IJBPAS, December, 2026, 15(12): 5713-5726

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

IN VIVO PHARMACOLOGICAL ASSESSMENT OF THE ANTIMALARIAL POTENTIAL OF *HEDYOTIS HERBACEA* AGAINST *PLASMODIUM* INFECTION

SURUSE PB[*1], KOLTE S[2], UPLANCHIWAR V[1], HIRADEVE S[1] AND DURAGKAR N[3]

1: Nagpur College of Pharmacy, Wanadongri, Hingna Road, Nagpur – 441110

2: Department of Pharmaceutical Sciences, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur, Maharashtra, India 440033

3: Central Indian Pharmaceuticals, MIDC, Hingna, Nagpur-441110

***Corresponding Author: Dr. Pravin B. Suruse: E Mail: pravinsuruse@gmail.com**

Received 18th May 2026; Revised 24th June 2026; Accepted 18th Aug, 2026; Available online 1st Dec. 2026

https://doi.org/10.31032/IJBPAS/2026/15.12.10633

ABSTRACT

The current study set out to evaluate the *in vivo* antimalarial activity of different extracts of *Hedyotis herbacea*, a plant that has long been used as a febrifuge to treat chronic malaria. The extracts contained triterpenoids, flavonoids, alkaloids, saponins, tannins, cardiac glycosides and steroids, according to a preliminary phytochemical screening. The average percentage of parasitemia and chemosuppression were calculated for each *Hedyotis herbacea* extract. The percentage of parasitaemia ranged from 9.83 ± 0.29% to 7.16 ± 0.29% after four days of treatment with 50, 100 and 200 mg/kg/day ethyl acetate extract of *Hedyotis herbacea*, whereas the control group showed 47.33 ± 2.52%. Ethyl acetate extract of *Hedyotis herbacea* had a chemosuppressive efficacy of 84.87% at a dose of 200 mg/kg/day, which was equivalent to the standard drug chloroquine (85.93%) and very significant (***$p < 0.001$). However, although the extract demonstrated a maximal chemosuppression of 76.42% at 200 mg/kg/day (**$p < 0.01$), its activity was slightly lower than that of the standard. The mean survival time (MST) for mice in the standard group that received chloroquine treatment was 28 days. According to the Curative (Rane) test, the

ethyl acetate extract of *Hedyotis herbacea* exhibited the strongest schizonticidal activity among all the tested extracts. Interestingly, as compared to the standard, the groups treated with 100 and 200 mg/kg/day of the ethyl acetate extract of *Hedyotis herbacea* demonstrated a substantial MST of 27.67 ± 0.58 days (***$p < 0.001$). These results imply that *Hedyotis herbacea* ethyl acetate extract has strong antimalarial properties.

Keywords: *Hedyotis herbacea*, antimalarial activity, Schizonticidal activity

INTRODUCTION

One of the major infectious parasite diseases spread by mosquitoes worldwide is malaria. *Plasmodium*-genus protozoa are the culprit[1]. Both tropical and non-tropical nations are seeing an increase in this infectious disease **[1]**. Malaria kills more than a million people annually, primarily children **[1]**. The prevalence of dengue fever is higher in urban regions, but malaria is more common in rural areas **[2]**. Since *Plasmodium falciparum* is growing resistant to existing treatments, it is imperative to design and develop safe, effective drugs with suitable pharmacokinetic characteristics **[3]**.

Numerous herbal treatments are believed to have outstanding therapeutic value and can be used to a variety of ailments. Various native plants are successfully utilized in natural medicine to treat various infectious disorders, either alone or in combination. Therefore, in order to expand the use of these plants, scientific research is required to assess their efficacy. There are limitless opportunities for the development of new medications due to the unmatched amount of chemical variety included in natural products made from medicinal plants, whether they are pure compounds or standardized extracts **[4]**. The goal of creating antimalarial phytomedicines from traditional medicine is to identify new therapeutic leads among a variety of traditional medicinal plants that can be investigated for the purpose of drug discovery and development. Choosing the lead that has the best chance of producing safe and effective antimalarial medications or phytomedicines is one of the most challenging jobs in the creation of novel antimalarial pharmaceuticals from traditional medicine **[5]**.

