Volume 5 • Issue 4 • October - December 2011

ISSN 0973-8258

IJGP

International Journal of Green Pharmacy

Official publication of
TIFAC CORE in Green Pharmacy

BRNSS Institutions | Learning Innovations Entrepreneurship
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ORIGINAL ARTICLE

An investigation of the antimalarial activity of *Artemisia vulgaris* leaf extract in a rodent malaria model

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Antimalarial activity of an organic extract of *Artemisia vulgaris* was evaluated in this study in terms of both antiparasitic and anti-disease activities. Antiparasitic activity of the extract at three doses (250, 500, 1000 mg/kg) was assessed *in vivo* using the *Plasmodium yoelii* rodent malaria model, using distilled water as the negative control and Coartem as the positive control. Oral administration of the extract in the 4-day suppressive assay at 500 mg/kg and 1000 mg/kg significantly ($P<0.01$) inhibited parasitaemia by 65.16% and 51.46%, respectively. Significant ($P<0.05$) antinociceptive activity was observed for the extract in the hot plate test, indicating a central, supra-spinally mediated response in relieving pain. Anti-disease activity was further corroborated by increased survival of infected mice treated with the 500 mg/kg dose. The *A. vulgaris* extract was tolerated well by mice over a period of 14 days (assay of sub-chronic toxicity), showing no overt signs of toxicity or stress. Hepatotoxicity (evaluated in terms of serum glutamic-oxaloacetic transaminase (SGOT) and serum glutamic-pyruvic transaminase (SGPT) levels), renotoxicity (in terms of serum urea and creatinine) and haematotoxicity (in terms of total RBC, WBC and differential leukocyte counts) were also ruled out. In conclusion, *A. vulgaris* leaf extract is orally active, non-toxic and as a weed it has the potential to be a cheap source of plant-based antimalarial in the future.

Key words: Antimalarial activity, *Artemisia vulgaris*, *Plasmodium yoelii*, Sri Lanka, toxicity

INTRODUCTION

An estimated 225 million cases of malaria occurred in 2009 resulting in an estimated 781,000 deaths.[1] Young children, pregnant women, and non-immune visitors to malarious areas are at greatest risk of severe or fatal illness.[2] Antimalarial drug resistance has emerged as one of the greatest challenges facing malaria control today, and it underlines the urgent need for alternative antimalarials.[2]

WHO has recommended artemisinin-based combination therapies (ACTs) as the first-line treatment for uncomplicated *P. falciparum* malaria since 2001 and during the past decade, most malaria-endemic countries shifted their national treatment policies to ACTs.[3] Currently these artemisinins (a family of highly effective compounds derived from the herb *Artemisia annua*) are the most potent weapons remaining in the modern antimalarial arsenal for effective malaria control.[4]

Access this article online	
Quick Response Code:	Website: www.greenpharmacy.info
	DOI: 10.4103/0973-8258.94347

Artemisia vulgaris L. (Indian wormwood or mugwort) is a semi-shrubby perennial weed that belongs to the family Asteraceae and grows in the hilly districts of India, Sri Lanka, temperate Europe and Asia, Thailand, and Java.[5]

A. vulgaris is a valuable medicinal plant used widely in traditional medicine.[6] It is also the only plant species found in Sri Lanka belonging to the genus *Artemisia*. However no study has been conducted so far in Sri Lanka to evaluate the antimalarial activity of a crude extract obtained from the leaves of this plant. This study was undertaken to fill this void.

MATERIALS AND METHODS

Collection of Plant Material

Leaves of *A. vulgaris* were collected from Nuwara Eliya (latitude: +6.97°, longitude: +80.77°) in the Central province of Sri Lanka, in March 2009. Following identification and authentication a voucher specimen (KK/01/2009) was deposited in the museum of the Department of Zoology, University of Colombo, Sri Lanka.

