

Anti-Oxidant Potential and Antimalarial Effects of *Acanthus polystachyus* Delile (Acanthaceae) Against *Plasmodium berghei*: Evidence for in vivo Antimalarial Activity

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Background: Malaria is among the most devastating and widespread tropical parasitic diseases which is more prevalent in developing countries. *Acanthus polystachyus* (Acanthaceae) leaves are traditionally used for the treatment of malaria in Ethiopia. This study aimed to investigate the in vivo antimalarial and in vitro antioxidant activity of the leaves extract of *Acanthus polystachyus*.

Materials and Methods: The leaves part of *A. polystachyus* were extracted using 80% methanol. The antioxidant effect of *Acanthus polystachyus* extract was evaluated by using (DPPH) diphenyl-2-picrylhydrazyl assay model. To evaluate the antimalarial effect of the *A. polystachyus* extract, *Plasmodium berghei* ANKA strain (chloroquine sensitive) was used to infect the mice in three different experimental protocol: suppressive, curative, and prophylactic test models.

Results: The leaves extract of *Acanthus polystachyus* significantly ($P < 0.05$ – 0.0001) suppressed the parasitemia in all experimental protocol. The extract exhibited a chemosuppression of 27.64%, 33.67% and 49.25% in 4-day suppressive test; 33.76%, 39.24% and 53.59% in curative test, and 25%, 32.84% and 50% in prophylactic tests at dose of 100, 200, and 400mg/kg, respectively. The extract also extended the mean survival time, prevented the bodyweight loss and body temperature drop, and packed cell volume significantly ($P < 0.05$) as compared to the negative control. Dose-dependent antioxidant activity was shown by the leaf extract of *A. polystachyus* with an IC_{50} value of 9.37 μ g/mL.

Conclusion: The current finding showed that the leaves extract of *Acanthus polystachyus* has revealed promising antimalarial effects. Thus, this finding supports the traditional use of *A. polystachyus* for the treatment of malaria.

Keywords: antimalarial activity, antioxidants, *Plasmodium berghei*, *Acanthus polystachyus*

Introduction

Malaria is a lethal disease caused by *Plasmodium* parasites transmitted to humans via bites of *Plasmodium*-infected female Anopheles mosquito. *P. falciparum* is the most fatal malaria parasite in Africa and accounts for most malarial deaths worldwide.^{1,2} About 219 million cases and 435, 000 deaths of malaria were reported in the world by 2017. Among these, 92% of the cases and 93% of the death report due to malaria were from Africa. Globally, more than two-third of the malaria deaths were reported in under-five children.³ According to WHO

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2019 malaria report, roughly 228 million cases and 405,000 deaths of malaria were reported worldwide.⁴ The cases of *P. falciparum* and *P. vivax* were 66% and 34%, respectively, in Ethiopia.⁵ According to a 2015 report, a total of 621,345 cases and 1561 deaths were reported in Ethiopia.⁶ Nearly 6% of global malaria cases and 12% of global deaths due to *P. vivax* have occurred in Ethiopia. About 75% of global malarial deaths were from four countries: India, Pakistan, Indonesia, and Ethiopia.³

In Ethiopia, malaria was the foremost cause of outpatient visits, accounting for 17% of all outpatient visits, and 8% of health facility admissions among all age groups and one of the top ten causes of inpatient deaths among children aged less than five years and in older individuals.⁷ The socioeconomic impact of malaria is striking, although the country's economy is based on agriculture and peak malaria transmission coincides with the planting and harvesting season. About 75% of the country is malinicious, with about 68% of the country's total population living in areas at risk of malaria.^{8,9}

Plant-based traditional medicines have been the core source for the management of malaria in different countries.¹⁰ Traditionally claimed anti-malarial plants are among the most potential areas to search antimalarials drugs in African flora. It is well known that about 80% of the Ethiopian population depend on herbal medicine for the management of numerous diseases, including malaria.¹¹ Although most of the medicinal plants that have potential anti-malarial activity have been studied, there still exist potentially useful medicinal plants awaiting evaluation for therapeutic applications against various groups of pathogens.¹²

Acanthus is a genus belonging to the family Acanthaceae. The Acanthaceae family has several pharmacological activities such as cytotoxic, immunomodulatory, anti-inflammatory, antiviral, insecticidal, antiplatelet, antipyretic, hepatoprotective, antifungal, and antioxidant activities.^{13,14} Traditionally, the leaves and root parts of *Acanthus polystachyus* have been used for the treatment of different illnesses such as pneumonia, bleeding, scorpion sting, stabbing pain, and anthrax.¹⁵ The leaves part of *Acanthus polystachyus* Delile have been used in the treatment of malaria in Ethiopia.^{16–18} So far in vivo antimalarial activity of *Acanthus polystachyus* has not been reported. Thus, this study is intended to evaluate the in vivo antimalarial and in vitro antioxidant activities of the leaves extract of *Acanthus polystachyus*.

Materials and Methods

Drugs and Reagents Used

Chloroquine (Addis Pharmaceuticals Factory, Ethiopia), giemsa (ScienceLab, USA), trisodium citrate (Deluxe Scientific Surgico, India), hydrochloric acid (Supertek Chemicals, India), halothane (Rotexmedica, Germany), normal saline (Epharm, Ethiopia), 2,2-Diphenyl-1-picrylhydrazyl, 3.5 (Sigma Aldrich, Germany), chloroform (Avishkar Lab Techchemicals, India), methanol (Avishkar Lab Tech Chemicals, India), ethyl acetate (Loba chemie, India), sulfuric acid (Supertek, India), acetic anhydride (Central Drug House, India), nitric acid (Supertek, India), ferric sulfate (BDH Ltd, UK), lead acetate (BDH Ltd, UK), benzene (Nice laboratory reagent, India), ferric chloride (Fisher Scientific Co, USA), Mayer's reagent (Avishkar Lab Tech Chemicals, India), ascorbic acid (Blulux Laboratories, India), Wagner's Reagent (BDH Ltd, UK) were used. All reagents were analytically graded and procured from certified local and international suppliers.