Hedyotis herbacea is an erect, glabrous annual or biennial herb that reaches a height of 4.5 to 7.5 cm. The branches are thin, divaricate, dichotomous, and many. The leaves are sessile, linear or linear-lanceolate, acute, glabrous, and typically have recurved margins. The bottom leaves are often wider than the upper ones, and they are 1.3-2 cm by

0.8-3 mm long. It is found in hilly areas all throughout India. Additionally, it can be found in Tropical Africa and the Malay Islands **[6-7]**. Ethano medical botanical investigations in the hills areas of Maharashtra have shown that a decoction of the entire plant is used to treat liver illnesses and as a substitute for low-grade fevers, such as recurrent fever with gastric irritation and nervous depression. An alkaloid and a significant amount of alkaline salts, primarily in the form of chlorides, including sodium, potassium, and calcium, are present in this herb **[6-7]**.

A review of the literature shows that *Hedyotis herbacea* has a broad range of medical uses and is a good febrifuge for chronic malaria. Therefore, evaluating the *in vivo* antimalarial activity of *Hedyotis herbacea* was the aim of the current investigation.

MATERIALS AND METHODS

Materials

We purchased Quinine hydrochloride and Giemsa stain from Sigma Chemicals in the US. Petroleum ether (60–80^{0}C), ethyl acetate, acetone and ethanol were the extraction solvents that were purchased from Loba Chemicals in Mumbai, India. Medico Remedies Private Limited, located in Mumbai, India, kindly donated atropine. Every additional chemical and reagent utilized in this inquiry was of analytical quality.

Methods

Authentication of *Hedyotis herbacea*

The entire plant of the *Hedyotis herbacea* was collected in Aamgaon, Gondia District, Maharashtra, India. *Hedyotis herbacea* herbarium specimens were authenticated by Department of Botany at Bhawabhuti Mahavidyalaya, Aamgaon, Maharashtra.

Successive solvent extraction of *Hedyotis herbacea*

In order to create a coarse powder that was separated on sieves, the entire plant material was washed, shade-dried, and ground in a milling machine. For extraction, Soxhlet equipment with a 5L capacity was utilized. Petroleum ether was used to completely defatten the coarse powder of *Hedyotis herbacea* at 60- 80^{0}C. Following deffation, the plant material was taken out of the assembly, dried and then put back into the soxhlet apparatus. The remaining plant material was then further extracted using solvents including ethanol, acetone and ethyl acetate that increased in polarity. A hydroalcoholic extract was obtained after 15 days of maceration using a solvent mixture of absolute ethanol and distilled water (1:1) **[8-9]**. All extracts were collected, concentrated using a thermostatic water bath and filtered

through Whatman filter paper (No. 44). Until they were required, the dried extracts were maintained in a BOD incubator in a sealed container **[10-11]**.

Screening for bioactive phytoconstituents

All extracts were examined for the presence of phytoconstituents, including alkaloids, flavonoids, tannins, steroids, saponins, triterpenoids, proteins and sugars **[12-14]**.

Standardization of *Hedyotis herbacea* extracts

Alkaloid content was determined by dissolving *Hedyotis herbacea* extract (1 mg) in DMSO, adding 1 ml of 2 N HCl, filtering and transferring to a separating funnel. Phosphate buffer and bromocresol green were added, then extracted with increasing volumes of chloroform. The combined extract was diluted to 10 ml with chloroform. Atropine standards (20–100 µg/ml) were created such that the absorbance of the test and standard solutions could be measured at 470 nm using a UV/Vis spectrophotometer and a reagent blank as a reference **[15-16[**.

Evaluation of acute oral toxicity of extracts

According to OECD-420 recommendations, a fixed dose was used in a stepwise process to conduct an acute oral toxicity investigation in mice **[17]**. Animals were grouped for acute toxicity testing of *Hedyotis herbacea* extracts, administered orally in Tween 80 at doses of 5, 50, 300, and 2000 mg/kg. Animals who had fasted overnight and had unrestricted access to water were given each dose in a volume of 1 ml/100 gm of body weight. For a total of 14 days, all animals were observed for indications of toxicity and impending mortality at least once during the first 30 minutes and then at regular intervals throughout the first 24 hours, paying particular attention during the first 4 hours and each day thereafter. After the test chemical was administered, food was also denied for a further three to four hours. For 48 hours and then once a day for 14 days, mice were observed for behavioral reaction and death. Body weight was recorded both before and after the trial ended. The vital organs from the test animals (including those died during the test or humanely killed) were excised immediately and fixed in 10% formalin for macro and micro changes in histopathology by the routine procedure **[18]**.