Preparation of the Plant Extract

Leaves of *A. vulgaris* were cleaned and air dried under the shade for 21 days and powdered using a mechanical

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Received: 24-07-2011; **Accepted:** 27-07-2011

grinder. Ground leaves (125 g) were soaked in 900 ml of an organic solvent mixture which consisted of diethyl ether, methanol, and petroleum ether at a 1:1:1 ratio at room temperature (~28°C) for 48 hours. The resulting dark green solution was filtered using a Buckner funnel. The leaves were then soaked in the organic solvent mixture for a second time for 48 hours. Extracted solution was then evaporated at reduced pressure using a rotavapour to obtain 20.204 g of the crude extract in the form of a paste.[7] This crude extract was dissolved in distilled water to obtain the required dosages which were determined using the human dose for the ACT drug; Coartem. Taking the metabolic rate of mice into consideration the animals were orally treated with 250, 500, and 1000 mg/kg of body weight of the extract resulting in the low, human equivalent, and high dose, respectively.

Experimental Animals

Healthy adult Institute of Cancer Research (ICR) mice weighing 25–30 g purchased from the Medical Research Institute, Colombo, were used in this study. Animals were housed in plastic cages under standard conditions and fed with pelleted food (Master Feed Ltd. Colombo, Sri Lanka) and drinking water *ad libitum*. Approval for this project was granted by the Ethical Review Committee of the Faculty of Medicine, University of Colombo, Sri Lanka (Reference no.: EC/09/010).

Parasite Isolates

Plasmodium yoelii parasite line maintained through serial passage of blood from mouse to mouse was used to assess the *in vivo* antimalarial activity of the *A. vulgaris* leaf extract.

Parasitic Inoculation

Parasitized mouse blood diluted in sterile saline to obtain a 0.2 ml suspension containing 10^7 parasitized RBC, was injected intraperitoneally into adult male ICR mice using a 25G needle.

Evaluation of Antiparasitic Activity on an Early Infection

The 4-day suppressive assay or preventive assay was used to evaluate the *in vivo* antimalarial activity of *A. vulgaris* using three oral doses of the extract at 250, 500, and 1000 mg/kg.[8] Coartem at 450 mg/kg body weight was used as the positive control while distilled water served as the negative control. ICR mice were inoculated (*N*=6) intraperitoneally with 10^7 infected RBC (in 0.2 ml saline) of *P. yoelii* on day zero. The *A. vulgaris* leaf extract and the controls were administered from day zero through day 3. On days 4 and 5, thin blood smears were prepared from tail blood of each mouse, stained with Giemsa and the number of parasitized RBC counted in random fields under oil immersion microscopy. Then the degree of parasitaemia (%) was calculated. The percentage inhibition of parasitaemia was also calculated according to the formula (A-B) 100/A, where A was the average parasitaemia in the control group and B the average parasitaemia in the *A. vulgaris* treated group. Parasitaemia levels in the treated group were then statistically compared with the controls.

Mice used for the 4-day suppressive assay were monitored daily from day 0 to day 7 for seven consecutive days and percentage survival was determined for each group.

Evaluation of Antiparasitic Activity on an Established Infection

Three groups (*N*=6) of male ICR mice were injected intraperitoneally with 10^7 *P. yoelii* infected RBC (in 0.2 ml saline) on day 0. Seventy-two hours following inoculation (day 3), thin blood smears were prepared by a tail bleed. The three groups were then orally administered with 500 mg/kg of the *A. vulgaris* leaf extract, Coartem as the positive control and distilled water as the negative control. Preparation of thin blood smears and treatment with the extract and the controls were carried out for two more days on days 4 and 5, to evaluate the effects of *A. vulgaris* on an established infection. Parasitaemia levels were then determined for each group.

Evaluation of Antinociceptive Activity

Hot plate and tail flick methods were used to assess antinociceptive activity of the *A. vulgaris* leaf extract.[9] Two groups (*N*=6) of healthy male ICR mice were used for the assay. The treated group received 500 mg/kg of the extract while the control group received distilled water. In the hot plate test, the mouse was placed inside the hot plate analgesia meter (Model MK 35A, Muromachi kikai Co. Ltd., Tokyo, Japan) at 52°C and time taken to lick the hind paw or jump was recorded. In the tail flick test, 2–3 cm of the tail from the tip was immersed in a hot water bath at 54°C and time taken to flick the tail was recorded. Reaction times of mice were measured at 1 hour intervals following treatment for 6 hours.