Plant Materials

Fresh leaves of *Acanthus polystachyus* were collected from Bahir Dar city, Ethiopia in February 2017. The botanical identification and authentication of the plant material were done by a botanist from Department of Biology, University of Gondar, Gondar, Ethiopia. The voucher specimen (D/W-0/0/1) was deposited in Herbarium at University of Gondar.

Methods of Extraction

The leaves of *Acanthus polystachyus* were washed with distilled water to remove dirt and dried at room temperature (25–27°C). The plant material was coarsely grounded into powder by the electrical mill. Then, the coarse powder of the plant material was macerated with methanol for about 72 hours (three times) and filtered via Whatman filter paper. The filtrate of the plant material obtained from the successive maceration was concentrated via a rotary evaporator followed by a hot air oven (40°C). Finally, the dried extract of *Acanthus polystachyus* was kept separately in a desiccator until used for the experiment. The extraction yield was calculated using the following formula:¹⁹

$$\% \text{extraction (yield)} = \frac{\text{weight of extract(g)}}{\text{weight of plant material(g)}} \times 100$$

Experimental Animals and Parasites

A total of 95 Swiss albino mice of both sexes (weighing 24–30g and age 6–8 weeks) were used for the experiment. The mice were kept at room temperature with a 12h light/12h dark cycle and given food and water ad libitum. After randomization into various groups and before initiation of the experiment, the mice were acclimatized to the laboratory conditions of the Department of Pharmacology, College of Medicine & Health Sciences, University of Gondar. Animal handling and care was carried out throughout the experiment according to international laboratory animal use and care guidelines.²⁰ The euthanasia procedure was conducted in a chemical fume hood to prevent inhalation of halothane by workers. Finally, cervical dislocation was used on mice to ensure that they have been properly euthanized. The *Plasmodium berghei* ANKA strain (Chloroquine sensitive) was obtained from EPHI_ Addis Ababa.²¹

Preliminary Phytochemical Screening of Leaf Crude Extract

Qualitative tests were done for the leaves extract of *Acanthus polystachyus* for the presence of phytoconstituents such as flavonoids, steroidal compounds, terpenes, alkaloids, saponins, tannins, and phenolic compounds, as described in a standard procedure. Test for alkaloids: extract (0.2 g) was dissolved in dilute hydrochloric acid. The solution was filtered and a few drops of Dragendroff's reagent added. The treated solutions were observed for any precipitation. Similarly, to a portion of filtered solution, a few drops of Mayer's reagent were added. The treated solutions were observed for any precipitation; test for flavonoids: five mL of ethyl acetate was added to a solution of 0.5 g of the extract in water. The mixture was shaken, allowed to settle and inspected for the production of yellow color in the organic layer which is taken as positive for free flavonoids; test for saponins: the hydro-alcoholic extract (0.5 g) was dissolved in 10 mL of distilled water in a test tube. The test tube was corked and shaken vigorously for about 30 sec. The test tube was allowed to stand vertically and observed over a 30-min period of time. If froth above the surface persists after 30 min the sample is suspected to contain saponins; test for tannins: a portion of the alcoholic extract was dissolved in water. The solution was clarified by filtration. 10% ferric chloride solution was added to the clear filtrate. This was observed for a change in color to bluish black; test for glycosides: 0.5 g of crude extracts was dissolved separately in 5 mL of methanol.

10 mL of 50% HCl was added to 2 mL of each extract in test tubes. The mixtures were heated in a boiling water bath for 30 min. 5 mL of Fehling's solution was added and the mixtures were boiled for 5 min to give a brick red precipitate as an indication for the presence of glycosides; test for Phenols: 0.5 g of each of crude extracts were put in a different test tube and treated with a few drops of 2% of FeCl₃; bluish green or black coloration were indicated the presence of phenols; test for steroids: 0.5 g of each crude extracts were dissolved in 5 mL of methanol. 1 mL of the extract was treated with 0.5 mL of acetic acid anhydride and cooled in ice. This mixed with 0.5 mL of chloroform and 1 mL of concentrated sulphuric acid was then added carefully by means of a pipette. At the separations level of the two liquids, reddish-brown rings were formed, as indication of the presence of steroids; test for terpenoids: 0.5 g of each crude powders were separately dissolved in 5 mL of methanol. 2 mL of the extract was treated with 1 mL of 2,4-dinitrophenyl hydrazine were dissolved in 100 mL of 2M HCl. Yellow-orange colorations were observed as an indication of Terpenoids; and test for anthraquinones: the extract of the plant material (equivalent to 100 mg) was shaken vigorously with 10 mL of benzene, filtered and 5 mL of 10% ammonia solution added to the filtrate. The mixture was shaken and observed for the presence of a pink, red or violet color in the ammonia (lower) phase that indicates the presence of free anthraquinones.^{22–24}

In-vitro Antioxidant Activity in DPPH Assay Model

The antioxidant activity of the leaves extract of *Acanthus polystachyus* was evaluated by using DPPH free radical scavenging assay. 0.004 gm of DPPH was dissolved in 100mL methanol in the dark and 3.9 mL of a 0.1 mM methanolic solution of DPPH was mixed with a 0.1mL methanolic solution of different concentrations of the extract ranging from 50 to 1000 µg/mL and incubated in the dark for thirty minutes at room temperature. Ascorbic acid served as a standard antioxidant for this experiment. After 30 minutes, the absorbance of the mixture and the control at 517 nm was read by a UV spectrophotometer.²⁵ The test was carryout in triplicate so that the average value was taken. The percent inhibition of free scavenging was calculated as:

$$\% \text{free radical scavenging} = \frac{\text{Abs Control} - \text{Abs Sample}}{\text{Abs Control}} \times 100$$

where Abs Control was the absorbance without sample, Abs samples was the absorbance of sample latex or ascorbic acid.