Experimental subjects, IAEC approval and animal husbandry

Standard laboratory conditions were employed to house swiss albino mice (20-25 g) of both sexes in a temperature-controlled environment (24 ± 1.0 ºC) with a light/dark cycle of 12:12 hours. Standard rat chow (Lipton, India) and water were freely available to the animals. The Committee for the Purpose

of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India, New Delhi, established the Institutional Animal Ethics Committee (IAEC), which approved all experimental protocols (IAEC approval No. 536/02/C/CPCSEA; dated 20.01.2002).

Assessment of the *in vivo* antimalarial activity of various extracts (The 4-day suppressive test by Peter)

The *in vivo* antimalarial activity of *Hedyotis herbacea* extracts was assessed using Peter's 4-day suppressive assay against chloroquine-sensitive *Plasmodium falciparum* in albino mice.

Experimental animals being injected with parasites

The National Institute of Malaria Research (NIMR), located in New Delhi, provided the chloroquine-sensitive *Plasmodium falciparum* (MRC-Pf-435), which was then kept in mice using the protocol outlined by Knight and Peters (1980).

According to the erythrocyte count and % parasitaemia, blood from a donor mouse that had already infected *Plasmodium falciparum* been diluted with isotonic saline to create the inoculum **[19]**. The final inoculum contained 5×10^7 parasitized erythrocytes with 29% parasitaemia**.** Mice were infected intraperitoneally with 0.2 ml of this inoculum on day zero **[20]**.

Investigation of schizonticidal potential in malaria

The schizonticidal activity of *Hedyotis herbacea* extracts was evaluated in mice following the standard four-day suppressive test. Swiss albino mice (n = 4 per group) were randomly assigned to 17 groups. Treatment with the respective *Hedyotis herbacea* extracts was initiated shortly after inoculation with *Plasmodium* parasites and continued once daily for four consecutive days **[21-23]**. Seventeen groups of mice were assigned **(Table 1)**.

Table 1: Experimental group of animals

Group	Treatment	Dose (mg/kg, p.o.)
1	Saline (control)	-
2-4	Petroleum ether extract of *Hedyotis herbacea*	50, 100, 200
5-7	Ethyl acetate extract of *Hedyotis herbacea*	50, 100, 200
8-10	Acetone extract of *Hedyotis herbacea*	50, 100, 200
11-13	Ethanolic extract of *Hedyotis herbacea*	50, 100, 200
14-16	Hydro-alcoholic extract of *Hedyotis herbacea*	50, 100, 200
17	Chloroquine (standard)	5

Estimation of percentage parasitaemia

% Parasitemia = (Parasitized RBC / (Parasitized RBC + Non-parasitized RBC)) × 100

The treatments were given from day 0 (day of inoculation) for four consecutive days (day 0 to day 3). On the fifth day (day 4), thin films were made from the tail blood of each mouse and the parasitaemia level was determined by counting the number of parasitized erythrocytes out of 200 erythrocytes in random fields of the microscope **[23-24]**.

Calculation of chemosuppression percentage

Comparing the test extracts to the control group, the average chemosuppressive effect was determined:

Chemosuppression Average = (A-B)/A×100

Where:

A = Mean percentage of parasites in control;

B = Mean percentage of parasites in test group

Assessment of schizonticidal effects in established malaria infection using the Curative (Rane) test

The approach outlined by Ryley and Peters (1970) was used to assess the test drug's therapeutic impact. A conventional inoculum consisting of 1 x 10^7 *Plasmodium falciparum* infected erythrocytes was administered intraperitoneally to mice on day 0. The mice were split into 16 groups, each with four animals, at random 72 hours after the vaccination. As previously mentioned, the corresponding therapies were given orally once every day for five days in a row. To assess parasitaemia levels, thin blood smears were made daily from each mouse's tail vein blood, stained with Giemsa, and examined under a microscope **[25-26]**. Animals were monitored for 28 days, from day 0 to day 27, in order to determine the mean survival time (MST) for each group. The mean survival time is calculated by dividing the number of animals in the group by the total number of survival days for the animals in the group **[27]**.

Statistical treatment of data

The mean ± standard deviation (SD) is used to express the data. GraphPad Prism software, version 6.04, was used to make statistical comparisons using one-way ANOVA. At $P < 0.05$, differences were deemed statistically significant.