Evaluation of Subchronic Toxicity

Twelve healthy adult male ICR mice were selected for the toxicological investigation. One group (*N*=6) was treated with 1000 mg/kg of the *A. vulgaris* leaf extract while the control group received distilled water. This was carried out daily (0900–1000 hours) for 14 consecutive days. The mice were closely observed on each day of treatment for mortality, overt signs of toxicity (salivation, diarrhea, yellowing of fur, loss of fur, postural abnormalities, behavioural change), stress (fur erection), and aversive behaviour (biting and scratching behaviour, licking of tail, paw and penis, intense grooming behaviour, vocalization). Body weight of mice was evaluated at weekly intervals during the treatment period.

On day 15, 0.3–0.4 ml of blood was collected from each mouse by a tail bleed under mild ether anaesthesia and aseptic conditions. Red blood cell (RBC), white blood cell (WBC), and differential counts (DC) were determined using standard techniques. Serum parameters (SGOT, SGPT, urea, creatinine) were determined using Randox kits (Randox Laboratories Ltd., Antrium, UK) and the spectrophotometer (Jasco V560, Jasco Corporation, Tokyo, Japan) according to the manufacturer's instructions.

On day 15 the mice were weighed and then sacrificed. Their liver, spleen, lungs, kidneys, and heart were immediately removed, blotted dry and weighed using an electronic balance (SHIMADZU LIBROREB series, SHIMADZU Corporation, Tokyo, Japan).

Statistical Analysis

Data were analyzed using the Minitab 15 statistical package for Windows. Data were expressed as mean±SEM. Statistical analysis was performed using the Mann–Whitney *U*-test. Significant values were set at $P \leq 0.05$.

RESULTS

Evaluation of Antiparasitic Activity on an Early Infection

Results obtained from the 4-day suppressive assay are summarized in Table 1. The group treated with 500 mg/kg of the extract and the positive control showed maximum (100%) survival [Figure 1].

Table 1: Antiparasitic activity of the *A. vulgaris* leaf extract in the 4-day suppressive assay

Treatment	% Parasitaemia		% Inhibition of parasitaemia (as compared with the control)	
	Day 4	Day 5	Day 4	Day 5
Control (DW)	49.88±3.55	71.08±5.32		
250 mg/kg	42.92±4.78	64.78±6.12	13.95	8.86
500 mg/kg	17.38±3.94**	27.81±5.79**	65.16	60.88
1000 mg/kg	24.21±6.93**	40.45±11.20*	51.46	43.10
Coartem	0**	0**	100	100

Values are expressed as means±S.E.M (*N*=6); *P<0.05 as compared with the control (Mann-Whitney *U*-test); **P<0.01 as compared with the control (Mann-Whitney *U*-test); DW – Distilled water

Evaluation of Antiparasitic Activity on an Established Infection

A daily increase in parasitaemia was observed in the negative control and the group treated with 500 mg/kg of the *A. vulgaris* extract. However a daily decrease in parasitaemia was observed in the positive control treated with Coartem [Table 2].

Evaluation of Antinociceptive Activity

When compared with the control, treatment of mice with 500 mg/kg of *A. vulgaris* extract significantly ($P \geq 0.05$) prolonged the reaction times (1st hour by 45.38%; 2nd hour by 17.39%; 3rd hour by 196.43%; 4th hour by 19.31%; 5th hour by 67.77%; 6th hour by 169.84%) in the hot plate test but not in the tail flick test [Table 3].

