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Antiparasitic Activity of *Piliostigma thonningii* and *Alstonia boonei* on Malaria-Infected Albino Mice

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Abstract

Background: Malaria is a significant parasitic disease in Nigeria and across Africa, posing a risk to everyone. **Objective:** The study assessed the in vivo antiplasmodial activity of the aqueous extracts of *Piliostigma thonningii and Alstonia boonei, and their combinations, against malaria infection in* albino mice. **Methodology:** The in vivo antiplasmodial activity of aqueous extracts of *Piliostigma thonningii* and *Alstonia boonei*, alone and in combination, was assessed against chloroquine-sensitive *Plasmodium berghei* in mice. Curative assays were conducted on established infections via orogastric administration (0.2-0.6 ml). Chloroquine and distilled water served as positive and negative controls, respectively. **Results:** Phytochemical analysis revealed the presence of various compounds such as saponins, flavonoids, alkaloids, phenolics, glycosides, anthraquinones, tannins, and steroids in both plant extracts. There was a significant reduction in parasitaemia loads after treatment, particularly with the combined extract, which exhibited the highest curative effect. No significant differences were observed in body weight (15.0±0.59, 17.4±0.65, 19.0±5.96, 17.6±2.27, 15.0±5.25, 19.0±5.96, 15.3±1.41, 14.5±0.41, 16.8±1.68, 15.1±1.00) and temperature (35.3±0.25, 36.2±0.12, 36.7±0.53, 36.3±0.92, 35.3±0.25, 36.3±1.14, 35.0±0.43, 35.3±1.35, 36.1±0.98, 36.9±1.55) among the treatment groups, respectively. Overall, the combined plant extracts demonstrated promising anti-malarial activity compared to individual extracts and the standard drug. **Conclusion**: In conclusion, the present study showed that the aqueous extracts of the two medicinal plants are potent in causing a reduction in malaria parasite load. The findings from this study showed that the two herbs (*Alstonia boonei* and *Piliostigma thonningii*) are promising antimalarial herbs and could be explored in the manufacture of potent antimalarial drugs. **Recommendations:** Future research could centre on isolating and identifying the specific bioactive compounds responsible for the antiplasmodial activity, investigating their mechanisms of action, and also evaluating the extracts against drug-resistant *Plasmodium* strains.

Keywords: Parasitaemia, Standard drug, Plant extract, saponins, anthraquinones, Chloroquine

Introduction

Malaria is a significant parasitic disease affecting humans globally, predominantly endemic to Africa, Asia, and the Americas. The burden of malaria morbidity falls significantly on Africa, where approximately 200 million cases (92%) were recorded in 2017 (WHO, 2017). The African region continues to share a disproportionately high burden of malaria, accounting for 95% of all malaria cases and 96% of deaths in 2020, with children under five years old accounting for 80% of all malaria-related deaths in the region (Tabuti, 2017; Akanbi *et al.,* 2019). The impact of malaria on African children after 2000 has been far greater than previously believed, as shown by applying the statistical approach. By 2020, half of the global population was at risk of contracting malaria. Certain groups face a higher risk of contracting malaria and developing severe disease, including patients with HIV/AIDS, pregnant women, infants, children under five, and individuals with weakened immune defences who travel to malaria-endemic regions, such as

migrant workers, mobile populations, and travellers (WHO, 2021).

Malaria is caused by Plasmodium parasites, which are transmitted to humans through the bites of infected female Anopheles mosquitoes. Five different species of parasites are responsible for human malaria, two of which*, P. falciparum*, pose particular dangers and are common in Africa. The predominant malaria parasite outside sub-Saharan Africa is *P. vivax*, which poses higher risks alongside *P. falciparum* (WHO, 2021; Dkhil *et al.,* 2021). The first symptoms of malaria, including fever, chills, and headache, typically emerge within 10 to 15 days following the bite of an infected mosquito. These symptoms can be moderate and challenging to diagnose as malaria. If untreated*, P. falciparum* malaria can lead to severe illness and death in less than 24 hours. Countries such as Nigeria (31.9%), the Democratic Republic of the Congo (13.2%), the United Republic of Tanzania (4.1%), and Mozambique (3.8%) together account for slightly more than half of all malaria deaths worldwide (provide references to the claim). As one of the primary diseases in developing countries, malaria caused 219 million cases worldwide (WHO, 2017). It is endemic in many tropical regions, with ongoing transmission occurring in 85 countries and territories (WHO, 2021). According to the World Health Organisation (WHO), there were 241 million cases and 627,000 deaths from malaria in 2020. This increase is compared to a 2019 forecast of 227 million cases and 558,000 deaths (WHO, 2022). The rise in morbidity and mortality can be traced back to service interruptions due to the COVID-19 pandemic and a revised methodology for calculating the malaria burden (WHO, 2022).