Acute Oral Toxicity Study

Acute oral toxicity test was done according to the OECD (Organization for Economic Cooperation and Development) 425 guideline for the crude extracts.²⁶ Following the acclimatization of the mice for one week, five female mice (weighing 25–28 gram, 6–8 weeks of age) were employed for the acute toxicity study. Following the period of fasting, the first one mouse was weighed and the test substance 2000mg/kg bodyweight of the extract of *Acanthus polystachyus* was administered by the oral route. The animal was observed at least once during the first thirty minutes, intermittently during the first 24 hours, with special attention given during the first four hours and daily thereafter, for a total of 14 days. Observations include changes in skin, eyes, mucous membranes, respiratory, circulatory, autonomic, and central nervous system changes, and behavioral patterns. The mouse in the sighting test did not show any sign of toxicity for 4 days and the toxicity test of the remaining four mice for methanolic crude extract was given and followed as usual for 14 days.^{27,28}

Grouping and Dosing of Animals

In all mouse models, the positive control group was received Chloroquine 25 mg/kg and the negative control group was received distilled water 10 mL/kg; while the test groups were received three doses (100mg/kg, 200mg/kg, and 400mg/kg) of the *Acanthus polystachyus* crude extract. The leaves extract of *Acanthus polystachyus* was determined based on the result of the acute oral toxicity test. The middle doses of the solvent fractions of *Acanthus polystachyus* were 1/10 of the limit dose, the higher doses of the solvent fractions *Acanthus polystachyus* were twice of the middle doses of the solvent fractions of *Acanthus polystachyus*, and a half does of the middle doses of *Acanthus polystachyus* were the lower doses of the extract.^{29,30}

Peter's Test (4-Day Suppressive Test)

By using chloroquine-sensitive rodent malaria parasite, namely; *Plasmodium berghei*, the standard four-day suppressive method was used in this study.³¹ Treatment of infected mice was started after 3 hours of infection on day 0 and continued daily for four days (ie, from day 0 to day 3). On the fifth day (day 4) blood

samples were collected from the tail snip of each mouse. Thin smears were prepared and stained with 10% Giemsa solution. Then, each stained slide was examined under the microscope with an oil immersion objective of 100x magnification power to evaluate the percent suppression of each extract with respect to the control groups.

Rane's Test/Curative Test

Evaluation of the curative antimalarial potential of the extract was done by using a method described previously.^{27,32} On the first day, blood containing approximately the inoculum of 1×10^7 *Plasmodium berghei* ANKA strain (Chloroquine sensitive) was injected into 25 Swiss Albino mice, intraperitoneally (IP). After 72 hours, the mice were randomly divided into 5 groups of 6 mice each. Subsequently, 3 different doses of the extract (100 mg/kg, 200 mg/kg, and 400 mg/kg/day), chloroquine phosphate (25 mg/kg/day), and distilled water (1 mL/100 g/day) were given orally to the respective groups. These treatments were administered once daily for 3 consecutive days. For each mouse, thin blood film stained with 10% Giemsa was prepared from the tail blood of each mouse daily for 5 consecutive days to monitor the levels of parasitemia. The mice were further observed for 30 days. Any death that occurred during this period was recorded and used to determine the mean survival time. Similar to the chemosuppressive test, the average % parasitemia suppression was calculated.

Prophylactic Activity Test

Evaluation of the prophylactic potential of the active compounds in a 4-day suppressive test of the extract was done by methods described previously.³³ 30 Swiss Albino mice were randomly divided into 5 groups of 6 mice each. They were administered orally with 100, 200, and 400 mg/kg/day of the crude extract, chloroquine phosphate (25 mg/kg/day), and distilled water (1 mL/100 g/day) for 3 consecutive days of the respective groups. On the fourth day, a standard inoculum of 1×10^7 *Plasmodium berghei* ANKA strain (Chloroquine sensitive) infected erythrocytes were administered by the IP route to each mouse. After 72 hours (on the seventh day), thin blood smears were prepared from the tail blood. Percentage parasitemia and the percentage of chemosuppression of parasitemia were calculated using the formula described in the chemosuppressive test.

Determination of the Level of Parasitaemia and % Suppression

Tail blood smearing of each mouse was made for both the suppressive and curative tests. After fixation with absolute alcohol and staining with 10% Giemsa at pH 7.2 for 15 min, the slides were washed gently with distilled water followed by air drying at room temperature. By using Olympus microscope oil immersion objective of power, the number of parasitized erythrocytes in random fields of the microscope was counted. Three separate fields on each of the slides were used to calculate percent parasitemia and percent suppression using the following formula:³⁴

$$\% \text{ Parasitemia} = \frac{\text{Number of parasitized RBC}}{\text{Total number of RBC}} \times 100$$

$$\% \text{ suppression} = \frac{\text{mean parasitemia of untreated} - \text{mean parasitemia of treated group}}{\text{mean parasitemia of negative control}} \times 100$$

Determination of Mean Survival Time

From the time of infection until death, the mortality of each mouse was monitored and recorded regardless of the group in which the mouse was allocated throughout the follow-up period (15 days). The mean survival time of mice of each group was determined using the following formula:³⁵

$$\text{MST} = \frac{\text{Sum of survival time of mice in the group (days)}}{\text{Total number of mice in that group}}$$

Determination of Packed Cell Volume

Blood was collected from the tail of each mouse in heparinized micro-hematocrit capillary tubes. The capillary tubes were filled to 3/4th of their height with blood and sealed at one end with sealing clay. The tubes were then placed in a micro-hematocrit centrifuge, with the sealed end outwards and centrifuged for 5 minutes at 11,000 rpm. The tubes were taken out of the centrifuge and PCV was determined using a standard Micro-Hematocrit Reader. It was measured before inoculating the parasite (day 0) and day 4 in Peter's 4-day suppressive test and D0 and D7 in the prophylactic activity test. In the case of Rane's test, PCV was measured on the third day after the infection was established, and on the last day of treatment on the seventh day. PCV is a measure of the proportion of RBCs

to plasma in the whole blood and determined using the relation shown below.²⁸

$$\text{PCV} = \frac{\text{volume of erythrocytes in given volume of blood}}{\text{Total blood volume}}$$

Monitoring of Body Weight and Temperature Changes

The body weight of each mouse in all groups was recorded on day 0 and after infection on day 4. The rectal temperature of mice was measured with a digital thermometer to see the effect of the extract on the prevention of body temperature reduction due to malaria.³⁶

Statistical Analysis

All data values are expressed as mean \pm SEM for six mice per group. Statistical analyses were carried out by using SPSS Statistical software version 24. Statistical significance of mean of parasitemia suppression, weight, PCV, and survival time differences between groups was computed by one-way ANOVA, followed by post hoc Turkey's Multiple Comparison Test. *P*-value of less than 0.05 was considered as statistically significant.

