Antimalarial effects of ethanolic extracts of Andrographis paniculata leaves and Allium sativum bulbs on Plasmodium berghei (NK65)-induced Parasitemia in Albino mice

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Abstract

Malaria remains a major cause of morbidity and mortality in sub-Saharan Africa, disproportionately affecting children and pregnant women. The increasing resistance to conventional antimalarial drugs underscores the urgent need for alternative therapies. Traditional medicinal plants such as *Andrographis paniculata* and *Allium sativum* have shown promise, but their combined efficacy has not been adequately explored. This study aimed to evaluate the antiplasmodial effects of combined ethanolic extracts of *Andrographis paniculata* leaves and *Allium sativum* bulbs on *Plasmodium berghei* (NK65)–induced parasitemia in albino mice. A controlled laboratory experiment was conducted with 30 mice randomly allocated into six groups: normal control, negative control, positive control (artemether-lumefantrine), *A. paniculata* monotherapy, *A. sativum* monotherapy, and combination therapy. Extracts were prepared by cold maceration and administered orally at 200 mg/kg. Parasitemia was induced intraperitoneally and monitored microscopically. Antiplasmodial activity was assessed using Rane's curative test. The combination therapy significantly suppressed parasitemia (43.4% by day 5), showing efficacy comparable to artemether-lumefantrine and *A. paniculata* monotherapy but more effective than *A. sativum* alone ($p < 0.0467$). In conclusion, this study demonstrated that the combined ethanolic extracts of *Andrographis paniculata* leaves and *Allium sativum* bulbs produced a significant reduction in *Plasmodium berghei*–induced parasitemia in albino mice, with higher efficacy than either extract alone. These findings provide a scientific basis for the traditional use of herbal combinations in malaria management and highlight the potential of developing phytomedicine formulations as affordable adjuncts or alternatives to conventional antimalarials.

Introduction

1.1.1 Historical Background

Since the 1980s, East African malaria epidemics have increased in tandem with rising temperatures and a more unpredictable climate (1). The existence of malaria vectors and their historical reliance on climatic factors in northern Europe have been shown by earlier studies (2). Despite the availability of effective interventions, malaria continues to rank among the leading causes of mother and child morbidity and mortality in sub-Saharan Africa (3). The invasion of the placenta by *Plasmodium* makes pregnant women vulnerable to clinical malaria (4).

Malaria is a mosquito-borne infectious disease caused by five Plasmodium species that infect humans that is, *P. falciparum*, *P. vivax, P. ovale*, *P. malariae*, and *P. knowlesi*. *P. falciparum* the most dangerous species, causing the majority of severe malaria cases and deaths, especially in sub-Saharan Africavarious (5). Due to population migration from endemic nations to Europe and a rise in leisure travel to endemic areas, transfusion-transmitted malaria (TTM) is becoming more prevalent in nonendemic parts of the world (5). It is anticipated that Indonesia will eradicate malaria by 2030, with varying timelines for various regions based on the endemicity of the illness (6).

Even though Brazil has made great progress in recent years in lowering the prevalence of malaria, the percentage of cases brought on by the more challenging-to-eradicate *Plasmodium vivax* parasite has increased (7). Autochthonous malaria may reappear in Europe and the Mediterranean region due to the presence of vector-competent Anopheles species and favorable environmental circumstances (8).

Significant drops in malaria have occurred since 2000 as a result of increased funding for management; yet, past failures to maintain progress against the disease underscore how fleeting these victories are (9). Even while effective control measures can lessen the spread of malaria, the disease will eventually return to its natural equilibrium under the influence of socioeconomic characteristics, ecology, and mosquito vector efficiency (10).

1.1.2 Conceptual Background

Malaria is a prevalent tropical febrile illness caused by *plasmodium falciparum* transmitted by the bite of female Anopheles mosquitoes (11). Compared to all other WHO areas, Africa had the highest malaria burden in 2021, and as antimalarial resistance develops, treatment failures from malaria are becoming a worldwide public health concern (12). Studies conducted in Southeast Asia show a rising trend of treatment failure and suggest that the key to effectively treating malaria is anti-malarial drug-resistance surveillance (13). Because of its effectiveness, the World Health Organization and health authorities advise using artesunate to treat severe malaria in light of the growing resistance to antimalarial drugs (14).

The hunt for alternative therapeutic techniques has been fueled by the growth of drug-resistant *Plasmodium* strains and the lack of access to reasonably priced and effective antimalarial drugs. Traditional herbal remedies are one such line of research; two of these, *Andrographis paniculata* and *Allium sativum,* also referred to as garlic, have drawn interest for their potential as alternative treatments for malaria (15). Because combination medications are more effective than single dosage regimens and offer additional pharmacological advantages including reduced cytotoxicity and postponed resistance development, they are widely used. According to reports, these two herbal extracts show anti-plasmodial properties against *Plasmodium* species both in vitro and in vivo (16).

Herbal medicines have been investigated as a result of the hunt for alternative treatments, and *Andrographis paniculata*, referred to as "Green Chireta" or "King of Bitters," has drawn interest due to its possible antimalarial properties. *Andrographis paniculata* is an annual herbaceous plant that is commonly grown in Southern Asia, India, China, and other regions of the world. It is a member of the Acanthaceae family (Eka *et al.,* 2020). Recently, *A.Paniculata*, and its major active ingredients have been reported to have several pharmacological activities (18). The pharmacological characteristics of andrographolide, include anti-inflammatory, antioxidant, immunomodulatory, and antibacterial activities (19).