With improved calculations by WHO, malaria now accounts for 7.8% of the global disease burden, compared to the previously reported 4.8%. The emergence of partial resistance to artemisinin in *Plasmodium falciparum* in the African region is concerning, alongside increasing reports of resistance to pyrethroid insecticides among Anopheles species. Licensing the Real-time Strategy (RTS), S malaria vaccine in 2021 represents a promising development (WHO, 2022).

A staggering 99% of malaria deaths are attributed to *Plasmodium falciparum* malaria (WHO, 2015; Alebie *et al.,* 2017). Nigeria, in particular, accounts for over half of all malaria cases in Africa (WHO, 2021). In southern Nigeria, malaria transmission occurs year-round, while in the north, it is more seasonal, as environmental conditions are less favourable for the growth of malaria parasites. Approximately 76% of the Nigerian population lives in high transmission areas, while 24% reside in low transmission regions. In the southern part of the country, transmission can last all year, whereas it is approximately three months or less in the northern regions. The primary vector for malaria across most of Nigeria is the Anopheles gambiae mosquito (USAID, 2020). The 2020 World Malaria Report indicates that Nigeria had the most cases of malaria worldwide (27% of cases worldwide) in 2019. It has the highest number of deaths (23 % of global malaria deaths) (WHO, 2019). Case numbers increased by 3.5% between 2016 and 2019, from 293 to 303 per 1000 of the population at risk. However, deaths decreased 16% at that same time, from 0.57 to 0.47 per 1000 of the population at risk (WHO, 2019). *Plasmodium falciparum*, a protozoan species found as the most common cause of malaria in the country, is regarded as a single infectious disease and the leading cause of mortality pathogen on the globe (Akanji *et al.*, 2016).

Artemisinin-combined therapies (ACT) were formally adopted as Nigeria's first-line treatment of uncomplicated malaria in 2005. However, Artemisinin-based combination therapies (ACT) are limited due to their high costs, limited production of artemisinin derivatives to Good Manufacturing Practices (GMP) standards, and toxicity (Boareto *et al.*, 2019). Neither the Cinchona nor *Artemisia annua* plants, from which the most potent drugs (quinine and artemisinin) were isolated, are indigenous to sub-Saharan Africa. Ethiopia is the home of a diverse flora used as traditional medicine. Few of them are scientifically studied as a source of drugs. The research on Ethiopian

medicinal plants has focused chiefly on producing checklists and inventories; only a few have been touched by modern research. Its principal component has been analysed and defined (Assefa *et al.,* 2019). Rat parasites have led us to design this study due to their genome sequence and pathology similarity to that of human parasites (Carlton *et al.,* 2021 The increasing resistance of malaria parasites, such as *Plasmodium falciparum*'s resistance to chloroquine, is a growing concern in developing countries (Trape *et al.*,2021). Due to the scarcity and affordability of orthodox medicine in many tropical countries, most populations now depend on traditional/ medical remedies from plants, which have been revealed to be a rich source of new drugs (Murshed *et al.*, 2024). The majority of the widely used anti-malarial drugs, such as quinine and artemisinin, have also been developed from plants. Some were produced chemically using plant-derived compounds as a pattern. Plants' most significant bioactive constituents are alkaloids, tannins, flavonoids, and phenolic compounds (Chiyaka *et al.*, 2019). According to the WHO, about 70% of the world's population relies on herbal preparations to treat diseases (WHO, 2019). Plants have been revealed to be a rich source of new anti-malarial traditional remedies, still offer new tracks for identifying promising antiplasmodial molecules, and a way to ensure that all people have access to care (Mamedov, 2019). According to an ethnobotanical survey in Nigeria, 98 species of plants are used in traditional medicine, singly or in combination, by different families to treat malaria and/or fever (Adebayo & Krettli, 2016). The plants used to treat and control malaria comprise more than half of Nigeria's medicinal species (Bankole *et al.*, 2016). Limited literature has shown that many of these plants have been scientifically investigated for anti-malarial properties in *vivo* (Bankole *et al.*, 2016). Herbal medicines have often justified their popularity for historical and cultural reasons and their lower costs. The term medicinal plant refers to various plants with medicinal properties. Plants are a rich source of compounds that can be used to develop drug synthesis (Rasool Hassan, 2020). The parts of medicinal plants used are seeds, roots, leaves, fruit, skin, flowers, or even the whole plant. The active compounds in most parts of medicinal plants have direct and indirect therapeutic effects, which enable them to be used as medicinal agents. In the body of these plants, certain materials that are referred to as active compounds (substances) are produced and stored, which have physiological effects on living organisms (Jamshidi-Kia *et al.,* 2024). Humans mainly depend on raw plant materials to meet medical needs, maintain health, and cure diseases (Jamshidi-Kia *et al.,* 2024), using their ethnobotanical information.

