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Ethnopharmacological Evaluation of Cucurbita pepo Leaf Extract Reveals Antimalarial Potential in Nigerian Traditional Medicine

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ABSTRACT

Medicinal plants are commonly used for malaria treatment across various regions of Nigeria, and exploring these plants can lead to the discovery of new therapeutic agents. This study investigated the antiplasmodial properties of selected plants traditionally used to treat malaria in Nsukka, Enugu State, Nigeria. Leaves from three plants—Cucurbita pepo, Hibiscus rosa-sinensis, and Pennisetum purpureum—were collected, and two types of extracts were prepared from each: 70% ethanol and a 1:1 mixture of dichloromethane and methanol. The safety of the extracts was evaluated through acute toxicity tests in mice and cytotoxicity assays using human hepatoma (HUH) cells. Their antiplasmodial activity was tested in vitro against chloroquine-sensitive Plasmodium falciparum (Pf3D7) and in vivo against chloroquine-resistant P. berghei ANKA using the 4-day suppressive test in mice. Additionally, the ethanol extract of C. pepo (CpE) was assessed for hemolytic effects on human red blood cells and tested in mice with established malaria infections. Parameters measured included parasitemia after treatment, hematological indices, organ weights (brain, kidney, liver, spleen), and survival. The results showed that all extracts were non-toxic, with no cytotoxicity observed up to 100 µg/ml and no acute or delayed toxicity in mice at 2000 mg/kg. CpE demonstrated strong in vitro antiplasmodial activity with an IC50 of 3.05 µg/ml. Oral administration at 500 mg/kg resulted in significant suppression of parasitemia by approximately 51% ($p < 0.01$). The extract did not induce significant hemolysis in human erythrocytes at concentrations up to 1 mg/ml. In mice with established infections, a 300 mg/kg dose of CpE significantly prevented anemia by maintaining hematocrit levels ($p < 0.01$). The extract also significantly increased red blood cell and platelet counts ($p < 0.05$), and hemoglobin levels were notably elevated at doses of 100 and 300 mg/kg. Moreover, CpE dose-dependently reversed the increases in liver and spleen weights observed in untreated, infected mice. These findings suggest that C. pepo is a promising candidate for further research to identify its bioactive compounds and elucidate its potential mechanisms of antimalarial activity.

Keywords: Antimalarial, Nigeria, Curcubita pepo, Medicinal plants, Ethnomedicine

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Introduction

Malaria continues to be a major public health concern in Africa, ranking among the most deadly diseases after diarrhea and pneumonia. Globally, it is the third-leading cause of death in children under five, with young children and pregnant women being particularly susceptible [1]. The heaviest burden is observed in sub-Saharan Africa, where the majority of malaria cases and fatalities occur [2]. In 2019, there were approximately 229 million malaria cases worldwide, slightly higher than the 228 million reported in 2018, with deaths increasing from 405,000 to 409,000 [1, 2]. Nigeria alone accounted for nearly 25% of the global malaria burden. Progress in controlling the disease has slowed in recent years, especially since 2020, due in part to disruptions in preventive and treatment services caused by the COVID-19 pandemic, which have contributed to higher infection rates and increased severity.

The disease is caused by protozoan parasites of the genus Plasmodium, among which Plasmodium falciparum is the most prevalent in tropical regions and is associated with the most severe forms of malaria. Infected individuals often experience fever, chills, nausea, and other flu-like symptoms. Severe cases can progress to life-threatening complications such as cerebral malaria, severe anemia, liver and spleen enlargement, hemoglobinuria, and renal failure. Populations with weakened immune systems, particularly children, are at greatest risk. The emergence of drug-resistant malaria strains has created significant challenges for both traditional and modern antimalarial therapies, particularly in rural and endemic regions. Developing new antimalarial agents with unique mechanisms of action is therefore essential. Historically, effective antimalarial drugs such as quinine and artemisinin were derived from Cinchona officinalis and Artemisia annua, respectively, underscoring the potential of plants as sources of new treatments [3].

In Nigeria, the use of traditional medicinal plants for malaria treatment remains widespread. The country possesses a rich diversity of plant species with ethnopharmacological applications. Ethnobotanical surveys in Nsukka Local Government Area have recorded numerous plants used for malaria and fever management, including Azadirachta indica, Carica papaya, Cymbopogon citratus, Mangifera indica, and Psidium guajava [4]. Among these, Cucurbita pepo L. (Cucurbitaceae), Hibiscus rosa-sinensis L. (Malvaceae), and Pennisetum purpureum Schumach (Poaceae) were cited, but their potential antimalarial activity has not been previously investigated, motivating their selection for the current study.

Hibiscus rosa-sinensis is a perennial evergreen shrub native to India, growing 1–3 meters in height, with a taproot system. Methanol extracts of its leaves have shown antibacterial activity and the ability to enhance the effects of antibiotics against resistant bacterial strains [5, 6]. Pennisetum purpureum, also called Napier or elephant grass, is a robust African perennial traditionally used to treat blennorrhea, with young shoot sap applied to the eyes for cataracts [7]. Cucurbita pepo, which includes pumpkins, squashes, gourds, and zucchini, has demonstrated anti-inflammatory and anticancer properties [8].