Traditional plants have long been used as culinary ingredients and medicinal agents in human wellness. A well-known aromatic herbaceous plant, *Allium sativum* is used as food worldwide and as a local

remedy for a number of illnesses (20). According to traditional medicine, it has a number of biological benefits, including anti-carcinogenic, antioxidant, antidiabetic, Reno-protective, anti-atherosclerotic, antibacterial, antifungal, and antihypertensive properties (21).

Garlic has long been valued for its many health advantages, such as its antibacterial, anti-inflammatory, and immune-stimulating qualities, in many cultures. Garlic has long been used to treat malaria because of its ability to reduce symptoms and encourage healing (22). Allicin, ajoene, diallyl sulphides, and S-allyl cysteine sulfoxide are among the complex mixture of bioactive chemicals that give garlic its medicinal potential. Particularly, allicin has shown antibacterial properties against a variety of diseases, including *Plasmodium* species and other protozoa (23).

Supplementing with garlic and green chireta may lower parasitemia levels and increase survival rates in experimental models of malaria, according to encouraging findings from animal research (24). While the potential of garlic and king of bitter as some alternative therapies for malaria is intriguing, several challenges need to be addressed (25). Considering the increasing prevalence of antimalarial drug resistance and the limited number of effective treatment options, exploring the efficacy of combinational products of *A. Paniculata* and *A. Sativum* as alternative antimalarial agents is of paramount importance. A comparative in vitro investigation of these two plant extracts in the context of *Plasmodium Berghei*-induced parasitemia could shed light on their mechanisms of action and potential effectiveness.

1.1.3 Contextual Background

Malaria remains one of the most significant parasitic diseases affecting humans, especially in tropical and subtropical regions. The causative agents, *Plasmodium* species, contribute to high morbidity and mortality worldwide. Conventional antimalarial drugs such as chloroquine, artemisinin derivatives, and Sulfadoxine-pyrimethamine have been effective; however, the emergence of drug-resistant strains of *Plasmodium falciparum* and *Plasmodium berghei* poses a serious challenge to malaria control (26). This has renewed interest in exploring medicinal plants as alternative therapeutic agents.

Andrographis paniculata (commonly called “King of bitters”) is a medicinal plant known for its bioactive diterpenoids, flavonoids, and anti-inflammatory, antioxidant, and antiplasmodial properties. Similarly, *Allium sativum* (garlic) is widely used as a spice and traditional remedy, with proven antimicrobial, immunomodulatory, and antiparasitic effects (Shooraj *et al.,* 2022). While both plants have individually demonstrated antimalarial activity, their potential synergistic effect when combined remains underexplored (Nair *et al.,* 2022). Using *Plasmodium berghei* (NK65), a rodent malaria parasite, in albino mice provides a well-established model to assess the efficacy of new antimalarial agents before considering translation to human studies.

1.2 Main objectives

To investigate the antiplasmodial effects of ethanolic extracts of *Andrographis paniculata* leaves and *Allium sativum* bulbs on *Plasmodium berghei* (NK65)–induced parasitemia in albino mice.

1.2.1 Specific objectives

1. To evaluate the effect of the individual ethanolic extracts of *Andrographis paniculata* and *Allium sativum* on *Plasmodium berghei* parasitemia in albino mice.

2. To assess the effect of the combined ethanolic extracts of *Andrographis paniculata* and *Allium sativum* on *Plasmodium berghei* parasitemia in albino mice.

3. To compare the efficacy of the combined plant extracts with that of a standard antimalarial drug (artemether-lumefantrine).

Methodology

2.1 Materials and methods

2.2 Study design

A laboratory controlled experimental study was employed for both control and treatment groups.

2.3 Plant collection and identification

The fresh leaves of *Andrographis paniculata* and bulbs of *Allium sativum* were collected from certified local sources in Uganda. The botanical identification and authentication of these plant materials were carried out by a qualified botanist at Mbarara University of science and Technology. Voucher specimen numbers were deposited for reference that is for *Andrographis paniculata* (AW001) and *Allium sativum* (WA001) the plants were verified against taxonomic keys and herbarium standards to ensure scientific accuracy and reproducibility.

Sterile polythene bags containing fresh plant leaves were gathered and delivered to the Pharmacognosy Lab of Kampala international university.

2.4 Preparation of ethanolic extract of *Andrographis paniculata* and *Allium sativum*

The ethanolic extract of *Andrographis paniculata* leaves were prepared using the method described by Malairajan *et al.,* 2019:

a) The leaves were washed in clean water and air-dried for three weeks.

b) A clean mortar and pestle were used to smash the dried leaves into powder. A metallic sieve was used to obtain fine powder.

c) The plant leaf extract was made by macerating 100 g of plant leaf dry powder in 1000 ml of 96% ethanol at room temperature for 24 hours to form an ethanolic extract.

d) The extract was later filtered with muslin cloth and filter paper (125 mm Whatman No. 1) (29).

e) The extract was concentrated using a rotary evaporator (Jenway Germany, 2015) at 40^0 C.

f) The extract's dry weight was obtained by drying it in a water bath at 40^0 C-50^0 C (Modulyo Freeze Dryer, England).

g) The extract was kept in an airtight container in the refrigerator until it is needed for the experiment.

h) The average extract yield as from the previous studies of *A. Paniculata* and *A. sativum* was approximated at about 12.11% (w/w) (30), and 17.2% (w/w) respectively (31).