Plants are now selected based on their use in indigenous medicine against fever and malaria (Budiarti *et al.,* 2020). To a great extent, this has reduced the unnecessary waste of time and resources encountered in the former approach. Nigeria boasts a diverse array of flora, with various indigenous communities relying on numerous plant species for their medicinal properties. In the southern region, characterised by lush rainforests and a humid tropical climate, many medicinal plants are utilised to combat malaria, which is persistently transmitted in these ideal conditions. Thus, the study's objectives were to determine the phytochemical analysis and antioxidant activities of *Piliostigma thonningii and Alstonia boonei*; determine the antiplasmodial activities of the two medicinal plants using *Plasmodium berghei* chloroquine-sensitive mice; and evaluate the parasitaemia load in experimental animals

MATERIALS AND METHODS

Study Area

The study was carried out in Osogbo, Osun State, Nigeria. Osogbo lies at a latitude of 7º49'N and a longitude of 4º37'E. An ethnobotanical survey was carried out among the traditional herb vendors in the major herbs-selling market at Oja-oba to identify the frequently and not frequently used antimalarial plants.

Plant Materials Collection and Authentication

The plants' samples were collected from their natural habitat. The medicinal plants used in this experiment were fresh leaves of *Piliostigma*

thonningii (Caesalpiniacea*), Alstonia boonei*. (Apocynaceae) which were collected from Osogbo in Osun State, Nigeria, respectively, in June 2021. Identification and authentication were carried out in the Herbarium of the Department of Botany in Obafemi Awolowo University (OAU) Ile Ile-Ife.

Plant Extraction

The plant sample stems were removed, leaving only the leaves, and washed. The leaves were air-dried at room temperature (30 ±0.5 °C) until they dried for 5 (five) weeks, pulverised to a coarse powder using a laboratory blender, sieved to a fine powder, and put into an already sterilised container, then labelled accordingly. The plant parts (5g) were extracted exhaustively by mixing with 100 ml of distilled water and allowed to boil for 45 minutes. The resulting extract was left to cool and filtered using Whatman filter paper. The filtrates were kept in a tightly sealed container, maintained in a refrigerator to protect from sunlight and moisture till use for phytochemical and other analysis (Afolabi &Abejide, 2020).

Phytochemical Analysis of Medicinal Plants (*alstonia boonei* and *piliostigma thonningii*)

Chemical evaluations for the detection of bioactive chemical components of the two medicinal plants were carried out in aqueous extracts using the standard procedures as described by Shahzad *et al.* (2022) and Jimoh *et al* (2021).

Test for Tannins

Each plant extract (0.5 g) was weighed into a beaker, stirred with 10 ml of distilled water, and then filtered. The presence of tannins was determined by adding a few drops of 1% ferric chloride solution to 2 ml of the filtrate, resulting in a blue-black, green, or blue-green precipitate.

Test for Steroids

For each plant extract (0.2 g), 2 ml of acetic acid was added, and the solution was cooled well in ice, followed by the careful addition of concentrated H_2SO_4. The development of colour from violet to blue or bluish-green indicated the presence of a steroidal ring, i.e., a glycone portion of cardiac glycoside.

Test for Terpenoids

A little portion of each plant extract was dissolved in ethanol. To it, 1 ml of acetic anhydride was added, followed by the addition of concentrated H_2SO_4. A change in color from pink to violet showed the presence of terpenoids.

Test for Saponins

One gram of each portion of the samples was boiled with 5 ml of distilled water and then filtered. About 3 mL of distilled water was added to the filtrate, and the resulting solution was shaken vigorously for about 5 minutes. Frothing, which persisted on warming, showed the presence of saponins.

Test for Flavonoids

Each plant extract (0.2 g) was dissolved in ethanol, warmed, and filtered. Three magnesium chips were added to the filtrate, followed by a few drops of concentrated HCl. A pink, orange, or red colour, which changed to purple, indicated flavonoids' presence.

Ferric Chloride Test for Flavonoids

0.5 g of each plant extract was boiled and filtered with distilled water. A few drops of 10 % ferric chloride solution were added to 2 ml of the filtrate. A green-blue or violet colouration indicated the presence of a phenolic hydroxyl group.

Test for Glycosides

One millilitre of concentrated H_2SO_4 was prepared in a test tube, and 5 ml of aqueous extract from each plant sample was mixed with 2 ml of glacial CH_3CO_2H containing one drop of $FeCl_3$. The resulting mixture was carefully added to 1 ml of concentrated H_2SO_4, ensuring that the concentrated H2SO4 remained beneath the mixture. A brown ring appeared to indicate the presence of a cardiac glycoside.