Results

The Percentage Yield of Plant Material Extraction

A total of 187 grams of dried leaves extract of *Acanthus polystachyus* was collected. In the preparation of the extract from the dried leaves of *Acanthus polystachyus Delile* (Acanthaceae), a yield of 14% was obtained.

Preliminary Phytochemical Screening

The phytochemical screening finding for the leaves extract of *Acanthus polystachyus* revealed the presence of tannins, phenols, flavonoids, terpenoids, glycosides, anthraquinones, saponins, and steroids. However, steroids and alkaloids were not present in the phytochemical screening (Table 1).

Antioxidant Activity of Hydromethanolic Leaves Extract of *A. polystachyus*

In the present study, the antioxidant activity of the leaves extract of *Acanthus polystachyus* was tested using DPPH free radical. The extract exhibited antioxidant activity as summarized in Figure 1. There was concentration-dependent free radical scavenging activity by the extract

Table 1 Phytochemical Constituent of Methanolic Crude Leaf Extract of *A. polystachyus*

| Secondary Metabolites | Test Results |
|-----------------------|--------------|
| Alkaloid | - |
| Flavonoids | + |
| Saponins | + |
| Tannins | + |
| Glycosides | + |
| Phenols | + |
| Steroids | - |
| Terpenoids | + |
| Anthraquinones | + |

Notes: (+): Indicates the presence of the constituents; (-): Indicates the absence of the constituents.

and the standard drug. Ascorbic acid was used as a standard drug for the determination of the antioxidant activity by DPPH method. The concentration of ascorbic acid and the *Acanthus polystachyus* varied from 50 to 1000 $\mu\text{g/mL}$. The percentage inhibitions of the extract were: 27.43%, 35.67%, 50.02%, 65.43%, 72.32%, 79.86%, and 82.54% at 50, 100, 200, 400, 600, 800, and 1000 $\mu\text{g/mL}$ concentration of the extract, respectively. Likewise, the percentage inhibitions of ascorbic acid were: 35.21%, 50.21%, 65.43%, 77.53%, 86.43%, 90.42%, 96.83% at 50, 100, 200, 400, 600, 800, and 1000 $\mu\text{g/mL}$ concentration of the standard drug, respectively. Furthermore, the IC_{50} values of the extract and

ascorbic acid were: 9.37 $\mu\text{g/mL}$, and 2.31 $\mu\text{g/mL}$, respectively (Figure 1).

Acute Toxicity Test

In acute oral toxicity test, the leaves extract of *A. polystachyus* revealed no mortality 2 gm/kg bodyweight concentration in mice. The mice did not also demonstrate any toxic effects like changes in behavioral activities such as anxiety, polyuria, diarrhea, seizures, and coma. Thus, the median lethal dose (LD_{50}) of the extract of *A. polystachyus* is $>2\text{gm/kg}$.

Effect of the Extract on Blood Parasitemia and Mean Survival Time in 4-Day Suppressive Test

The current finding revealed that all tested doses (100mg/kg, 200mg/kg and 400mg/kg) of *A. polystachyus* extract exhibited significant parasitemia suppression when compared with the negative control group. The maximum suppression (49.25%, $p < 0.0001$) was revealed at the highest dose of the crude extract (400mg/kg) then by the middle dose of the extract (200mg/kg) (33.67%, $p < 0.0001$) and 100mg/kg (27.64%, $p < 0.01$) (Table 2). All doses of the extract (100mg/kg, 200mg/kg and 400mg/kg) were shown significant ($p < 0.001$, $p < 0.0001$, and $p < 0.0001$, respectively) variance in mean survival time when compared with the negative control group (Table 2).

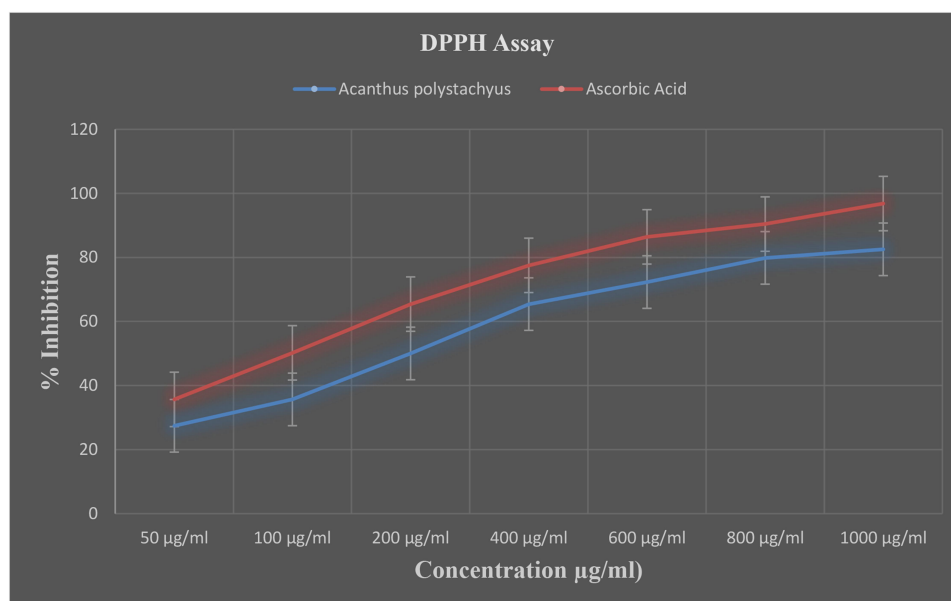


Figure 1 Free radical scavenging activity of the leaf extract of *Acanthus polystachyus*.

