaemia by AVELE are summarized in Table 1 and Fig. 1. Accordingly, 500, 750 and 1000 mg/kg doses of AVELE significantly ($p \leq 0.01$) inhibited parasitaemia on average by 79.3, 79.6 and 87.3%, respectively. Figure 2 illustrates the dose response of AVELE on D5. The IC_{50} value was calculated to be 412.1 mg/kg.

Effect of AVELE on established infection: The curative assay resulted in no significant difference (p >0.05) of

Table 1. Antiparasitic activity of the *A. vulgaris* ethanolic leaf extract (AVELE) on an early infection in the 4-day suppressive assay

Treatment	Percent parasitaemia			Percent inhibition of parasitaemia		
	Day 4	Day 5	Day 6	Day 4	Day 5	Day 6
Control (5% EtOH)	10.47 ± 1.74	26 ± 1.43	36.79 ± 2.77			
250 mg/kg	9.28 ± 1.81	23.84 ± 2.06	35.69 ± 2.91	11.3	8.3	3
500 mg/kg	2.293 ± 0.863*	3.69 ± 1.27*	9.58 ± 2.11*	78.1	85.8	74
750 mg/kg	2.663 ± 0.175*	2.88 ± 0.16*	8.97 ± 0.11*	74.5	88.9	75.6
1000 mg/kg	0.925 ± 0.0839*	1.708 ± 0.231*	8.47 ± 1.03*	90	93.1	78.8
Coartem® (450 mg/kg)	0*	0*	0*	100	100	100

Values are expressed as mean ± SEM (n = 6); **p* <0.01 as compared with the control (Mann-Whitney U-test).

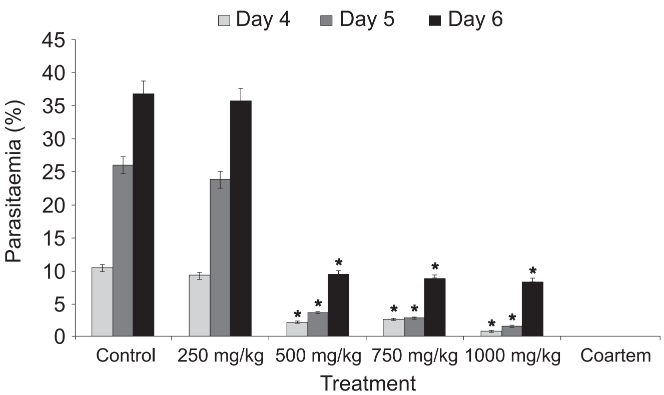

Fig. 1: Antiparasitic effect of *A. vulgaris* ethanolic leaf extract on *P. berghei* in the 4-day suppressive assay [**p* <0.01 as compared with the control (Mann-Whitney U-test)].

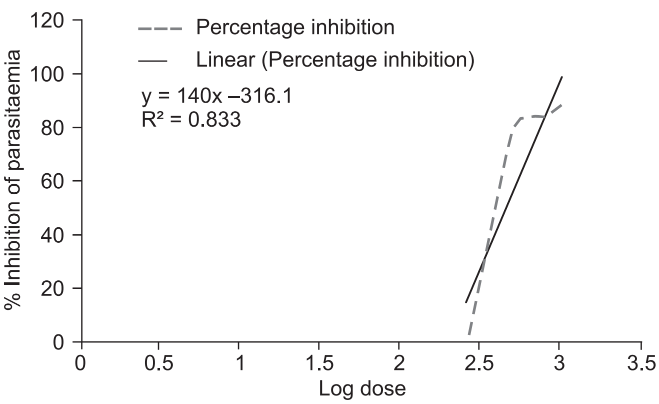

Fig. 2: Dose response curve of parasitaemia inhibition on Day 5.

parasitaemia between the 1000 mg/kg high dose of AVELE treated and the control (5% ethanol) groups. Conversely, the positive control (Coartem®) group showed a marked significant decrease of parasitaemia compared with the control (Table 2).

Table 2. Antiparasitic activity of the AVELE on an established infection in the curative assay

Treatment	Percent parasitaemia		
	Day 3	Day 4	Day 5
Control (5% EtOH)	1.56 ± 1.20	11.24 ± 3.80	25.24 ± 3.64
1000 mg/kg	1.27 ± 0.23	9.31 ± 2.23	21.87 ± 2.44
Coartem® (450 mg/kg)	2.40 ± 1.44	1.99 ± 0.32*	0.32 ± 0.08*

Values are expressed as mean ± SEM (n = 6); **p* <0.01 as compared with the control (Mann-Whitney U-test).