Test for Alkaloids

0.2 g of each plant extract was added to a beaker, stirred with 5 ml of 1 % aqueous HCl on a water bath, and then filtered. From the filtrate, 1 mL was transferred to two test tubes. To the first portion, a few drops of Dragendorff's reagent were added; the occurrence of orange-

red precipitate was taken as positive. To the second 1 ml, Mayer's reagent was added, and the appearance of buff-coloured precipitate indicated the presence of alkaloids.

Test for Cardiac Glycosides (Keller-Killani Test)

Five millilitres of each plant extract were combined with two millilitres of glacial acetic acid and one drop of ferric chloride solution. Concentrated sulfuric acid in the amount of one millilitre was added. The cardenolides' deoxysugar trait was identified by a brown interface ring. Below the brown ring, a violet ring may emerge, and in the thin layer of acetic acid, a greenish ring may slowly form.

Test for Anthraquinone Borntrager

A 0.5 g of the plant extract was put in a beaker and shaken, with the benzene layer separated and half of its volume of 10% ammonia solution added. A pink, red, or violet colouration in the ammoniacal phase indicated the presence of anthraquinone.

Test for Phenolic Compounds

The plant extracts (500 mg) were dissolved in 5 mL of distilled water. A few drops of neutral 5 % ferric chloride solution were added to this. A dark green colour indicated the presence of phenolic compounds.

In-vitro antioxidant assay of medicinal plant extract

The extract's antioxidant capacity was assessed in vitro by determining the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (Jimoh *et al.,* 2021) of the individual and various combinations of the extracts. The aqueous extract of two leaves or samples was prepared at different concentrations (2, 4, 6, 8, and 10 ml) and was mixed with 1.0 ml of reagent solution (0.004 g of DPPH in 100 ml of methanol). The control contained only DPPH solution in place of the sample, while methanol was used as the blank. The mixture was vigorously shaken and incubated at room temperature for 30 minutes. After 30 minutes, the decrease in absorbance of the test mixture (due to quenching of DPPH free radicals) was read at 517 nm against ascorbic acid (control). The scavenging effect was determined (Jimoh *et al*., 2021).

Quantitative determination of reducing sugar concentration

The concentration of the extracts varied accordingly (2, 4, 6, 8, and 10 ml). The reducing sugar concentration was determined by adding 1 ml of 3,5-dinitrosalicylic acid (DNS reagent) to 1 ml of the extract and boiling for 5 minutes. The reducing sugar was determined at 540 nm using a JENWAY6300 Spectrophotometer (Jimoh *et al*., 2021).

Collection of experimental animals and *plasmodium berghei* donor mice

The mice used for the experiment were obtained from the Institute for Advanced Medical Research and Training (IAMRAT), College of Medicine, the University of Ibadan (UCH), Oyo State. The weight and temperature of the mice were recorded, and the mice were grouped on the same day. Fifty-five (55) albino mice weighing 18 to 24 g of either sex and aged 6–8 weeks were kept in the Animal House, Biological Sciences Department, Fountain University, Osogbo, Osun State, Nigeria. The animal attendants practised hygiene by constantly cleaning and removing faeces from cages every 4(four) days. According to current ethical standards for laboratory animals, the animals were handled and cared for under conventional laboratory circumstances. After 1 (one) week of acclimatisation to the experimental environment, before the actual experiment, the animals were fed on a rodent diet and given free access to drinking water.

Malaria parasite and preparation of inoculum

Donor chloroquine-sensitive *Plasmodium berghei*-infected mice collected from IMRAT were subjected to chloroform in a container, and the blood was collected by heart puncture. The blood was taken from donor mice and diluted with normal saline. An aliquot of 0.2 ml of (2x 107 parasitised erythrocytes) of this suspension was injected intraperitoneally into experimental mice. Each animal received an inoculum of about 10 million parasites per kilogram body

weight, which is expected to produce a steadily rising infection in mice.

Treatment Regime

In-vivo evaluation of antimalarial activity of the plant extracts

The experimental animals were inoculated intravenously with 2×10^7 red blood cells infected with the Chloroquine (CQ) sensitive *P. berghei* ANKA strain. The first day of inoculation was regarded as Day 0. Post-infection treatment was given after three days of inoculation. The standard four-day suppressive method was employed in studying the plant extracts' antiplasmodial activities. Forty mice were randomly selected into twelve groups of three per cage, except for the negative control group, which had 5 per group. Three separate dosages of 0.2, 0.4, and 0.6 ml of the test extracts were produced. Each extract was administered as a single dose per day. The CQ, as a standard drug and vehicle (distilled water), was through orogastric administered to the mice. Furthermore, the treatment was done for five days.