RESULT AND DISCUSSION

Screening for bioactive phytoconstituents

Triterpenoids, flavonoids, alkaloids, saponins, tannins, cardiac glycosides, and steroids have all been found in various *Hedyotis herbacea* extracts. Because *Hedyotis herbacea* extracts include bioactive phytoconstituents, their phytochemical profile substantiates their

ethnopharmacological claims and, consequently, demonstrates its preclinical applications in the treatment of malaria. Several visualizing agents were used to confirm the presence of phytoconstituents. When p-anisaldehyde and sulfuric acid reagent were sprayed, the red and green color indicated the presence of steroids and triterpenoids. When Dragendorff's reagent was used as a visualizing agent, the presence of orange color spots indicated the detection of alkaloids. Blue - greenish color was observed with 5% $FeCl_3$ solution indicates the presence of tannins **[28]**.

Analytical standardization of *Hedyotis herbacea* Extracts

Using the BCG approach and the standard curve (y = 0.004x + 0.044, R2 = 0.980), the amount of alkaloids in *Hedyotis herbacea* extracts was calculated and represented as Atropine equivalents (mg AE/g extract). The approach offers great sensitivity and stability, and the maximum concentration in the ethyl acetate extract was 50.48 mg AE/g. The standard calibration curve of Atropine is shown in **Figure 1**.

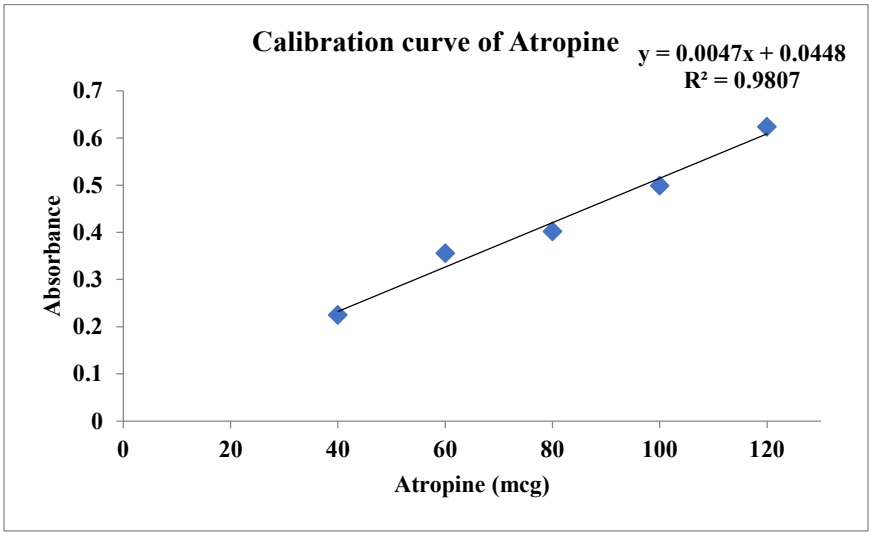


Figure 1: Standard calibration curve of Atropine

Evaluation of acute oral toxicity of extracts

According to the findings of the acute toxicity tests, *Hedyotis herbacea* test extracts were safe up to a dose of 2000 mg/kg administered orally. Mortality, toxicity markers and gross behavior did not significantly change during the study **[29-31]**.

Assessment of the *in vivo* antimalarial activity of various extracts (The 4-day suppressive test by Peter)

The percentages for chemosuppression and parasitemia are inversely correlated. Better antimalarial potency is demonstrated by increased chemosuppression, which is indicated by lower parasitemia percentage values. The average percentages of parasitemia and chemosuppression for each of the *Hedyotis herbacea* extracts were calculated. The percentage parasitaemia varied between 9.83 ± 0.29 and 7.16 ± 0.29% after four days of treatment with ethyl acetate extract of *Hedyotis herbacea* at dosages of 50, 100, and 200 mg/kg/day, while the control group showed parasitaemia of 47.33 ± 2.52%. In comparison to the standard (85.93%), the percentage chemosuppression with a 200 mg/kg/day dose of *Hedyotis herbacea* ethyl acetate extract was highly significant (84.87%; with statistical significance *** $p < 0.001$). With a maximal percentage of chemosuppression at 200 mg/kg/day dose (76.42%; with statistical significance ** $p < 0.01$), the ethyl acetate extract of *Hedyotis herbacea* produced less significant effects than the standard. As a reference standard, chloroquine was employed **(Table 2)**.