Notes: Each value of percentage inhibition of α -amylase is presented as means \pm SEM, $n = 3$.

Abbreviation: DPPH Assay, diphenyl-2-picrylhydrazyl assay model.

Table 2 Effect of *A. polystachyus* on Percentage Parasitemia and Survival Time of *P. berghei* Infected Mice in the 4-Day Suppressive Test

| Group | % Parasitemia | % Suppression | Survival Time (Days) |
|------------|--|---------------|--|
| NC | 39.80±1.23 | – | 6.80±0.45 |
| 25mg/kg CQ | 0.00±2.01 ^{a****} | 100 | 30.00±0.87 ^{a****} |
| 100g/kg | 28.80±0.99 ^{a**} , ^{b****} | 27.64 | 10.20±0.62 ^{a****} , ^{b****} |
| 200mg/kg | 26.40±1.30 ^{a****} , ^{b****} | 33.67 | 11.20±1.01 ^{a****} , ^{b****} |
| 400mg/kg | 20.20±1.33 ^{a****} , ^{b****} | 49.25 | 13.60±0.77 ^{a****} , ^{b****} |

Notes: Values are presented as Mean ± SEM (Standard Error Mean). Statistical significance denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$; ^aCompared to NC, ^bCompared to CQ25mg/kg.

Abbreviations: CQ, chloroquine diphosphate; NC, negative control.

Effect of the Extract on Packed Cell Volume, Body Weight, and Rectal Temperature in the 4-Day Suppressive Test

As summarized in Table 3, the crude extract at the doses of 200mg/kg and 400mg/kg significantly ($p < 0.05$) prevented the loss of packed cell volume as compared to the negative control group. However, there was no significant difference at 100mg/kg of crude extract ($p > 0.05$) as compared to the negative control.

The lower (100mg/kg), and the middle (200mg/kg) doses of *A. polystachyus* failed to protect the weight loss as compared to the negative control group. However, the higher (400mg/kg) dose of the extract and the standard drug exhibited a significant ($p < 0.05$) protection of body weight loss as compared to the negative control group. The extract also produced a dose-dependent effect on preventing a drop in body temperature on day 4 after the infection. The middle (200mg/kg) and highest (400mg/kg) crude extract-treated group had showed significant ($p < 0.01$, $p < 0.0001$, respectively) activity in prevention against rectal temperature reduction when compared to negative control group. Similarly, chloroquine 25mg/kg had shown significant ($p < 0.001$) activity in prevention against body temperature reduction when compared to the negative control (Table 3).

Effect of the Extract on Blood Parasitemia and Mean Survival in Curative Test

In the curative test, the extract of *A. polystachyus* showed a dose-dependent parasitemia suppression (33.76%, 39.24%, and 53.59% at 100mg/kg, 200mg/kg and 400mg/kg, respectively). As summarized in Table 4, the extract of *A. polystachyus* exhibited a significant ($p < 0.0001$) curative action at the doses of 100mg/kg, 200mg/kg, and 400mg/kg on the 7th day from a thin blood smear. The mean survival time in the curative test was significant at the middle (200mg/kg, $p < 0.001$) and highest dose (400mg/kg, $p < 0.0001$) of crude extract as compared to the negative control. Similarly, the mean survival time of mice treated with the standard drug significantly ($p < 0.0001$) extended when compared to the untreated group (Table 4).

Effect of the Extract on Packed Cell Volume, Body Weight, and Rectal Temperature in the Curative Test

All doses of the extract of *A. polystachyus* (100mg/kg, 200mg/kg, and 400mg/kg) significantly ($p < 0.0001$) prevented the PCV reduction when compared to the untreated group (Table 5). Similarly, chloroquine 25 mg/kg significantly ($p < 0.0001$) prevented the packed cell volume reduction when compared to the negative control group. Excluding

Table 3 Effect of *A. polystachyus* on the Body Weights, PCV, and Temperature of *P. berghei* Infected Mice in the 4-Day Suppressive Test

| Group | Temperature | | | Wight | | | Packed Cell Volume | | |
|------------|-------------|------------|-----------------------------|------------|------------|-------------------------|--------------------|------------|-----------------------------|
| | D0 | D4 | % Change | D0 | D4 | % Change | D0 | D4 | % Change |
| NC | 36.02±0.97 | 34.66±0.56 | 3.75±0.77 | 31.02±1.45 | 28.78±1.21 | 7.14±0.73 | 56.20±1.44 | 52.40±1.03 | 6.74±2.01 |
| 25mg/kg CQ | 36.20±1.03 | 36.06±1.00 | 0.37±1.03 ^{a****} | 30.32±0.97 | 29.98±0.34 | 1.18±1.00 ^{**} | 51.00±0.95 | 51.60±0.56 | -1.28±1.31 ^{a****} |
| 100g/kg | 37.08±0.59 | 36.16±0.85 | 2.47±0.99 ^{b*} | 30.54±1.02 | 29.28±0.98 | 4.20±0.59 | 52.80±1.32 | 51.20±1.60 | 2.94±0.98 |
| 200mg/kg | 36.50±0.61 | 36.04±0.32 | 1.27±0.57 ^{a*} | 24.74±0.54 | 23.68±0.58 | 4.53±0.32 | 56.80±0.88 | 55.60±0.89 | 2.10±0.49 ^{a*} |
| 400mg/kg | 35.72±0.39 | 35.76±0.44 | -0.13±0.81 ^{a****} | 26.28±0.77 | 25.66±0.36 | 2.45±0.51 ^{a*} | 56.00±0.64 | 54.80±1.22 | 2.10±0.76 ^{a*} |

Notes: Values are presented as Mean ± SD (Standard Deviation). Statistical significance as compared to treatment control denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$; ^aCompared to NC ^bCompared to CQ.

Abbreviations: CQ, chloroquine diphosphate; NC, normal control; D0, a day infection initiated; D4, 5th day of infection.