Determination of body weight (g) and temperature (°c) before and after the experiment

The mice's body weight and temperature were measured according to Dada and Oloruntola's instructions (Cheesbrough, 2014). Weighing each mouse in each group with a delicate digital balance allowed us to determine their body weight, and a digital thermometer was used to measure the mice's rectal temperatures. The extracts' antimalarial activity was determined using Peter's 4-day suppressive test (Adebayo *et al.,* 2020).

$$\text{mean body weight} = \frac{\text{Body weight of the mice in a group}}{\text{Total number of mice in that group}} \quad \text{-- (1)}$$

It was done before and after treatment.

Table 1: Experimental plan for test animals and control

Group names	Experimental plan
GRP 1A	Group of mice treated with 0.2ml of *Piliostigma thonningii*
GRP 1B	Group of mice treated with 0.4ml of *Piliostigma thonningii*
GRP1C	Group of mice treated with 0.6ml of *Piliostigma thonningii*
GRP2A	Group of mice treated with 0.2ml of *Alstonia boonei extract*
GRP2B	Group of mice treated with 0.4ml of *Alstonia boonei extract*
GRP2C	Group of mice treated with 0.6ml of *Alstonia boonei extract*
GRP3A GROUP 3 SYNERGY GROUP, i.e., a mixture of extracts	Group of mice treated with 0.2ml of Piliostigma *thonningii* and *Alstonia boonei,* the two extracts of the same concentration
GRP3B	Group of mice treated with 0.4ml of *Piliostigma thonningii* and *Alstonia boonei* extract
GRP3C	Group of mice treated with 0.6ml of *Piliostigma thonningii* and *Alstonia boonei* extract
GRP4	The negative control group were given distilled water (uninfected group
GRP5	The positive control group were given Chloroquine (CQ)
GRP6	And the last group was infected but not treated. But was given distilled water and food alone

(A-green, B-blue and C-red) for identification.

Plate 1: inoculated mice (A-green, B-blue and C-red) for identification.

Parasitaemia Determination

A drop of blood was collected from each infected mouse for parasitaemia screening by tail nip. The blood was placed on a clean glass slide and smeared to make a thick and thin film, fixed with methanol and stained with Giemsa. When dried, the film was viewed under an oil immersion lens×100 magnification on a microscope. The parasitaemia density was evaluated by counting the parasitised red blood cells. Using the Trans *et al.* (2019) approach, the difference between the mean values of the experimental and control groups (designated as 100%) will be calculated and expressed as a percentage suppression of parasite growth, as shown in the equation below. The following formula was used to determine the percentage of parasitaemia suppression.

$$\%\text{parasitaemia} = \frac{\text{Total number of parasitised red blood cells}}{\text{Parasitised red blood cells}} \times 100 \text{ ------- (2)}$$

$$\%\text{chemo suppression} = \frac{\text{Parasitaemia of control group} - \text{parasitaemia in study group}}{\text{Parasitaemia of control group}} \times 100 \text{ ------ (3)}$$

Statistical Analysis

Mean parasitaemia was determined in the control and treated groups. The mean parasitaemia was compared using the student's t-test. Per cent suppression of parasite growth of the treated and control groups was compared using one-way ANOVA and two-tailed Student's t-test (GraphPad Prism 4.0, GraphPad Software, San Diego, USA) at $p<0.05$ considered.

Results

Qualitative phytochemical analysis of medicinal plants (*Alstonia boonei* and *Piliostigma thonningii*)

The qualitative phytochemical analysis of the two aqueous extracts (Table 2) showed the presence of secondary metabolites such as saponins, flavonoids, alkaloids, phenolics, glycosides, anthraquinones, as well as cardiac glycosides, tannins, steroids, while phylobatannins and Fehling were absent. Phytochemical studies of *P. thonningii* and *A. boonei* revealed the presence of diverse chemical classes of compounds that possibly accommodate the various activities of these medicinal plants, which are helpful for the treatment of malaria.

Antioxidant assay 2,2-Diphenyl-1-PicrylHydrazyl (DPPH - nm) radical scavenging activity of the two medicinal plant extracts

A. boonei and P. thonningii extracts were investigated for potential free radical inhibitors and degree of reactive oxygen species (ROS) such as hydrogen peroxide, superoxide radical anion, hydroxyl radical, nitric oxide and singlet oxygen. The result shows the DPPH radical scavenging activity (% Inhibition) of the antimalaria herbal extract across the concentrations; the scavenging activity level of the two herbal extracts was numerically higher than that of the standards (Ascorbic acid). As the concentration increases, the scavenging property of the herbal extract also increases. *Alstonia boonei* herbal extract shows higher radical scavenging activity than *Piliostigma thonningii* extract. (Table 3).