Evaluation of Sub-chronic Toxicity

Treatment with the *A. vulgaris* leaf extract at 1000 mg/kg did not elicit any overt signs of toxicity and no deaths were

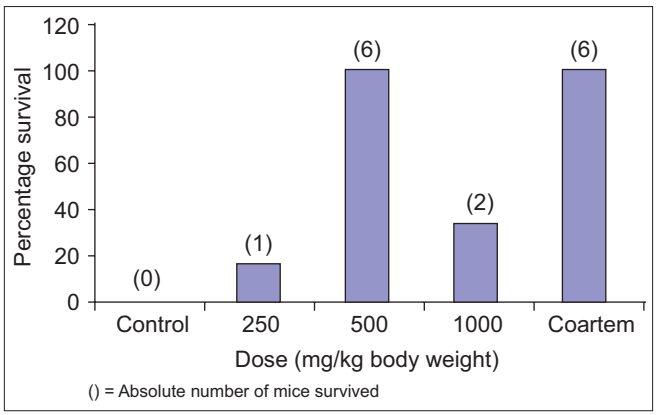

Figure 1: Effect of oral administration of the *A. vulgaris* leaf extract on survival of mice infected with *P. yoelii* in 4-day suppressive assay

Table 2: Antiparasitic activity of the *A. vulgaris* leaf extract in the curative assay

Treatment	% Parasitaemia		
	Day 3	Day 4	Day 5
Control (DW)	45.85±11.20	62.24±11.80	85.24±9.64
500 mg/kg	39.47±4.23	51.90±9.23	72.87±11.34
Coartem 450 mg/kg	49.40±5.44	38.37±9.32	15.32±7.81

Values are expressed as means±S.E.M (*N*=6); DW – Distilled water

Table 3: Reaction times of mice in the hot plate and tail flick tests following oral administration of the *A. vulgaris* leaf extract (500 mg/kg) and distilled water

Experimental group	Reaction times (seconds)					
	1st hour	2nd hour	3rd hour	4th hour	5th hour	6th hour
Hot plate test						
Control group (DW)	4.87±0.73	5.75±1.05	3.08±0.59	5.18±1.10	2.73±0.73	3.05±0.88
Test group (500 mg/kg)	7.08±0.78*	6.75±1.20*	9.13±2.29*	6.18±1.46*	4.58±1.42*	8.23±2.17*
Tail flick test						
Control group (DW)	2.32±0.37	2.25±0.26	2.02±0.18	2.60±0.35	2.58±0.38	2.20±0.27
Test group (500 mg/kg)	2.45±0.32	2.47±0.21	2.38±0.23	2.72±0.39	2.62±0.24	2.35±0.18

Values are expressed as means±S.E.M (*N*=6); *$P \geq 0.05$ as compared with the control (Mann-Whitney *U*-test); DW – Distilled water

encountered. Body weight and organ weights did not show a significant difference (P >0.05) between the treated group and the control group [Table 4]. Haematological (total RBC, WBC and differential leukocyte counts) and serum (SGOT, SGPT, urea and creatinine) parameters also did not vary significantly (P >0.05) between the treated group and the control group [Table 5].

Table 4: Effect of oral administration of the *A. vulgaris* leaf extract on body weight and organ weights in mice

Parameters	Control (DW)	1000 mg/kg extract
Body weight (g)		
Pre-treatment	30.83±0.53	31.19±0.68
1st week	33.26±0.49	32.96±0.61
2nd week	36.59±0.64	34.88±0.56
Organ weights (mg)		
Heart	168.50±3.15	159.50±3.29
Lungs	184.33±6.18	212.67±9.08
Spleen	145.83±8.62	155.17±4.45
Liver	1999.8±89.1	1828.0±70.9
Kidneys	650.0±14.6	556.3±18.6

Values are expressed as means±S.E.M (N=6); DW – Distilled water

Table 5: Effect of oral administration of the *A. vulgaris* leaf extract on haematological and serum parameters in mice