This study was designed to assess the antimalarial activity of leaf extracts from H. rosa-sinensis, P. purpureum, and C. pepo, which are traditionally employed by communities in Nsukka for malaria management.

Materials and Methods

Selection, collection, and identification of plant materials

The plants examined in this study, illustrated in **(Figure 1)**, were sourced from different regions in Nigeria. Leaves of Hibiscus rosa-sinensis and Cucurbita pepo were gathered from Orba in Enugu State (6.8541° N, 7.4625° E) and their identities confirmed by experts at the International Centre for Ethnomedicines and Drug Development (InterCEDD) in Nsukka. Specimens were formally documented and added to the InterCEDD herbarium for reference. In contrast, Pennisetum purpureum was collected from Niger State (9.2179° N, 7.1898° E) and authenticated by the Plant Taxonomy Unit of the National Institute for Pharmaceutical Research and Development (NIPRD). A representative specimen was preserved in the NIPRD herbarium to ensure accurate future reference.

Figure 1. Plant species examined for antimalarial activity in this study: starting from the top left and moving clockwise—Cucurbita pepo, Hibiscus rosa-sinensis, and Pennisetum purpureum.

Extraction of plant materials

Freshly collected leaves of the three plants were first air-dried in the shade to preserve their phytochemical properties and then ground into a fine powder using an electric hammer mill. For each plant, two types of extracts were prepared, resulting in a total of six extracts. In the first extraction, 50 g of powdered plant material was soaked in 700 ml of a 1:1 mixture of dichloromethane and methanol. Separately, another 50 g portion was

macerated in 700 ml of 70% ethanol. Both mixtures were left to stand for 48 hours at room temperature, with occasional agitation to improve solvent penetration. After maceration, the solutions were filtered first through a fine mesh cloth and then through Whatman filter paper (10 µm) to remove particulate matter. The filtrates were concentrated to dryness on a water bath set at 55 °C, and the resulting yields were recorded. Finally, the dried extracts were transferred to airtight containers and stored in a refrigerator at 4 °C until further use.

HPLC fingerprint of C. pepo extract

To characterize the chemical profile of the extracts, high-performance liquid chromatography (HPLC) was employed using a system from Shimadzu Corporation (Kyoto, Japan). Separation was carried out on a VP-ODS column (150 mm × 4.6 mm, particle size 5 µm). The mobile phase consisted of 0.2% formic acid in water as solvent A and 20% acetonitrile as solvent B, and the analysis was performed under isocratic conditions with a flow rate of 0.6 ml/min. Extract solutions were prepared at 20 mg/ml in water, and 20 µL of each was injected for analysis. Detection of compounds was achieved using a UV detector set at 254 nm. Pure reference compounds—including rutin, morin, gallic acid, caffeic acid, and ferulic acid (Fluka, Germany)—were prepared in methanol at 50 µg/ml and analyzed under the same conditions to allow comparison. The HPLC run was conducted with 80% solvent A and 20% solvent B, with the column maintained at 40 °C and a total runtime of 20 minutes, according to standard protocols [9].

Quantification of phenolics in C. pepo extract

A calibration curve for gallic acid was constructed using a series of dilutions ranging from 0.0016 to 1 mg/ml. For each dilution, 1 ml was mixed with 2.5 ml of 10% Folin–Denis reagent and allowed to react at room temperature for 2 minutes. Subsequently, 2 ml of a 75 g/L sodium carbonate solution was added, and the mixture was incubated in a water bath at 50 °C for 15 minutes, followed by cooling in an ice bath for 3 minutes. The absorbance of each sample was measured at 760 nm using a Cary 60 UV-Vis spectrophotometer (Agilent, Santa Clara, USA). In parallel, serial dilutions of Cucurbita pepo extract (1–0.25 mg/ml) were prepared and processed in the same manner as the gallic acid standards. All measurements were performed in triplicate. The total phenolic content of the extract was calculated by referencing the standard gallic acid calibration curve, described by the equation $y = 1.474x – 0.001782$ ($R^2 = 0.9978$).

Malaria parasite

For the experiments, two malaria parasite systems were employed to assess antimalarial activity. A chloroquine-resistant rodent malaria, Plasmodium berghei ANKA, was maintained in mice through periodic inoculations to preserve its infectivity. In parallel, the chloroquine-sensitive human malaria parasite, Plasmodium falciparum Pf3D7 (MRA 102), was cultured under laboratory conditions to provide a consistent in vitro model for testing.