Quantitative determination of the reducing sugar concentration of the plant extracts

The result showed a reduction in sugar activity in the anti-malaria herbal extract. The herbal extract's activity levels were numerically higher than the standards across the concentrations studied. As the concentration increases, the herbal extract's sugar property also increases (Tables 4 and 5).

Determination of body weight (g) and temperature (^{o} C) of experimental mice before and after the experiment

Body weight and temperature analysis of experimental mice (Table 6) showed that the mice treated with 0.4ml of P. thonningii and 0.4ml of A. boonei had a statistically significant ($p < 0.05$) final weight gain compared to the control and all other treatment groups subjected to different concentrations of the two herbal extracts. Similarly, there were no significant differences in weight gain between the mice administered with 0.2ml, 0.6ml of P. thonningii, and 0.4ml of the combined extract.

The mice treated with 0.2 ml, 0.4 ml, and 0.6 ml of *A. boonei*, as well as 0.2 ml and 0.6 ml of the combined extract, showed no significant differences ($p>0.05$) in final weight gain compared to the control. Additionally, there were no significant differences ($p>0.05$) in the final body temperature of the mice across different concentrations of the herbal extracts when compared with the control. The results indicated no significant difference ($p>0.05$) in body weight and temperature compared with the control after treatment. In summary, all five groups exhibited comparable values to the control in final weight gain, suggesting that the extract doses do not negatively affect body weight. Weight loss might be attributed to decreased appetite, reduced food intake, or disrupted metabolic functions associated with malaria. Regarding temperature, there was a general increase across all treatment groups, including the control. However, the observed temperatures remained within normal ranges. Furthermore, no significant differences in body weight or temperature were observed among the treatment groups, indicating the safety of the plant extracts.

Table 2: The qualitative phytochemical composition of the aqueous extracts of *Piliostigma thonningii (Ewe Abafe) and Alstonia boonei (Ewe Ahun),* respectively.

Phytochemicals	*Piliostigmathonningii* (Ewe Abafe)	*Alstonia boonei* (Ewe Ahun)
Flavonoid	+	+
Alkaloid	+	+
Phenol	+	+
Tannin	+	+
Reducing sugar	+	+
Steroid	+	+
Terpenoid	+	+
Saponin	+	+
Fehling	-	-

Glycoside	+	+
Phylobatannins	-	-

Remarks: + = Presence of phytochemical, - = Absence of phytochemical.

Table 3: 2,2-DiPhenyl-1-Picryl Hydrazyl (DPPH - nm) radical scavenging activity of (GRP1) *Piliostigma thonningii (Ewe Abafe) and (GRP2) Alstonia boonei (Ewe Ahun)* extracts.

Tested material	20%	40%	60%	80%	100%
Piliostigma thonningii	27724	31000	31400	32400	33325
Alstonia boonei	34925	37900	41775	42175	45800
Ascorbic acid	1475	1350	1325	1275	1225

Note: *Piliostigma thonningii*=plant extract for group 1, *Alstonia boonei*=plant extract for group 2, Ascorbic acid= standard

Table 4: Reducing sugar activity of the medicinal plants *(Piliostigma thonningii and Alstonia boonei)*.

Tested material	20%	40%	60%	80%	100%
Piliostigma thonningii	0.341	0.585	0.618	0.757	0.934
Alstonia boonei	0.294	0.559	0.722	0.823	1.026
Glucose	0.272	0.214	0.277	0.254	0.262

Note: *Piliostigma thonningii*=plant extract for group 1, *Alstonia boonei*=plant extract for group 2, Glucose= standard

Table 5: Reducing sugar concentration of group 1and group 2 extracts.

Tested material	20%	40%	60%	80%	100%
Piliostigma thonningii	3.410	5.850	6.180	7.570	9.340
Alstoniaboonei	2.940	5.590	7.220	8.230	10.260
Glucose	2.720	2.140	2.770	2.540	2.620

Note: *Piliostigmathonningii*=plant extract for group 1
Alstonia boonei=plant extract for group 2
Glucose= standard

Table 6: Body weight and temperature analysis of experimental mice during treatment with the aqueous extract of the two medicinal plants.

	CONTROL	GRP1A	GRP1B	GRP1C	GRP2A	GRP2B	GRP2C	GRP3A	GRP3B	GRP3C
Initial weight (g)	24.8±0.06[a]	22.5±0.97[a]	22.8±1.12[a]	23.8±1.65[a]	21.9±2.79[a]	23.2±1.55[a]	23.0±1.91[a]	24.2±0.56[a]	24.0±1.12[a]	22.1±2.37[a]
Final weight(g)	15.0±0.59[c]	17.4±0.65[b]	19.0±5.96[a]	17.6±2.27[b]	15.0±5.25[c]	19.0±5.96[a]	15.3±1.41[c]	14.5±0.41[c]	16.8±1.68[b]	15.1±1.00[c]
Initial temp (^{o}C)	34.4±0.31[a]	35.9±0.98[a]	34.2±1.53[a]	35.8±0.86[a]	36.3±1.14[a]	35.0±0.43[a]	22.1±2.37[b]	35.4±0.58[a]	34.7±0.51[a]	35.0±0.22[a]
Final temp(^{o}C)	35.3±0.25[a]	36.2±0.12[a]	36.7±0.53[a]	36.3±0.92[a]	35.3±0.25[a]	36.3±1.14[a]	35.0±0.43[a]	35.3±1.35[a]	36.1±0.98[a]	36.9±1.55[a]