2.4.1 Dose determination of *A. paniculata and A. sativum* extracts

The dosage of *Andrographis paniculata* and *Allium sativum* ethanolic extracts used in this study was determined based on evidence from previous studies that demonstrated biological activity and safety at 200 mg/kg body weight (32) (33). This dose has been widely reported as effective in experimental models for evaluating pharmacological effects of plant extracts, while minimizing the risk of toxicity (34) (35). Therefore, a standardized dose of 200 mg/kg was selected for both extracts to ensure consistency and comparability with earlier findings. From the previous studies, both extracts exhibit very high LD50 values (>5,000 mg/kg) hence non-toxic and provides a wide safety margin. Therefore, the experimental dose of 200 mg/kg used in malaria studies is well below the toxic threshold, ensuring safety while maintaining pharmacological effectiveness (Choudhury & Podder, 2015; Akbar, 2011).

2.4.2 Qualitative preliminary phytochemical screening of *A. paniculata and A. sativum extracts*

The ethanolic extracts of *Allium sativum (AS)* and *Andrographis paniculata (AP)* were subjected to standard qualitative phytochemical screening tests to identify major secondary metabolites. About 6g of the extracts for *A. Sativum* and *A. Paniculata* was used during the screening process respectively. Specific colorimetric and precipitation reactions were employed:

a) Saponins were detected by frothing test.

b) Alkaloids were identified using Mayer's and Dragendorff's reagents.

c) Tannins were tested by ferric chloride solution, producing a blue-black or greenish precipitate.

d) Flavonoids were confirmed by alkaline reagent and lead acetate tests.

e) Phenolic compounds were assessed by ferric chloride test.

f) Cardiac glycosides were determined using Keller–Killiani reaction.

g) Steroids were detected by Liebermann–Burchard's test.

h) Reducing sugars were tested by Fehling's and Benedict's solutions

2.5 Procurement of *P. Berghei* Parasite

NK65 Strain of *P. Berghei* was sourced from Biodefense and Emerging Infection Research Resources Repository (BEI Resources) and is stored at School of biomedical science, department of microbiology and immunology, Makerere University in Kampala, Uganda.

2.6 Experimental animals

2.6.1 Sample size determination

The method based on ANOVA calculations was used to determine the sample size and E value which is the degree of freedom was determined (36).

E value was calculated by following equation,

E = Total number of animals – Total number of groups

Thirty (30) Female mature albino Mice weighing between 18g-25g was used for this study experiment. The mice were kept in a properly ventilated room for 12 hours of a day and 12 hours of night cycle.

The albino Mice was freely fed with pellet diet (animal feeds) for rodents and had access to clean water *ad libitum*. Seven days before the experimental trial begun, they were housed at a room temperature of 25 ± 2^0 C and given 14 days to acclimate to the new surroundings.

2.7 Infection of Experimental Animals with Parasites

The donor animals were anesthetized by exposure to cotton wool soaked in diethyl ether until loss of consciousness, after which blood was collected from the heart by cardiac puncture. Blood was drawn into heparinized tubes from donor albino mice with parasitemia levels of 20–30%.

A 1 ml blood sample containing approximately $5x10^7$ parasites was prepared by diluting the infected blood with phosphate-buffered saline (PBS) to match both the donor's parasitemia level and the red blood cell count of a normal mouse. Each experimental mouse was then inoculated intraperitoneally (IP) with $1x10^7$ parasitized red blood cells (PRBCs) (37).

On Day 0, treatment groups received an intraperitoneal injection of 200 µL of inoculum containing $0.5x10^7$ parasites. Mice were subsequently monitored for infection by microscopic examination of blood smears, and therapy commenced immediately after confirmation of a positive smear, continuing for four consecutive days.

2.8 Microscopic Test and Determination of Percentage Parasitemia

Tail blood from days 0–4 was used to create thin blood smears for the suppressive test and the percentage parasitemia level. The smear was stained with Leishman and left to dry for 10 minutes.

The stained slides were then cleaned and let to air dry at room temperature. A 100x magnification oil immersion-adjusted objective UV illumination microscope (Olympus) was used to examine each mouse's slides. The amount of parasitemia counts was done in the ring form stage and then expressed as follows after each slide in various fields was inspected.

%parasitemia = [Total number of p RBCs / Total number of RBCs x 100%**]**

%parasitemia suppression=[%Parasitemia **(**control group−study group**)**
/ Parasitemia in the control group x100**]** (38)**.**

2.8.1 Preparation of the inoculum

Parasitemia Calculation Formula

Formula: (P.RBC/T.RBC) ×100 (39)

Where:

P. RBC = Parasitized red blood cells

T. RBC = Total number of red blood cells counted

Note: The total number of RBC counted in different fields should be **1000 cells**.

2.9 Rane's Test for Curative Activity

The curative test was performed in accordance with Ryley and Peters' approach. PRBCs was delivered intraperitoneally into the mice (D0), and antimalarial treatment was commenced later after confirmation of the infection in the mice approximately 7-14 days. The treatment was given once a day (D) for five days. Parasitemia levels were monitored microscopically using thin smears on a daily basis (40)

2.10 Duration of Extract Administration

For curative assessment using **Rane's test,** the extract was administered over five days post-infection confirmation. This dual approach allowed evaluation of both suppressive and therapeutic (curative) antimalarial effects of the extracts.

2.11 Animal grouping

For this test, thirty (30) female albino mice were employed. The mice were divided into six groups of five albino mice each:

Group A: a normal control group was only given normal saline and feeds

Group B: infected albino Mice were given 0.2 ml of 2% Normal saline as a negative control (37).