Table 4 Effect of *A. polystachyus* Crude Extract on Percentage Parasitemia and Survival Time of *P. berghei* Infected Mice in Rane's Test

| Group | % Parasitemia (\pm SEM) | | | | | % Suppression | MST |
|------------|----------------------------|--|--|--|--|---------------|--|
| | D3 | D4 | D5 | D6 | D7 | | |
| NC | 20.60 \pm 1.32 | 25.80 \pm 0.56 | 33.20 \pm 0.43 | 39.80 \pm 1.54 | 47.40 \pm 1.23 | – | 6.80 \pm 0.98 |
| 25mg/kg CQ | 21.20 \pm 0.870.77 | 13.40 \pm 1.32 ^{a****} | 6.20 \pm 1.09 ^{a****} | 2.00 \pm 0.87 ^{a****} | 0.00 \pm 0.34 ^{a****} , b**** | 100 | 30.00 \pm 0.00 ^{a****} |
| 100g/kg | 21.20 \pm 1.21 | 22.60 \pm 0.98 ^{b****} | 25.20 \pm 0.88 ^{a****} , b**** | 27.00 \pm 0.96 ^{a****} , b**** | 31.40 \pm 0.88 ^{a****} , b**** | 33.76 | 8.60 \pm 1.23 ^{b****} |
| 200mg/kg | 22.00 \pm 0.73 | 21.20 \pm 1.11 ^{a**} , b**** | 22.60 \pm 0.53 ^{a****} , b**** | 24.60 \pm 1.23 ^{a****} , b**** | 28.80 \pm 0.59 ^{a****} , b**** | 39.24 | 10.00 \pm 0.88 ^{a****} , b**** |
| 400mg/kg | 21.20 \pm 0.59 | 20.60 \pm 0.53 ^{a**} , b**** | 22.00 \pm 0.66 ^{a****} , b**** | 20.40 \pm 0.74 ^{a****} , b**** | 22.00 \pm 0.91 ^{a****} , b**** | 53.59 | 12.40 \pm 1.43 ^{a****} , b**** |

Notes: Values are presented as Mean \pm SEM (Standard Error Mean). Statistical significance denoted by * p <0.05, ** p <0.01, *** p <0.001, and **** p <0.0001; ^aCompared to NC, ^bCompared to CQ25mg/kg.

Abbreviations: CQ, chloroquine diphosphate; NC, negative control.

Table 5 Effect of *A. polystachyus* Crude Extract on the Body Weights, PCV, and Temperature of *P. berghei* Infected Mice in Rane's Test

| Group | Temperature | | | Wight | | | Packed Cell Volume | | |
|------------|------------------|------------------|----------------------------------|------------------|------------------|-----------------------------------|--------------------|-------------|-----------------------------------|
| | D3 | D7 | % Change | D3 | D7 | % Change | D3 | D7 | % Change |
| NC | 37.20 \pm 0.21 | 35.66 \pm 0.86 | 4.13 \pm 0.76 | 26.20 \pm 0.76 | 24.20 \pm 0.66 | 7.74 \pm 1.34 | 56.60 \pm | 47.80 \pm | -15.52 \pm 1.32 |
| 25mg/kg CQ | 37.00 \pm 0.66 | 36.96 \pm 0.10 | 0.08 \pm 0.32 ^{a****} | 27.64 \pm 0.54 | 27.72 \pm 1.41 | -0.43 \pm 0.78 ^{a****} | 55.00 \pm | 53.60 \pm | -2.45 \pm 1.44 ^{a****} |
| 100g/kg | 36.46 \pm 0.25 | 35.52 \pm 0.34 | 2.57 \pm 0.92 ^{b*} | 27.26 \pm 0.82 | 25.94 \pm 0.98 | 4.89 \pm 0.77 ^{b**} | 55.80 \pm | 52.60 \pm | -5.75 \pm 0.98 ^{a****} |
| 200mg/kg | 36.62 \pm 0.54 | 36.00 \pm 0.76 | 1.69 \pm 1.01 ^{a*} | 27.22 \pm 1.22 | 26.46 \pm 1.11 | 2.79 \pm 0.92 ^{a**} | 57.00 \pm | 54.40 \pm | -4.55 \pm 1.09 ^{a****} |
| 400mg/kg | 36.74 \pm 0.88 | 36.46 \pm 0.34 | 0.76 \pm 0.47 ^{a**} | 26.46 \pm 1.01 | 26.48 \pm 0.49 | -0.15 \pm 0.62 ^{a****} | 55.60 \pm | 54.00 \pm | -2.84 \pm 0.79 ^{a****} |

Notes: Values are presented as Mean \pm SD (Standard Deviation). Statistical significance as compared to treatment control denoted by * p <0.05, ** p <0.01, *** p <0.001, and **** p <0.0001; ^aCompared to NC, ^bCompared to CQ.

Abbreviations: CQ, chloroquine diphosphate; NC, normal control; D0, a day infection initiated; D4, 5th day of infection.

the lower dose (100mg/kg), both the middle (200mg/kg) and higher (400mg/kg) doses of the extract exhibited a significant (p <0.01 and p <0.0001, respectively) increment of body weight as compared to the untreated group. Similarly, the standard drug showed significant (p <0.0001) increment of body weight change between the seventh and fourth day as compared to the untreated group. The extract at the dose of 200 mg/kg, 400 mg/kg, and chloroquine 25 mg/kg significantly (p <0.05, p <0.01, and p <0.001, respectively) prevents the drop of temperature compared to the untreated group. Though, the lower dose (100mg/kg) of the extract failed to prevent the drop of temperature as compared to the negative control group (Table 5).

Effect of the Crude Extract on Blood Parasitemia and Mean Survival Time in Prophylactic Test

The crude extract of *A. polystachyus* showed a significant parasitemia suppression (25%, p <0.001; 32.84%, p <0.0001;

and 50%, p <0.0001), at 100mg/kg, 200mg/kg, and 400mg/kg doses the extract, respectively, as compared to the negative control. Likewise, the standard drug significantly (p <0.0001, 100%) suppressed the parasitemia level when compared with the negative control group (Table 6). Regarding the mean survival time, mice that received the middle dose (200 mg/kg) and the higher dose (400mg/kg) of the extract had longer MST (p <0.001, p <0.0001, respectively) as compared to the negative control group. However, the lower dose (100 mg/kg) of the extract of *A. polystachyus* did not show a significant difference in MST when compared with the negative control (Table 6).