Table 2: *In vivo* assessment of the schizonticidal effects of *Hedyotis herbacea* extracts

Extracts	Dose (mg/kg/day)	% Parasitaemia	% Chemosuppression
Petroleum ether	50	27.83 ± 0.77	41.20
Petroleum ether	100	26.33 ± 0.29	44.37
Petroleum ether	200	27.83 ± 0.77	41.20
Ethyl acetate	50	9.83 ± 0.29	79.23***
Ethyl acetate	100	8.66 ± 0.77	81.70***
Ethyl acetate	200	7.16 ± 0.29	84.87***
Acetone	50	25.33 ± 0.29	46.48
Acetone	100	25.83 ± 0.77	45.43
Acetone	200	26.50 ± 1.13	44.01
Ethanol	50	14.00 ± 0.87	70.42
Ethanol	100	12.33 ± 0.29	73.95**
Ethanol	200	11.16 ± 0.29	76.42**
Hydro alcohol	50	23.83 ± 0.77	49.55
Hydro alcohol	100	23.33 ± 0.29	50.71
Hydro alcohol	200	24.33 ± 0.29	48.60
Chloroquine (Standard)	5	6.67 ± 0.29	85.93***
Normal Saline solution (Control)	0.2 ml	47.33 ± 2.52	-

For a sample size of N = 4, all results are shown as mean ± standard deviation (SD). * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Evaluation of schizonticidal activity in established infection (Curative or Rane test)

The Ryley and Peter (1970) approach was used to assess each extract's schizonticidal activity. The average survival days of mice during a 28-day period (days 0–27) were used to compute the mean survival time for each group. The more the extract's Mean Survival Time (MST), the more effective it is. The

untreated control group had the lowest Mean Survival Time (MST), which was 8 days. MST was 28 days for the mice in the standard group that received chloroquine treatment. According to the Curative or Rane test, the ethyl acetate extract of *Hedyotis herbacea* exhibited the strongest schizonticidal activity among the other extracts. The groups that received 100 and 200 mg/kg/day of *Hedyotis herbacea* ethyl acetate extract demonstrated a substantial MST of 27.67 ± 0.58 days (*** $p < 0.001$) when compared to the standard **(Table 3)**.

Table 3: Mean survival time of mice treated with various extracts of *Hedyotis herbacea*

Extracts	Dose (mg/kg/day)	Mean survival time (days)
Petroleum ether	50	10.66 ± 0.58
Petroleum ether	100	9.67 ± 0.58
Petroleum ether	200	9.0 ± 1.00
Ethyl acetate	50	26.00 ± 1.00***
Ethyl acetate	100	27.67 ± 0.58***
Ethyl acetate	200	27.67 ± 0.58***
Acetone	50	10.68 ± 1.53
Acetone	100	11.00 ± 1.00
Acetone	200	10.00 ± 1.32
Ethanol	50	20.33 ± 0.58
Ethanol	100	20.66 ± 1.53
Ethanol	200	20.00 ± 2.65
Hydro alcohol	50	11.68 ± 0.58
Hydro alcohol	100	12.67 ± 2.09
Hydro alcohol	200	13.74 ± 0.58
Chloroquine (Standard)	5	28 ± 0.00***
Normal Saline solution (Control)	0.2 ml	7.66 ± 0.58

For a sample size of N = 4, all results are shown as mean ± standard deviation (SD). * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

According to the results, ethyl acetate (EA) extracts of *Hedyotis herbacea* have potent antimalarial effects. The antimalarial activity of these extracts may be attributed to their bioactive phytoconstituents, particularly alkaloids and flavonoids, which have been reported to exhibit antimalarial effects **[32]**.

CONCLUSIONS

As a source of antimalarial drugs, the current investigation validated the prudent use of specific medicinal herbs. Significant *in vivo* antimalarial activity was observed with the selected medicinal plant extracts. The chemicals that were isolated from bioactive extracts were discovered to be alkaloidal and to possess antimalarial properties. Clinical studies must be conducted in order to create a well-known formulation that the general public will find beneficial if these extracts and constituents are to be employed for therapeutic purposes.

To determine how the plant extracts work, pharmacodynamic investigations ought to be conducted. Since the cost of therapy is

increasing, creating affordable solutions would undoubtedly provide a better choice and chance to treat malaria, the most prevalent infectious and dangerous parasitic illness spread by mosquitoes.

CONFLICT OF INTEREST

Regarding this work, the authors have disclosed no conflicts of interest.

ACKNOWLEDGEMENTS

The authors owe the management and principle of Nagpur college of pharmacy, Nagpur, for providing the facilities needed to carry out the study.

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