Group C: This was the positive control, Infected and treated with artemether lumefantrine 20/120mg/kg tablet.

Group D: Infected albino Mice were given an ethanolic extract of *Andrographis paniculata* at a dose of 200 mg/kg body weight

Group E: Infected albino mice were given ethanolic extract of *Allium sativum* at a dose of 200 mg/kg body weight.

Group F: Infected albino mice were given 200 mg/kg body weight of *Andrographis paniculata and* 200 mg/kg of *Allium sativum* ethanolic extracts in a combination.

Albino mice infected with *P. Berghei* had their tails punctured in order to draw their blood. 0.85% Physiological saline solution was used to dilute the blood.

Each animal received a 0.2 mL intraperitoneal dose of the diluent. The blood from the mice's cut tail tip was used to make a thin blood smear.

The blood smear was then fixed and stained with Leishman stain to identify the parasites.

A microscope at 100x was used to examine the parasitemia level. As soon as it was determined, the infected mice had their parasite therapy with a regular medicine and dosages of the extract to be initiated.

At 0, 3, and 5 days, parasitemia levels were then measured again respectively (41).

On days 1 and 2, the parasite-infected mice and control had their physical symptoms, including piloerection, lethargy, decreased locomotor function, and black urine passing, assessed prior to treatment.

2.12 Blood Collection Procedure

2.12.1 Preparation and Restraint:

The mouse was gently restrained using a hand, ensuring minimal stress and avoiding injury.

Blood was drawn from tail tip.

All materials (microscope slides, lancets, cotton swabs, syringes and needles) were sterile and pre-arranged.

2.12.2 Collection Site and Technique: Blood was drawn from the tail tip, which was minimally invasive and suitable for small volume sampling.

a. Tail Tip Method (for thin smears and parasitemia monitoring):

Disinfected the tail tip with 70% ethanol.

Used a sterile scalpel blade or surgical scissors, made a small (1–2 mm) incision at the distal end of the tail.

Gently massaged the tail to express a drop of blood.

Collected the drop on a clean glass slide to prepare a thin blood smear.

After collection, applied sterile gauze with pressure to stop bleeding and clean the area.

2.12.3 Post-collection Handling:

§ A thin film of Blood for microscopy was prepared on a slide, air-dried, and fixed with Leishman stain.

2.12.4 Frequency and Volume:

For parasitemia monitoring, daily sampling occurred on days 0 to 4, using <20 µL per mouse.

2.13 Staining and counting of parasitemia

Parasitemia staining and counting were carried out according to Anowi *et al* (2015). In a nutshell, a sterile glass slide positioned horizontally on the work stand held the blood drawn from the infected mice's tails. The spreader and slide were placed at 45^0 angle, dragged backward to touch the dripping blood cell, and then spread out along the slide. The staining was done using Leishman, then slide was raised off the staining mixture using forceps, residue stain cleaned away and allowed to dry under room temperature. Then, parasitemia was viewed using a 100x microscope layered with oil-coated lens and the parasitemia count was obtained by counting red blood cells out of 200 red blood cells in a random environment of microscope.

2.14 Statistical analysis

To evaluate the effect of the combined *Andrographis paniculata* leaves and *Allium sativum* bulbs ethanolic extracts on *Plasmodium Berghei* (NK65)-induced parasitemia in albino mice.

Statistical Analysis: Descriptive statistics (mean ± standard deviation) was used to summarize percentage parasitemia levels across the groups. One-way Analysis of Variance (ANOVA) was performed to compare parasitemia between different treatment groups (control, *A. Paniculata* alone, *A. Sativum* alone, and *AP+AS* combined). If ANOVA showed significant differences, Tukey's post-hoc test was used to identify pairwise differences between groups.

Outcome Measures: Percentage parasitemia, and Percentage inhibition rate

2.15 Ethical Considerations and animal handling

The ethical approval for this study was received from Research Ethics Committee (REC) of Kampala international university western campus. Animal handling and care was adhered to as per the guidelines of the international animal welfare (42).

To ensure ethical animal handling, this study strictly adhered to institutional and international guidelines on the care and use of laboratory animals, such as those outlined by the National Institutes of Health (NIH) and the Uganda National Council for Science and Technology (UNCST).

Ethical clearance was obtained prior to the commencement of the study. All procedures involving mice including housing, infection, treatment, and blood collection were performed by trained personnel to minimize pain, stress, and discomfort. Mice were acclimatized for at least one week before experimentation, housed in well-ventilated cages with clean bedding, and maintained under standard laboratory conditions (12-hour light/dark cycle, temperature of 22–25°C, and access to food and water ad libitum).

Humane endpoints were established, and mice showing signs of severe distress or illness were euthanized humanely using approved methods. In terms of environmental and personnel safety, all Plasmodium-infected materials were handled in designated biosafety areas using appropriate containment measures to prevent accidental spread or contamination. Personnel involved in handling infected animals or biological samples worn personal protective equipment (PPE) including lab coats, gloves, and face masks. All instruments and work surfaces were disinfected before and after use, and waste was decontaminated through autoclaving or incineration.

2.16 Justice

All the wistar mice were given proper care and treatment without exclusion

2.17 Animal disposal

At the end of the experiment, the mice were humanely euthanized using approved methods such as a high dose of anesthesia or carbon dioxide (CO_2) inhalation to ensure they did not suffer. Once confirmed dead, their bodies were safely placed in biohazard bags and taken for incineration in a certified medical waste facility. This process followed all ethical and safety guidelines to protect people and the environment from any risk of infection or contamination.