Effect of the Crude Extract on Packed Cell Volume, Body Weight and Rectal Temperature in Prophylactic Test

All doses of the extract of *A. polystachyus* (100mg/kg, 200mg/kg, and 400mg/kg) significantly (p <0.01, p <0.001, and p <0.0001, respectively) prevented the loss of body

Table 6 Effect of *A. polystachyus* on Percentage Parasitemia and Survival Time of *P. berghei* Infected Mice in the Prophylactic Test

| Group | % Parasitemia | % Suppression | Survival Time (Days) |
|------------|--|---------------|--|
| NC | 40.80±2.71 | – | 6.60±0.51 |
| 25mg/kg CQ | 0.00±0.00 ^{a****} | 100 | 30.00±0.00 ^{a****} |
| 100g/kg | 30.60±1.60 ^{a**} , ^{b****} | 25 | 8.80±0.37 ^{b****} |
| 200mg/kg | 27.40±1.21 ^{a****} , ^{b****} | 32.84 | 10.60±0.68 ^{a****} , ^{b****} |
| 400mg/kg | 20.40±1.25 ^{a****} , ^{b****} | 50 | 12.80±0.86 ^{a****} , ^{b****} |

Notes: Values are presented as Mean ± SEM (Standard Error Mean). Statistical significance denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$; ^aCompared to NC; ^bCompared to CQ25mg/kg.

Abbreviations: CQ, chloroquine diphosphate; NC, negative control.

weight when compared with the negative control group. Similarly, chloroquine 25 mg/kg significantly ($p < 0.0001$) prevented weight loss as compared to the negative control. The higher (400mg/kg) dose of the crude extract showed a significant ($p < 0.01$) effect on preventing a drop in body temperature as compared to the negative control. However, a drop in body temperature was not significantly prevented at 100mg/kg and 200mg/kg doses of the crude extract as compared to the negative control (Table 7). The standard drug still had a significant effect on preventing a drop in body temperature compared to the negative control. All doses of the crude extract (100 mg/kg, 200mg/kg, and 400mg/kg) showed a significant ($p < 0.01$, $p < 0.0001$ and $p < 0.0001$), respectively, effect on preventing the loss of packed cell volume when compared with the negative control group. Likewise, the standard drug showed a significant ($p < 0.0001$) prevention of packed cell volume reduction when compared to the negative control group (Table 7).

Discussion

In-vivo models are usually applied in antimalarial studies since they allow the possible prodrug effect and likely boosting of the immune system of the body against the pathogen.^{37,38} The study conducted currently explored the phytochemical screening, antioxidant activity, acute oral

toxicity study, and in vivo antimalarial activities of the crude extract of *A. polystachyus*.

In acute oral toxicity test, leaves extract of *A. polystachyus* revealed no mortality at the 2 gm/kg bodyweight concentration in mice. The mice did not also demonstrate any toxic effects like changes in behavioral activities such as anxiety, polyuria, diarrhea, seizures, and coma which received the extract. Thus, the median lethal dose (LD₅₀) of the extract of *A. polystachyus* is >2gm/kg. This finding supports the tough evidence of the non-toxic outcome of the plant.³⁹ This extract is therefore safe and this could explain the safe use of this plant by the local people who have been using it in the traditional treatment of malaria in Ethiopia.

Medicinal plants with antimalarial activity considered to be active should reduce parasitemia at least by 30%, which supports the finding of parasite inhibition in the current study.⁴⁰ In 4-day suppressive test, the extract of *A. polystachyus* showed significant parasite suppression: 27.64% ($p < 0.001$), 33.67% ($p < 0.0001$), and 49.25% ($p < 0.0001$) at doses of 100, 200 and 400mg/kg/day, respectively. In the curative test, significant ($p < 0.0001$) parasitemia suppression: 33.76%, 39.24%, and 53.59% at 100mg/kg, 200mg/kg and 400mg/kg, respectively. In prophylactic test, 25%, $p < 0.001$; 32.84%, $p < 0.0001$; and

Table 7 Effect of *A. polystachyus* Crude Extract on the Body Weights, PCV, and Temperature of *P. berghei* Infected Mice in the Prophylactic Test

| Group | Temperature | | | Wight | | | Packed Cell Volume | | |
|------------|-------------|------------|----------------------------|------------|------------|--|--------------------|------------|----------------------------|
| | D0 | D4 | % Change | D0 | D4 | % Change | D0 | D4 | % Change |
| NS | 36.60±0.48 | 34.06±0.52 | -6.88±1.77 | 29.80±1.18 | 26.88±0.93 | -9.55±1.89 | 57.60±0.87 | 48.00±1.23 | 16.63±0.76 |
| 25mg/kg CQ | 36.08±0.41 | 36.48±0.33 | 1.14±1.13 ^{a****} | 28.80±0.83 | 28.99±0.86 | 0.66±0.67 ^{a****} | 55.60±1.86 | 55.40±0.98 | 0.24±0.31 ^{a****} |
| 100mg/kg | 36.14±0.22 | 35.24±0.27 | -2.49±0.46 | 29.36±0.42 | 28.10±0.45 | -4.28±0.82 ^{a*} , ^{b*} | 57.20±1.28 | 54.60±0.59 | 4.53±0.97 ^{a**} |
| 200mg/kg | 36.48±0.44 | 35.58±0.48 | -2.46±0.77 | 31.48±0.96 | 30.66±0.80 | -2.54±0.52 ^{a****} | 54.80±1.27 | 53.80±1.32 | 1.76±1.32 ^{a****} |
| 400mg/kg | 36.02±0.38 | 35.68±0.59 | -0.96±0.92 ^{a**} | 30.78±1.75 | 30.40±1.34 | -1.31±0.82 ^{a****} | 55.60±1.07 | 55.20±0.98 | 0.74±1.04 ^{a****} |

Notes: Values are presented as Mean ± SD (Standard Deviation). Statistical significance as compared to treatment control denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$; ^aCompared to NC; ^bCompared to CQ.