Parameters	Control (DW)	1000 mg/kg extract
Serum parameters		
SGOT (U/L)	29.167±0.518	27.634±0.694
SGPT (U/L)	13.340±0.183	12.978±0.202
Urea (mg/dl)	46.62±5.11	42.87±4.98
Creatinine (mg/dl)	1.84±0.18	0.99±0.13
Haematological parameters		
RBC counts (×10⁶)	6.11±0.54	6.01±0.52
WBC counts	1749±46.8	1612±83.2
Neutrophils (%)	40.00±0.71	32.40±0.6
Eosinophils (%)	4.60±0.51	81.80±0.58
Basophils (%)	4.60±0.51	4.20±0.37
Monocytes (%)	5.40±0.81	8.00±0.71
Lymphocytes (%)	49.00±2.81	53.60±1.47

Values are expressed as means±S.E.M (N=6). SGOT – Serum glutamic-oxaloacetic transaminase; SGPT – Serum glutamic-pyruvic transaminase; DW – Distilled water

DISCUSSION

In this study, a rodent malaria model was used to evaluate the pathogenesis of *P. yoelii* and the potential antimalarial activity of the *A. vulgaris* leaf extract. Due to similarities between *P. yoelii* and human malaria parasites like *P. falciparum* and *P. vivax*, *P. yoelii* can be used as an alternative *in vivo* model to human malarias.[10] This model is sensitive, reliable, widely used, and scientifically validated. Therefore it could be deduced that any antiparasitic activity of the *A. vulgaris* leaf extract against the *P. yoelii* malaria model may be indicative of a possible activity against falciparum and vivax malaria.

Oral administration of *A. vulgaris* at 500 mg/kg elicited the greatest antiplasmodial activity on an early infection and suppressed parasitaemia by 65.16% on day 4 and by 60.88% on day 5 in the 4-day suppressive assay. Survival time of mice in the group treated with the *A. vulgaris* leaf extract ascompared with the controls further corroborated these findings and demonstrated the ability of the extract to reduce the disease burden in mice.

According to the results obtained, the mid-dose of 500 mg/kg demonstrated more antiparasitic activity compared to the high dose of 1000 mg/kg in the suppressive assay. There may be several reasons for this. One is an effect known as receptor desensitization in pharmacology.[11] Persistent or increased exposure of receptors to a drug or an agonist can result in the reduction or the eventual loss of receptor activated function, due to changes in structure and/or chemistry of receptors. Downregulation of receptors is also cited as a reason for the reduction in activity of pharmacological drugs at higher doses.[12] Production of a metabolite that acts as an antagonist to the active ingredients of the plant extract is another reason suggested for the reduction in activity at a higher dose.[13]

Oral administration of *Tinospara crispa* and *Zanthoxylim rhoiflium* aqueous extracts have shown similar results against *P. yoelii*, demonstrating 54% and 78% suppression, respectively.[14] Oral administration of *Barringtonia acutangula* aqueous root extract, a local medicinal plant used by traditional healers against malaria in Sri Lanka, has demonstrated ~60% parasitaemia suppression as well. [15] This shows that the antiparasitic activity observed in the *A. vulgaris* leaf extract in this study is on par with the antiparasitic activity observed in some traditional herbal preparations that have been used to treat malaria for many years.

A phytochemical screening carried out previously on a crude extract of *Artemisia vulgaris* has established the presence of alkaloids, coumarins, flavonoids, saponins, sterols, tannins, and terpenes.[16] In a separate phytochemical analysis of the leaves of *A. vulgaris*, researchers have established the presence of two sesquiterpene lactones; yomogin and 1,2,3,4-diepoxy-11(13)-eudesmen-12,8-olide.[17] Different families of sesquiterpene lactones have been reported from the genus *Artemisia*.[18] Prominent amongst them is artemisinin obtained from *A. annua*; the potent antimalarial agent that provides the basis for artemisinin-based combination therapy (ACT). A sesquiterpene lactone called tehranolide derived from *Artemisia diffusa* has also demonstrated antimalarial properties.[7] Furthermore, antiplasmodial activity has also been attributed to courmarin in a previous study.[19] The

presence of these compounds in *A. vulgaris* may therefore explain the antimalarial activity observed in this study.