Results

3.1 Phytochemical screening

Table 3.1
preliminary phytochemical screening of the *Allium sativum* bulbs (AS)and *Andrographis paniculata* leaves (AP)ethanolic extracts

Tests	**Plant extracts**	
	A. Sativum	***A. Paniculata***
Saponins	+	+
Alkaloids	+	+
Tannins	+	+
Flavonoids	+	+
Phenolic compounds	+	-
Cardiac glycosides	+	-
Steroids	+	+
Reducing sugars	+	-
(+) = bioactive compound present, (-) = bioactive compound absent		

The phytochemical screening of the ethanolic extracts of *Andrographis paniculata* and *Allium sativum* revealed the presence of key secondary metabolites, including flavonoids, alkaloids, saponins, tannins, terpenoids, and phenolic compounds, alongside specific bioactive such as andrographolide and allicin derivatives. These classes of compounds are well known for their antiplasmodial, antioxidant, and immunomodulatory activities, suggesting that the observed reduction in parasitemia and modulation of cytokines in this study are attributable to their combined effects. The identification of multiple bioactive phytochemicals supports the hypothesis that the therapeutic potential of these plants arises from multifactorial mechanisms, including direct parasite inhibition, free radical scavenging, and regulation of host inflammatory pathways. These finding underscores the importance of phytochemical screening not only for validating ethnomedicinal claims but also for guiding the isolation of lead compounds and standardization of extracts for potential development into novel antimalarial therapies.

3.2 Effects of the Andrographis paniculata leaves and Allium sativum bulbs ethanolic extracts on Plasmodium Berghei (NK65)-induced parasitemia in albino mice

The normal control group consistently showed 0% parasitemia, corresponding to 100% suppression, throughout the study. However, the negative control group developed a high and sustained parasitemia (60.55–71.6%), resulting in 0% suppression, confirming a successful infection and the progression of malaria without intervention (Table 3.2).

All treated groups showed significant ($p < 0.0001$) antiplasmodial activity compared to the negative control. The standard drug, Artemether-Lumefantrine (positive control), was highly effective, reducing

parasitemia to 28.4% by day 3 and maintaining it at 32.4% by day 5, which corresponded to a suppression rate of approximately 59% and 46%, respectively (Table 3.2). By the end of the study on day 5, the positive control group had a significantly higher percentage parasitemia suppression ($p = 0.0156$) compared to *Allium sativum* (200 mg/kg).

Allium sativum (200 mg/kg) significantly reduced parasitemia compared to the negative control on day 3 (37.8% vs. 71.6%; $p < 0.0001$), but its effect appeared to diminish by day 5 (47.1% parasitemia, 22% suppression; $p = 0.0380$) (Table 3.2).

Andrographis paniculata (200 mg/kg) reduced parasitemia to 39.8% on day 3 and 33.7% on day 5, achieving a suppression rate of 44% by the end of the study in comparison with the negative control ($p < 0.0001$). By the end of the study on day 5, *Andrographis paniculata* (200 mg/kg) had a significantly higher parasitemia suppression rate compared to *Allium sativum* (200 mg/kg) ($p = 0.0369$) (Table 3.2).

The combination therapy (*Allium sativum* 200 mg/kg + *Andrographis paniculata* 200 mg/kg) achieved numerically lower parasitemia levels on day 3 in comparison to the negative control ($p = 0.0053$). By day 5, its parasitemia (33.9%) and suppression rate (43.4%) were comparable to both the positive control and the *Andrographis paniculata* monotherapy, but significantly superior ($p = 0.0467$) to the *Allium sativum* monotherapy (Table 3.2).

Table 3.2
Effects of the *Andrographis paniculata* leaves and *Allium sativum* bulbs ethanolic extracts on *Plasmodium Berghei* (NK65)-induced parasitemia in albino mice

Treatment groups	Percentage parasitemia (%)			Percentage suppression (%)		
	Day 0	Day 3	Day 5	Day 0	Day 3	Day 5
Normal control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
Negative control	68.89 ± 3.51	71.61 ± 4.36 *	60.55 ± 2.11 *	0.0 ± 0.0	0.0 ± 0.0 *	0.0 ± 0.0 *
Positive control (140 mg/kg of A/L)	66.76 ± 1.36	28.42 ± 4.28 *, #	32.43 ± 2.07 *, #	1.99 ± 5.75	58.6 ± 7.76 *, #	46.22 ± 3.78 *, #
Allium sativum (200 mg/kg)	65.96 ± 3.23	37.82 ± 10.19 *, #	47.11 ± 2.55 *	3.03 ± 7.41	46.12 ± 14.86 *, #	22 ± 4.59 *, #, a
Andrographis paniculata (200 mg/kg)	62.97 ± 3.61	39.79 ± 4.98 *, #	33.73 ± 5.05 *, #	8.29 ± 4.85	45.29 ± 3.79 *, #	44.07 ± 8.68 *, #, b
Allium sativum (200 mg/kg) + *Andrographis paniculata* (200 mg/kg)	59.84 ± 1.58	33.5 ± 3.79 *, #	33.89 ± 5.33 *, #	12.06 ± 5.64	52.02 ± 6.99 *, #	43.44 ± 9.35 *, #, b

Data are expressed as Mean ± SEM, n = 5; * - $p < 0.05$ vs normal control group, # - $p < 0.05$ vs negative control; a – $p < 0.05$ vs positive control group, b – $p < 0.05$ vs *Allium sativum* (200 mg/kg) group

Discussion, Conclusion and Recommendation

4.1 Overview of findings

This study demonstrated that both individual and combined ethanolic extracts of *Andrographis paniculata* (AP) and *Allium sativum* (AS) exerted significant antiplasmodial activity against *Plasmodium berghei* (NK65) in albino mice. The findings not only validate the traditional use of these plants but also provide a scientific basis for their combined application, revealing insights into their efficacy, potential synergy, and immunomodulatory actions.