Abbreviations: CQ, chloroquine diphosphate; NC, normal control; D0, a day infection initiated; D4, 5th day of infection.

50%, $p < 0.0001$, at 100mg/kg, 200mg/kg, and 400mg/kg doses of the extract, respectively, as compared to the negative control. Secondary metabolites such as terpenoids, flavonoids, saponins, alkaloids, tannins, glycosides, phenols, and anthraquinone have been implicated for their antiprotozoal and antimalarial activities in many pharmacological studies.^{41–44} The current phytochemical screening result revealed that the methanolic extract of *A. polystachyus* leaves possesses phytochemical constituents namely saponins, tannins, terpenoids, phenols, flavonoids, glycosides, steroids, and anthraquinones. Thus, the significant parasitemia suppression detected in the current study could be due to the presence of these phytoconstituents. The present finding is in line with previous similar studies.^{45–47} Furthermore, it has been conveyed that antioxidant activity can inhibit heme polymerization (the unpolymerized heme is very toxic for the *Plasmodium* species).⁴⁸ In the current study, the leaves extract of *A. polystachyus* showed potent antioxidant activity. Hence, the observed antimalarial activity of the extract might be due to the antioxidant activity of *A. polystachyus* leaves extract. In the present study, the percentage suppression was higher in the curative test than in prophylactic test. This may be because of a rapid hepatic metabolism or clearance of the bioactive compounds accountable for ant plasmodial activities because of the administration of the extract initially for 4 days before the inoculation of the parasite. Compared to the standard drug, the extract of *A. polystachyus* showed less antimalarial activity. This could be because of low selectivity, unpurified/crude nature, poor bioavailability, slow absorption, other pharmacokinetics and pharmacodynamics parameters of the extract of *A. polystachyus*.

In all experimental protocol, the mean survival time was extended by the extract of *A. polystachyus* demonstrating suppression of the parasitemia which was due to the *P. berghei* infection. However, all doses of the extract (100, 200, and 400 mg/kg) failed to cure the *P. berghei* infection. The bioactive compound of medicinal plants that prolonged survival time greater than twelve days, it is considered as active.⁴⁹

Bodyweight loss is one of the most common features in *P. berghei* infected mice.⁵⁰ Therefore, a potential antimalarial plant product is expected to preserve these parameters in *p. berghei* infected mice due to the rise in the level of parasitemia.^{28,41,51} In the curative test and prophylactic test model, the middle (200mg/kg) and the higher (400mg/kg) doses of the extract exhibited significant

($p < 0.05$) protective effect against weight loss as compared to the negative control. This effect could have been contributed from the overall improvement of packed cell volume and parasite clearance among extract-treated groups.^{27,52} However, the lower doses of the extract did not prevent body weight loss in all experimental protocol, although there was significant suppression of parasitemia. This indicates the contribution of other factors for these reductions beyond malaria infection. The weight loss could be due to catabolic action on stored lipids or anorexigenic effect of the extract that may lead to reduced food intake because of the presence of appetite-suppressant phytoconstituents in the extract.⁵³ Thus, the extract of *A. polystachyus* may possess appetite-suppressing activity which could be attributed to flavonoids, saponins, and phenolic compounds.^{45,54,55} Notably, the present finding is in agreement with the previous finding.⁴⁵ However, the finding of the current study on body weight was not consistent with previous similar studies.^{52,56,57}

Hematological abnormalities and body temperature reduction are the common features of *P. berghei* infected rodents.^{58,59} *P. berghei* infection increases erythrocyte fragility and significantly reduces packed cell volume in mice.⁶⁰ In all test models, the higher doses of the crude extract (200 and 400mg/kg) showed a significant ($p < 0.05$) protective effect against temperature reduction as compared to the negative control. The possible mechanism for the prevention of body temperature reduction could be the reduced level of parasitemia as a reduction of temperature is directly related to the increased level of parasitemia. The packed cell volume was determined to assess the effectiveness of the extract of *A. polystachyus* in preventing hemolysis as a result of a rising parasitemia level. The basic causes of anemia in humans and rodents comprise, the clearance of uninfected RBC, dyserythropoiesis, erythropoietic suppression, and the clearance and/or destruction of infected RBCs. In the negative control group, the parasitemia level was increased, while the hematocrit packed cell volume was decreased noticeably from day to day till the death of the mice, which was also reported in previous similar studies.^{61,62}

In vivo antimalarial activity can be classified as very good, good, and moderate if the plant extract showed the percent of parasite suppression $\geq 50\%$ at the dose of 100mg, 250mg, and 500mg/kg body weight per day, respectively.⁶³ According to this classification, the leaves extract of *A. polystachyus* revealed a moderate antimalarial activity.

Conclusion

The results obtained from the present study revealed that the extract of *A. polystachyus* has antiplasmodial activity as evidenced by their ability to suppress *P. berghei* infection in mice in a dose-dependent manner. The results also revealed free radical scavenging activity by the extract which may contribute to the antimalarial activity. This confirms the traditional antimalarial claim of *A. polystachyus*. Thus, the extract of *A. polystachyus* leaves might serve as a new source for the development of a novel antimalarial agent. However, further fractionation, isolation, and characterization of the extract are required to identify the accountable lead compound (s) with the potential of antimalarial activity.

Data Sharing Statement

The data sets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics Approval

Ethical clearance was obtained from the research and ethics committee, department of pharmacology, University of Gondar to conduct the study in animal model with a Reference number of SOP 3/81/10. Experimental procedures were done on Swiss albino mice according to the internationally accepted laboratory animal use and care guideline (Guide for the care and use of laboratory animals). However, no consent was needed for this study.

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Disclosure

The authors report no conflicts of interest for this work.

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