Values mean ± standard deviation; there is no significant difference amongst the treatment group ($p \geq 0.05$).
NOTE: Initial (Before Treatment), Final (After Treatment) a, b and c are superscripts within the same rows that are significantly different.
GRP1: Group of mice treated with of *Piliostigma thonningii*, GRP2: Group of mice treated with of *Alstoniaboonei extract*, GRP3*:* Group of mice treated with *Piliostigmathonningii and Alstoniaboonei*, Control: Group of mice treated with Chloroquine, (A=0.2, B=0.4 and C=0.6ml of extracts)

Table 7: Comparative antiplasmodial activity of *piliostigma thonningii*, *alstonia boonei*, the combined recipe of plant aqueous extracts and chloroquine (a standard drug) against *plasmodium berghei* infected mice.

	CONTROL	GRP1A	GRP1B	GRP1C	GRP2A	GRP2B	GRP2C	GRP3A	GRP3B	GRP3C
Mg/KG	10	200	400	600	200	400	600	200	400	600
Before treatment	5.40±0.34[b]	2.40±0.24[e]	4.40±0.24[c]	5.40±0.25[b]	3.80±0.37[d]	4.50±0.26[c]	6.00±0.34[a]	3.20±0.37[d]	3.80±0.95[d]	5.90±0.49[a]
After treatment	0.00±0.00[e]	1.20±0.56[b]	1.30±0.52[b]	1.30±0.32[b]	1.00±0.32[c]	0.40±0.24[d]	1.60±0.40[a]	0.80±0.37[c]	0.40±0.24[d]	0.40±0.24[d]
% Curative	100	50	70	76	74	91	73	75	89	93

Values are presented as means ± standard deviation. Values that carry different superscript letters along the row are significantly different; 5%. Concentration unit is in mg/kg.200-0.2, 400-0.4 and 600-0.6ml. Statistically significant differences ($P<0.05$) compared to control group are indicated by a,b,c,d,e and f superscript
GRP1: Group of mice treated with of *Piliostigma thonningii*
GRP2: Group of mice treated with of *Alstonia boonei extract*
GRP3: Group of mice treated with *Piliostigma thonningii and Alstonia boonei*
Control: Group of mice treated with Chloroquine. (A=0.2, B=0.4 and C=0.6ml of extracts)

Parasitaemia determination

Initially before treatment, the comparative antiplasmodial activity of *Piliostigma thonningii*, *Alstonia boonei* and the combined aqueous extract (Table 7) showed that the mice treated with 0.6ml of *A. boonei* and 0.6ml of the combined extract had a higher statistical significant parasitaemia counts or antiplasmodial activity when compared with the control and other group, whereas, the mice treated with 0.6ml of *P. thonningii* had no statistical significant ($p > 0.05$) antiplasmodial activity compared with the control. However, the mice treated with every other extract concentration had marked statistically significant differences ($p<0.05$) compared with the control. After treatment, the experimental mice administered with 0.6ml of *A. boonei* had a higher statistically significant parasitaemia count ($p<0.05$) when compared with the control. They were significantly different from every other treatment group. Numerically, it was observed that the two medicinal plants showed significant ($p<0.05$) chemosuppression of parasite growth on day four (4) post-infection in mice infected with *P. berghei*. The survival rate of the mice that received extracts increases as the dose increases to 0.6 mL. Animals treated with *Piliostigma thonningii* extract show the highest parasitaemia level before and after treatments. In contrast, the mice treated with *Alstonia boonei* showed a marked reduction in the parasitaemia count compared to the first extract, except for the group treated with 0.6 ml of *Piliostigma thonningii* (76%). The combined group showed significantly higher ($p < 0.05$) parasitaemia count, i.e., a higher curative percentage (75, 89, and 93%), and no parasitaemia count was observed in the control group after treatment (Table 7).

Discussion

Malaria is a parasitic disease that can be lethal, especially in tropical regions of Africa and Asia. It is thought to kill more people than AIDS, and children under the age of five are most susceptible (WHO, 2018). In Africa, a herbal that often consists of two or more plants consumed as a concoction/extract is used to treat or prevent malaria (Oludele & Olufunso, 2018). Herbal medicine is becoming popular as an alternative to orthodox medicine. Still, scientific validation of their efficacies in treating diseases is relatively unknown (Lotfian *et al.,* 2019).