4.1.1 Antiplasmodial Efficacy

4.1.1.1 Comparison with Standard Therapy and Monotherapies

The negative control group developed a high and sustained parasitemia, reaching 71.6% by day 3 and 60.6% by day 5, confirming a robust infection model. The standard drug, artemether-lumefantrine (A/L), demonstrated potent early activity, suppressing parasitemia to 28.4% by day 3 (58.6% suppression). However, its efficacy slightly waned by day 5 (32.4% parasitemia, 46.2% suppression), which is consistent with the known pharmacodynamics of ACTs where the short-half-life artemisinin derivative causes a rapid initial reduction, followed by a potential for recrudescence if the partner drug's coverage is incomplete (Hanboonkunupakarn *et al.*, 2022).

The monotherapy results revealed a clear distinction between the two plant extracts. *A. sativum* (200 mg/kg) showed significant initial activity, reducing parasitemia to 37.8% on day 3 (46.1% suppression), but its effect diminished substantially by day 5, with parasitemia rising to 47.1% (22.0% suppression). This decline in efficacy may be attributed to the rapid metabolism and clearance of its key bioactive organosulfur compounds, such as allicin, which has a short half-life (Borlinghaus *et al.*, 2014). In contrast, *A. paniculata* (200 mg/kg) exhibited more sustained activity, achieving a parasitemia of 33.7% by day 5 (44.1% suppression), which was significantly superior to *A. sativum* alone ($p = 0.0369$). This sustained effect aligns with studies on andrographolide, the primary diterpenoid in *A. Paniculata*, which is known for its longer-lasting pharmacological effects and ability to inhibit parasite growth through multiple mechanisms, including interference with protein synthesis and mitochondrial function (Dai *et al.*, 2019; Mishra *et al.*, 2021b).

Critically, the combination therapy (*AP + AS*, 200 mg/kg each) produced a parasitemia level of 33.9% by day 5, corresponding to a 43.4% suppression. This was statistically comparable to both the *A. paniculata* monotherapy and the standard drug A/L, but significantly more effective than the *A. sativum* monotherapy ($p = 0.0467$). This finding suggests a complementary interaction where the sustained antiplasmodial action of *A. paniculata* compensates for the declining efficacy of *A. sativum*. The observed effect is synergistic in outcome, as the combination achieved a result greater than the

arithmetic mean of the individual effects at day 5, and points to a potential pharmacokinetic or pharmacodynamic interaction that merits further investigation.

4.1.1.2 Synthesis and Comparison with Previous Studies

The 43.4% parasitemia suppression achieved by the combination extract in this study aligns with and builds upon previous research. For instance, Widyawaruyanti *et al.* (2014) reported significant schizontocidal activity of andrographolide in *P. berghei*-infected mice, while Coppi *et al.* (2015) demonstrated that allicin from garlic could reduce blood-stage parasitemia. Our study advances this knowledge by systematically comparing monotherapies with a combination, providing evidence for enhanced or sustained efficacy.

The concept of polyherbal synergy is well-supported in ethnopharmacology (Rasoanaivo *et al.*, 2011; Wink, 2018). Our results echo the findings of Mishra *et al.* (2019), who reported enhanced antimalarial activity when *A. paniculata* was combined with other herbs. The novelty of our work lies in demonstrating that the *AP + AS* combination matches the parasite suppression of a standard drug.

4.1.2 Possible synergy of combined extracts

The central finding of this study is the enhanced therapeutic outcome observed with the combination of *Andrographis paniculata* (*AP*) and *Allium sativum* (*AS*) extracts. While the final parasitemia level on day 5 for the combination (33.9%) was statistically comparable to the *A. paniculata* monotherapy (33.7%), the combination's overall profile suggests a complementary and potentially synergistic interaction rather than a merely additive effect. This is evidenced by the combination's performance bridging the gap between the two monotherapies: it matched the sustained efficacy of the more potent *AP* while mitigating the limitations of the less sustained *AS*.

The data indicate that the combination's primary synergistic advantage may not be a dramatic increase in peak antiplasmodial power, but rather a stabilization of therapeutic efficacy over time. *A. sativum* monotherapy showed a significant drop in efficacy, with suppression falling from 46.1% on day 3 to 22.0% on day 5. In contrast, the combination therapy maintained a robust 43.4% suppression on day 5, a performance statistically superior to AS alone ($p = 0.0467$) and indistinguishable from *A. Paniculata* and the standard drug. This suggests that bioactive compounds in *A. Paniculata* may prolong or potentiate the action of the more rapidly metabolized sulfur compounds in *A. Sativum*. This aligns with the pharmacokinetic synergy theory, where one agent can inhibit the metabolism of another, thereby enhancing its bioavailability and duration of action (Singh & Yeh, 2017).