Coartem® produced by the Swiss pharmaceutical company Novartis is the ACT drug indicated for the treatment of acute uncomplicated malaria infections due to *P. falciparum* or mixed infections including *P. falciparum*. One tablet contains 20 mg of artemether (semisynthetic chiral acetal derivative of artemisinin) and 120 mg of lumefantrine (racemic mixture of a synthetic fluorene derivative). Since this study was evaluating the antimalarial activity of a plant species suspected of having artemisinin or a compound similar to it, this particular drug was used as the positive control instead of the standard chloroquine treatment that would be used otherwise. Dosage was determined according to the human dose. Adults and children weighing 35 kg and above receive the standard 3 days treatment schedule with a total of 8 tablets daily (taken in two doses of 4 tablets each in the morning and evening) where the total course comprises 24 tablets.[20] This results in a maximum dosage of 32 mg/kg of the tablet per day. 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. [21] Since a specific dosage was not available for *A. vulgaris*, dosages for the crude extract were determined using widely used dosages for traditional herbal medicinal preparations.

The ability of a malaria therapeutic agent to relieve pain demonstrates its antidisease activity or the ability to reduce the disease pathology of malaria. This study established a moderately long-acting antinociceptive activity of *A. vulgaris* using the efficacious 500 mg/kg dose. This was performed using the standard hot plate and tail flick tests. Acute thermal pain is modelled by these two tests. Mechanisms of pain perception involved in these two tests are different. The hot plate test stimulates a central, supra-spinally mediated response while the tail flick test acts via a central, spinally mediated mechanism.[9] Results obtained showed a statistically significant antinociceptive activity in the hot plate test and not in the tail flick test. This is evidence for a pain-relieving activity of *A. vulgaris* and also its mode of action, as it can be deduced that this extract relieves pain through a centrally mediated pathway that involves the brain and not the spinal cord.

Toxicity has been singled out as the main drawback of traditional herbal antimalarial preparations. The *A. vulgaris* extract however was tolerated well by mice who did not show any overt signs of toxicity, stress, or aversive behaviour. Hepatotoxicity was evaluated in terms of SGOT and SGPT levels and the results ruled out any liver toxicity. Renotoxicity, evaluated through urea and creatinine levels was also absent. RBC, WBC, and DC also showed no significant alteration in mice treated with the plant extract and therefore haematotoxicity was also ruled out. Weights and morphology of vital organs were also not significantly affected suggesting a possible lack of general toxicity.

The plant extract used in this study, prepared using the leaves of *A. vulgaris* displayed less antimalarial activity compared to Coartem. Furthermore the curative assay carried out using the 500 mg/kg dose to study the effect of *A. vulgaris* on an established infection, did not show efficacious results as the parasitaemia increased steadily in the test group while in the positive control group treated with Coartem the parasitaemia reduced continually until it reached 0% after 8 days. This may suggest that the active compound was not present in sufficient concentration in the crude extract to eliminate an established infection. However the results obtained in this study are extremely encouraging for a crude plant extract and highlight the need for further *in vivo* and *in vitro* studies to fully understand the potential of *A. vulgaris* as a therapeutic agent against malaria and also its mode of action.

In conclusion, the results demonstrated that the *A. vulgaris* leaf extract is orally active with moderate antiparasitic and antidisease activity and is non-toxic, and as a weed it has the potential to be a cheap source of plant-based antimalarial in the future.

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How to cite this article: Kodippili K, Ratnasooriya WD, Premakumara S, Udagama PV. An investigation of the antimalarial activity of *Artemisia vulgaris* leaf extract in a rodent malaria model. Int J Green Pharm 2011;5:276-81.

Sources of Support: Financial assistance by the University of Colombo, Sri Lanka; Prof. Anura Weerasinghe, Professor of Physiology of the University of Rajarata, Sri Lanka provided the rodent malaria species; Dr. Gawrie Galappaththy of the National Anti-Malaria Campaign, Sri Lanka provided Coartem, the positive control drug, used in this study., **Conflict of Interest:** None declared.