The qualitative phytochemical analysis of the aqueous extracts via research has shown the presence of secondary metabolites capable of treating malaria. Antimalarial and therapeutic properties of the medicinal plants have been attributed to the presence of different classes of secondary metabolites or phytochemical compounds in them, and the leaves were the popular plant part used for the experimental malaria research due to the availability of several active compounds (Asafo-Agyei *et al.,* 2019). Phytochemicals such as flavonoids, alkaloids, terpenes, saponins, cardiac glycosides, among others, have exhibited antiplasmodial activity through various mechanisms of action. The observed absence of phylobatannins in the two herbal extracts is in line with the report of Misganaw (2022).

Antioxidants are chemicals that prevent or lessen the effects of free radicals. Free radicals are waste or toxic substances produced by cells, such as reactive oxygen species (ROS), including hydrogen peroxide, superoxide radical anion, hydroxyl radical, nitric oxide, and singlet oxygen. Specific plant-based products are thought to be rich in antioxidants (Uddin *et al.,* 2020; Jayasri *et al.,* 2018). It has been reported by Atanu *et al.* (2021) that *Alstonia boonei* and *Carica papaya* are usually used to treat malaria. Statistically, there is an increase in the scavenging abilities of two herbal extracts when compared with the standards (Ascorbic acid) in this study.

The observed higher statistically significant final weight gain in the mice treated with 0.4 mL of *P. thonningii* and 0.4ml of *A. boonei* is contrary to the report of Omoya *et al. (*2018), which used *Alchornea laxiflora* extract on *plasmodium berghei-infected* mice. Furthermore, no significant difference ($p>0.05$) in the loss of body weight as compared with the control, which implies that the

extract at this dose does not harm body weight. However, it might be due to loss of appetite, reduced food intake, and disturbed metabolic functions associated with malaria (Afolabi and Abejide, 2021). The crude extract did not cause hyperthermia in the experimental mice, as there were no significant differences ($p>0.05$) in final body temperature compared with the control group. This follows the previous reports by Omoya et al. (2019) and Afolabi & Abejide (2021).

The leaf extracts of *Alstonia boonei* that showed higher parasitaemia suppression than other extracts could be due to the presence of phytochemical compounds such as terpenoids, alkaloids, phenols, and flavonoids, which are thought to be responsible for the antiplasmodial activity of most plants (Omoya *et al.,* 2019). The aforementioned cited researchers, as well as Odugbemi and Akinsulire (2022) and Gbadamosi *et al*. (2021), confirmed the traditional use of *A. boonei* for treating malaria in Southwestern Nigeria. They also reported the antimalarial activity of various fractions of the *A. boonei*. The significant decrease in parasitaemia observed in this study was dose-dependent. The highest chemo-suppression observed in the chloroquine treatment group may be due to the parasite's sensitivity to chloroquine and to the purified preparation used, compared with the crude extract used. Similar observations were reported by Olandunmoye *et al* (2011) and Alaribe *et al. (*2020). Adewale *et al.* (2021) disclosed that various researchers proclaimed that most herbal extracts not only achieved chemosuppressive but also total curative effects on malaria parasites. The observed result is in tandem with the findings of Afolabi and Abejide (2021), who used Alstonia boonei and Morinda lucida in mice infected with Plasmodium berghei and reported similar results.

Conclusion: The present study showed that the aqueous extracts of the two medicinal plants are potent in reducing malaria parasite load. The findings from this study showed that the two herbs (*Alstonia boonei* and *Piliostigma thonningii*) are promising antimalarial herbs and could be explored in the manufacture of potent antimalarial drugs.

Recommendations: Identifying and isolating bioactive components from these medicinal plants (*Alstonia boonei* and *Piliostigma thonningii*) is a promising area that can be investigated to develop non-toxic, effective antimalarial drugs, especially for children. Moreover, herbal extracts with antipyretic, nutritional supplement, and antimalarial effects, or a combination of those extracts, will be potent in eliminating malaria infection.

Scientific implications of the study: If the plant extracts demonstrate significant antimalarial activity, they could serve as a source of novel antimalarial compounds. Further research can focus on isolating and characterising the specific bioactive compounds responsible for the observed effects.

Limitations to the study: Albino mice infected with *Plasmodium berghei* are a common model, but there are some physiological and immunological differences between mice and humans infected with *Plasmodium falciparum* (the primary cause of human malaria). This can affect drug pharmacokinetics (how the body processes the drug), pharmacodynamics (how the drug affects the parasite).

Conflict of interest: No conflict of interest

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