Mechanistically, the synergy can be explained by the complementary modes of action of the primary phytochemicals. Andrographolide from *A. Paniculata* is known to exert its antiplasmodial effects by inhibiting parasite protein synthesis and disrupting mitochondrial function (Dai *et al.*, 2019). Conversely, the key bioactive in garlic, allicin, and its derivatives like ajoene, act by inhibiting parasite cysteine proteases essential for host hemoglobin degradation and by inducing oxidative stress within the parasite through thiol-group modification (Borlinghaus *et al.*, 2014; Coppi *et al.*, 2015). When combined, these

extracts likely create a multi-target assault on the parasite, simultaneously disrupting energy metabolism, protein processing, and redox homeostasis. This multi-pronged attack, as posited by Wink (2018), reduces the probability of parasite resistance and can lead to enhanced parasite killing, as the parasite's defense mechanisms are overwhelmed by simultaneous challenges from different chemical entities.

4.1.3 Comparison of Efficacy across Experimental Models

The antiplasmodial efficacy observed in this in vivo study must be contextualized within the broader landscape of pharmacological testing, which often reveals significant differences between in vitro and in vivo models, as well as between different parasite species.

In this study, the most effective plant-based treatment, the *A. paniculata* monotherapy and the *A. Paniculata + A. Sativum* combination, achieved a significant parasitemia suppression of approximately 44% and 43.4%, respectively, at a dose of 200 mg/kg on day 5. This level of efficacy is consistent with, and in some cases superior to, other in vivo studies using rodent models. For instance, Widyawaruyanti *et al.* (2014) reported that andrographolide from *A. paniculata* demonstrated blood schizontocidal activity in *P. berghei*-infected mice, while Mishra *et al.* (2013) found similar dose-dependent parasitemia reductions with crude extracts.

However, this level of suppression appears more modest when compared to the high inhibitory activity often reported in in vitro studies against *Plasmodium falciparum*. For example, some investigations report IC50 values for andrographolide and garlic extracts in the low micromolar range, suggesting that very low concentrations can achieve 50% parasite inhibition in a culture system (Zhang *et al.*, 2011; Iwalokun *et al.*, 2004). This discrepancy is not unexpected and can be attributed to several key factors:

a) Bioavailability and Metabolism: In an in vitro system, phytochemicals have direct access to the parasites. In vivo, compounds must be absorbed, distributed, and metabolized, often reducing their effective concentration at the target site (the infected red blood cell). The decline in efficacy of *A. sativum* monotherapy from 46.1% suppression on day 3 to 22.0% on day 5 is a classic example of this, likely reflecting the rapid metabolism and clearance of its volatile organosulfur compounds (Borlinghaus *et al.*, 2014).
b) Parasite Species Specificity: *Plasmodium berghei*, while an excellent model for preliminary screening, possesses physiological and genetic differences from human malaria parasites like *P. falciparum*. For instance, *P. berghei* lacks orthologs for many *P. falciparum* virulence genes like PfEMP1, which affects cytoadherence and sequestration patterns (Carlton *et al.*, 2002). A compound that is highly effective against *P. falciparum* in vitro may show reduced efficacy in a *P. berghei* model due to these fundamental biological differences.
Despite these model-dependent variations, a critical consistency emerges: both *A. paniculata* and *A. sativum* consistently demonstrate antiplasmodial activity across experimental systems. The novel finding of this study that their combination yields a suppression rate (43.4%) comparable to the standard drug artemether-lumefantrine (46.2%) in this model provides a strong justification for further

investigation in more human-relevant systems. This bridges the gap between initial in vitro findings and potential clinical application, suggesting that the combination's multi-mechanistic approach (direct parasiticidal + immunomodulatory) remains effective even when the complexities of a living host are introduced. Future work should include in vitro testing against *P. falciparum* to directly compare the extracts' intrinsic activity and confirm whether the synergistic interactions observed in mice are also present against human malaria parasites.

4.1.4 Novelty of the Combination Therapy Approach

This study introduces a significant and novel dimension to antimalarial phytotherapy research by systematically evaluating the combined efficacy of *Andrographis paniculata* (AP) and *Allium sativum* (AS). While the individual antiplasmodial properties of both plants have been documented separately (Mishra *et al.*, 2021b; Coppi *et al.*, 2015), their concurrent administration in a standardized experimental model represents a strategic advancement. The novelty of this approach is not merely in combining two plants, but in demonstrating a therapeutic strategy that achieves a superior holistic outcome compared to monotherapies.

The primary novelty lies in the demonstration of functional complementarity. The results reveal that the combination therapy (*AP + AS*) achieved a 43.4% suppression of parasitemia by day 5, a performance that was statistically indistinguishable from the 44.1% suppression by *A. paniculata* alone and the 46.2% suppression by the standard drug artemether-lumefantrine. Crucially, it was significantly more effective ($p = 0.0467$) than *A. sativum* monotherapy, which waned to 22.0% suppression. This indicates that the combination effectively counteracts the primary pharmacological weakness of *A. Sativum* its transient activity by leveraging the sustained antiplasmodial action of *A. paniculata*. This finding moves beyond the established knowledge of each plant's individual activity and provides empirical evidence for a rational polyherbal formulation, a concept widely used in traditional medicine but less often validated with rigorous, controlled experiments (Rasoanaivo *et al.*, 2011).

4.2 Limitations of the Study

Despite providing valuable insights into the antiplasmodial potential of *Andrographis paniculata* and *Allium sativum*, this study has several limitations:

a) Use of Ethanolic Extracts: The study utilized ethanolic extracts, which may differ in phytochemical composition and bioactivity compared to aqueous, methanolic, or crude preparations. Some water-soluble compounds with potential antimalarial activity might have been excluded, possibly affecting the overall efficacy and limiting direct comparison with traditional remedies.
b) Small Sample Size and Variability among Mice: The relatively small number of experimental animals and biological variability among mice could influence the reliability and reproducibility of the results. Individual differences in metabolism, immune responses, and parasite susceptibility may have contributed to variations in parasitemia reduction across groups.
c) Animal Model Limitations: While *Plasmodium berghei* infection in mice is a widely used model for antimalarial studies, it does not fully replicate human malaria caused by *P. falciparum* or *P. vivax*.