Evaluation of chronic toxicity

The AVELE was well-tolerated by mice over a period of 30 days. Chronic administration of 1000 mg/kg dose, showing no overt signs of toxicity or stress. Hepatotoxicity (evaluated in terms of serum AST and ALT), renotoxicity (in terms of serum urea and creatinine) and hematotoxicity (in terms of RBC, WBC total and DC counts) were also ruled out (Table 3). In addition, the body weights, organ weights, food and water intake of the test group did not significantly differ from those of the control group (*p* >0.05).

Table 3. Effect of oral administration of AVELE on hepatological, renological and hematological parameters in mice

Parameter	Control (5% EtOH)	1000 mg/kg extract
Serum parameters		
AST (U/L)	39.067 ± 0.418	37.534 ± 0.594
ALT (U/L)	12.540 ± 0.283	12.978 ± 0.232
Urea (mg/dl)	44.55 ± 7.11	41.87 ± 3.98
Creatinine (mg/dl)	2.44 ± 0.28	1.79 ± 0.18
Hematological parameters		
RBC count cells/mm^3	$6.23\times10^6 \pm 5.43\times10^5$	$6.11\times10^6 \pm 5.26\times10^5$
WBC count cells/mm^3	1649 ± 45.8	1608 ± 73.2
Neutrophils (%)	35.01 ± 0.81	32.45 ± 0.68
Eosinophils (%)	3.60 ± 0.81	1.40 ± 0.58
Basophils (%)	4.10 ± 0.71	4.20 ± 0.37
Monocytes (%)	6.40 ± 0.81	7.99 ± 0.95
Lymphocytes (%)	59.00 ± 1.81	55.60 ± 2.47

Values are expressed as Mean ± SEM (n=6); No significant deviations (Mann-Whitney U-test; *p* >0.05) were observed between the test and the control groups with respect to the serum and hematological parameters tested.

DISCUSSION

The relentless pursuit of novel antimalarials is a worthy exercise. Currently, first line chemotherapeutics against malaria are fixed dose ACT, which are presumed to act against the blood stages of all human malaria parasite[16]. Several previous studies had identified potential antimalarial phyto candidates. Oral administration of *Tinospara crispa* and *Zanthoxylum rhoiflium* aqueous extracts have demonstrated 54 and 78% parasitaemia suppression, against *P. yoelii*, respectively[17]. Oral administration of *Barringtonia acutangula* aqueous root extract, a local medicinal plant used by traditional practitioners against malaria in Sri Lanka, demonstrated a 60% suppression of parasitaemia in a *P. yoelii* murine model[14].

Artemisia vulgaris is not commonly used to treat malaria but is reported to possess many other ethnopharmaceutical properties[4–5]. Nevertheless, encouraged by a pilot study where we reported that the only *Artemisia* species found in Sri Lanka, *A. vulgaris*, possesses moderate antiplasmodial activity in a *P. yoelii* rodent model[6], a comprehensive study was undertaken to further evaluate, antiplasmodial properties of this abundant weed in a *P. berghei* ANKA mouse model. The lethal strain of *P. berghei* ANKA, is a reliable, widely accepted and scientifically validated alternative *in vivo* rodent model to human malaria that causes severe malaria in mice. The experimental infection elicits structural, physiological and life cycle analogies with the human disease caused by *P. falciparum*[7] including cerebral malaria[18]. Therefore, any potential antimalarial activity of the AVELE manifested in the *P. berghei in vivo* model may be presumed as potential activity against falciparum malaria, though such interpretation should be made with caution.

Though, the present study resulted in high antiparasitic activity with *A. vulgaris*, in our previous preliminary study, the 4-day suppressive assay demonstrated only moderate antiparasitic activity of an aqueous leaf extract of *A. vulgaris* when administered orally, against *P. yoelii* rodent malaria parasites[6]. It was assumed that the hydrophobic nature of the paste may have accounted for this observation. However, in the present study, 5% ethanol as the solvent provided a homogenous suspension which was used for the *in vivo* assays, and we assumed that lead to most of the oil components of the leaf to be extracted.

The high antiparasitic activity observed in the AVELE (87.3%) evidenced in the current study, shows much promise as an antimalarial compared with the antiparasitic activity observed in several other tested extracts and some time tested traditional herbal preparations used as antimalarials[14, 17, 19]. Few recent studies demonstrated several clinical effects of the use of an *Artemisia* tea against malaria. While artemisinin is not readily soluble in water, it appears to be sufficiently so at high temperatures to provide antimalarial effects[20].