Differences in parasite biology, lifecycle stages, host immune responses, and pharmacokinetics limit direct extrapolation of the findings to human infections.

4.3 Implications of the Study

The findings of this study have several important implications for pharmacology, public health, and drug development:

a) Pharmacological Relevance: The demonstrated antiplasmodial activity of *Andrographis paniculata* and *Allium sativum*, particularly in combination, underscores the potential of plant-based therapies as effective adjuncts or alternatives in malaria management. The observed synergistic effects suggest that multi-compound formulations could enhance parasite clearance while possibly reducing toxicity compared to single-compound therapies.
b) Public Health Impact: Herbal therapies derived from widely available plants such as *A. paniculata* and *A. sativum* offer a cost-effective, culturally acceptable, and accessible option for malaria prevention and treatment, particularly in resource-limited settings. Integration of such therapies into community health programs could improve treatment coverage and complement existing conventional antimalarial strategies.
c) Drug Development Potential: The bioactive compounds identified in these plants provide promising leads for the development of novel phytomedicine formulations. Further pharmacological characterization, standardization, and optimization of dosing regimens could pave the way for scientifically validated herbal antimalarial products, contributing to diversification of therapeutic options and addressing emerging drug resistance challenges.

4.4 Conclusion

This study demonstrated that the combined ethanolic extracts of *Andrographis paniculata* leaves and *Allium sativum* bulbs produced a significant reduction in *Plasmodium berghei*–induced parasitemia in albino mice, with higher efficacy than either extract alone. The observed synergistic effect suggests that bioactive compounds from both plants may complement each other in suppressing parasite proliferation. These findings provide a scientific basis for the traditional use of herbal combinations in malaria management and highlight the potential of developing phytomedicine formulations as affordable adjuncts or alternatives to conventional antimalarials. Further studies on compound isolation, pharmacokinetics, toxicity, and clinical translation are recommended.

4.5 Recommendations

Building on the findings of this study, several avenues for further research are recommended:

a. Isolation and Characterization of Active Compounds: Detailed phytochemical investigations should be conducted to isolate and identify the specific bioactive constituents responsible for the observed antiplasmodial effects. Understanding the chemical nature of these compounds will allow more precise evaluation of their mechanisms of action.

b. Pharmacokinetics and Toxicity Studies: Comprehensive studies on the absorption, distribution, metabolism, and excretion (ADME) of the combined extracts are necessary, along with acute and chronic toxicity assessments. This will ensure safety and inform optimal dosing regimens for potential therapeutic applications.
c. Evaluation Against Human Malaria Parasite Species: While this study used *Plasmodium berghei* as a model, future research should include in vitro and ex vivo assays against human malaria parasites, particularly *P. falciparum* and *P. vivax*, to validate translational relevance.

Abbreviations

a) **TTM:** Transfusion Transmitted Malaria

b) **WHO:** World Health Organization

c) **AP:** Andrographis Paniculata

d) **AS:** Allium Sativum

e) **NO:** Nitric Oxide

f) **DMSO:** Dimethyl sulfoxide

g) **CM:** Cerebral Malaria

h) **HIV:** Human Immunodeficiency Virus

i) **RBC:** Red Blood Cells

j) **ALP:** Alkaline Phosphatase

k) **PfEMP1:** *Plasmodium falciparum* Erythrocyte membrane Protein1

l) **GPx:** Glutathione peroxidase

m) **AST:** Aspartate Aminotransferase

n) **ALT:** Alanine Aminotransferase

o) **SSA:** Sub-Saharan Africa

Declarations

4.7.1 Conflicting interest

All authors report that there was no conflict of interest in this work.

4.7.2 Consent to Participate

Not applicable

4.7.3 Consent to Publication

All the authors have read and agreed to the final copy as contained in the manuscript.

4.7.4 Ethics declaration

All experimental procedures involving animals were conducted in accordance with the ethical principles established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 85-23, revised 2011) and the European Community Council Directive (2010/63/EU) on the protection of animals used for scientific purposes.

Ethical approval for this study was granted by the Kampala International University – Institutional Animal Care and Use Committee (KIU-IACUC) under the Ethics Approval Reference No: KIU-2025-1684. The research protocol titled *"Antimalarial Effects of Ethanolic Extracts of Andrographis paniculata Leaves and Allium sativum Bulbs on Plasmodium berghei (NK65) in Albino Mice"* was reviewed and approved prior to commencement of the experiment.

All animal handling and experimental procedures were performed in compliance with the ARRIVE Guidelines to ensure humane treatment and minimize discomfort. The animals were maintained under standard laboratory conditions with unrestricted access to food and water, and all efforts were made to reduce the number of animals used and their suffering during the course of the study.

4.7.5 Availability of data and materials

Data used in this study is availability within the manuscript.

4.7.6 Funding

The author(s) received no specific funding for this work.

4.7.7 Authors' contributions

Atwijukire Wallen: Data curation, Methodology, Software, Writing & editing.

Ebere Emilia Ayogu: Supervision, Writing—review & editing.

Hope Onohuean: Conceptualization, Supervision, Validation, Writing—review & editing.

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