The antiparasitic activity of AVELE was dose dependent. This can be attributed to increased exposure of the receptor that may result in amplification of the receptor function[21]. Though chloroquine was used as the standard positive control in similar studies[22], we used Coartem® as the positive control since this study evaluated antimalarial activity of a plant species presumably containing artemisinin or a related compound. The mice were treated with 450 mg/kg, about 15 times the human dose, which is within the acceptable range for mice due to their high metabolic rate[6]. The results obtained also underlined the potency of Coartem® as the positive control drug which achieved 100% parasitaemia suppression throughout the study. Coartem® is a fixed-dose ACT drug and each tablet contains 20 mg artemether and 120 mg lumefantrine. Artemether has a rapid onset of action and is rapidly eliminated, whereas lumefantrine is eliminated more slowly[23]. Consequently, the combination rapidly clears parasites. Further, this combination of two drugs with independent modes of action confers some mutual protection. However, the synthetic drugs used in ACTs usually have much longer half lives than the artemisinins, but these are still vulnerable to resistance[24]. A North American team has suggested the use of *Artemisia* plant materials more directly in compacted form and in combination with an ACT partner; in this way other *in plant* constituents may enhance the overall activity of the drug[25].

On the contrary, the curative assay carried out in the current study for the 1000 mg/kg did not exhibit effective results. The parasitaemia increased steadily, in contrast to the group treated with the positive control (Coartem®) whereas the parasitaemia reduced continually until it attained zero percent after eight days. It may be presumed that the concentration of the active compound in the crude extract may not have sufficed to eliminate an established infection. Artemether, one of the two antimalarials contained in Coartem®, is a synthetic derivative of artemisinin that is even more potent than artemisinin. Such potency against a lethal strain of a rodent malaria parasite cannot be expected from a crude plant extract. Yet the results with the prophylactic treatment of AVELE were very encouraging for a crude plant extract and highlight the need for future *in vitro* and *in vivo* investigations using the isolated active component of *A. vulgaris* to substantiate its potential as a therapeutic agent against malaria and also to elucidate its mode of action.

Several studies have attempted to elucidate the mechanism of action of artemisinin. When the malaria parasite infects an erythrocyte, it consumes hemoglobin and liberates heme, an iron-porphyrin complex. The iron reduces the peroxide bond in artemisinin generating reactive oxygen radicals that damage the infected blood cell which will be disposed of by the host's immune system[26]. Several observations suggest that artemisinins inhibit the *P. falciparum* sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA), encoded by the gene denoted PfATP6 which is crucial for the completion of the asexual reproductive cycle of the parasite[27]. Whether, a similar mode of action is operative for the reduction of parasitaemia with *A. vulgaris* leaf extract remains to be investigated.

Whether it is for chemotherapy or chemoprophylaxis, toxicity of drugs prescribed for malaria is a closely monitored factor. Toxicity has been singled out as the main drawback of traditional herbal antimalarial preparations[6]. Thirty days chronic oral administration of the 1000 mg/kg dose, showed no overt signs of toxicity or stress. Weights and gross morphology of vital organs (liver, spleen, kidneys, heart and lungs) were also not affected. Also, continuous weight gain of animals was observed. Thus, the *A. vulgaris* extract was well-tolerated by mice.

The discovery of artemisinin from a plant source has ensured that the interest in developing plant based traditional antimalarials has not waned over the years. Furthermore, there is concern that artesunate resistance is developing in Southeast Asia[28]. Various independent research attempts corroborate the emergence of artemisinin resistance. Reports of cure rates below 75% after artemether-lumefantrine treatment in Cambodia[29] and production of artemisinin-resistant strains *in vivo* in the rodent malaria models[30–31] also reinforce this fact. Thus, the need for continuous search for phytotherapy for malaria seems obvious.

Leaves of *A. vulgaris* in Pakistan were found to contain artemisinin in the range of 0.05 to 0.15%[32]. The phytochemical analysis of *A. vulgaris* reported from Philippines[4] established that this plant contains two sesquiterpene lactones, yomogin and 1,2,3,4-diepoxy-11(13)-eudesmen-12,8-olide, and a novel aromatic compound. The discovery of a new antimalarial agent, a sesquiterpene lactone compound termed as 'tehranolide' is reported from the plant species *A. diffusa*[8]. Phytochemical and TLC analysis of *A. vulgaris* leaf extract in the present study also suggested the presence of alkaloids, coumarines, and a group that contains higher alcohols, phenols, steroids and essential oils. Furthermore, antiplasmodial activity has also been attributed to coumarin in a previous study[33]. As such, the plausibility of extracting a compound other than artemisinin that demonstrates antimalarial activity from other *Artemisia* plant species cannot be ruled out.

In conclusion, this study for the first time demonstrated that the oral administration of a crude leaf extract of *A. vulgaris*, possess potent and safe antimalarial effects, in terms of antiparasitic activity in a *P. berghei* ANKA lethal murine malaria model. Activity directed fractionation and further research on antiparasitic activity of the purified components may hopefully lead to fascinating scientific drug discovery. More importantly, *A. vulgaris* is a weed distributed in the hilly parts of Sri Lanka in high abundance, and thus has the potential to be developed into a cheap source of plant based antimalarial in the future.

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