In vitro cultivation of Plasmodium falciparum

The chloroquine-sensitive Plasmodium falciparum strain Pf3D7 was sourced from MR4 and cultured in the Malaria Drug Discovery Laboratory at ICGEB, New Delhi, following a modified protocol based on Trager and Jensen (1976). Parasites were maintained at 4% hematocrit in a complete medium composed of RPMI 1640 (16.2 g/L), sodium bicarbonate (0.2%), Albumax I (0.5%), hypoxanthine (50 mg/L), and gentamicin (10 mg/L), using freshly obtained O+ erythrocytes. Cultures were incubated at 37 °C in a controlled gas environment consisting of 5% O_2, 5% CO_2, and 90% N_2 to support parasite growth

Preparation of extract stock solutions

Stock solutions of each extract were prepared at a concentration of 25 mg/ml using DMSO. For the assay, 10 µL of these stock solutions were combined with 90 µL of parasite culture medium and then serially diluted to achieve the desired working concentrations. A 4 µL aliquot of each working dilution was added to wells of a 96-well microplate for testing. Chloroquine served as a positive control, with its stock solution prepared in sterile distilled water.

Antiplasmodial assay of extracts against P. falciparum

The antiplasmodial activity of the extracts was evaluated using a SYBR Green I fluorescence-based assay [10]. Parasite cultures were first synchronized with 5% sorbitol and then seeded in 96-well plates at 2% hematocrit and

1% parasitemia. Test concentrations of the extracts ranged from 0.78 to 100 µg/ml, with 0.4% DMSO serving as the negative control and 40 nM chloroquine as the positive control. After 48 hours of incubation under standard culture conditions, 100 µL of SYBR Green I lysis solution [0.2 µL of 10,000× SYBR Green I per mL of lysis buffer containing 20 mM Tris (pH 7.5), 5 mM EDTA, 0.008% saponin, and 0.08% Triton X-100] was added to each well. Plates were incubated in the dark at 37 °C for 1 hour before measuring fluorescence at 485 nm excitation and 530 nm emission. Based on IC50 values, extracts were categorized as highly active (<5 µg/ml), promising (6–15 µg/ml), moderately active (16–30 µg/ml), low activity (31–50 µg/ml), or inactive (>50 µg/ml) [11, 12].

Cytotoxicity test

Human hepatoma (HUH) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5–10% fetal bovine serum (Gibco) and seeded into 96-well plates at a density of 10^4 cells per 100 µL per well, following a modified version of the method described by Mosmann (1983). After 24 hours of incubation at 37 °C in a 5% CO_2 atmosphere, test samples were added and incubated for an additional 24 hours under the same conditions. Cell viability was assessed using the MTT assay: 20 µL of MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 5 mg/mL in phosphate-buffered saline) was added to each well and incubated for 3 hours. The medium was then removed, and 200 µL of DMSO was added to solubilize the formazan crystals. Absorbance was measured at 570 nm using a multi-well plate reader. All experiments were performed in triplicate, and CC50 values were calculated from concentration-response curves. DMSO at 0.4% and 10% served as negative and positive controls, respectively.

Animals

Healthy Swiss albino mice of both sexes, weighing 15–25 g, were acclimated for two weeks under laboratory conditions at the animal facility of the Department of Pharmacology and Toxicology, NIPRD, Abuja. The animals were housed in ventilated plastic cages at approximately 25 °C, provided with standard rodent feed, and given unrestricted access to clean drinking water.

Ethical approval

Prior to the study, all procedures involving animals received clearance from the NIPRD Animal Care and Ethics Committee (Ref: NIPRD/05:03:05/007). The experiments were performed in accordance with the National Institutes of Health guidelines for laboratory animal care (NIH Publication No. 85-23, 2011) and aligned with the internal standard operating procedures of NIPRD (NIPRD/05.03.0.5–1) to ensure ethical and humane treatment.

Acute toxicity

The acute oral toxicity of the plant extracts was assessed following the OECD guideline 425 [13]. A total of 24 female mice were used to evaluate the six extracts. After overnight fasting with unrestricted access to water, the mice were weighed, coded, and randomly assigned to six groups of four animals each. In each group, one mouse served as a control and received the respective vehicle without any extract. Ethanol extracts were dissolved in distilled water, whereas dichloromethane/methanol (1:1 v/v) extracts were suspended in coconut oil. Each group received a single oral dose of 2000 mg/kg body weight of the assigned extract. Following administration, mice were closely monitored for 2 hours for behavioral and autonomic changes, and observations for signs of toxicity or mortality continued for 14 days.

In vivo antimalarial study

Parasite inoculation

Eight days following infection, a donor mouse was used to prepare Giemsa-stained thin blood smears from the tail vein to determine parasitemia. The donor was then anesthetized, and blood was collected via cardiac puncture. The blood was diluted with physiological saline to achieve a concentration of approximately 10^7 parasitized erythrocytes per 0.2 ml, based on the parasitemia of the donor [14]. Experimental mice were subsequently inoculated intraperitoneally with 0.2 ml of this diluted infected blood.

In vivo suppressive test of extracts

The schizonticidal activity of the extracts was evaluated using the 4-day suppressive test in *Plasmodium berghei*-infected mice [15]. A total of 48 male and female mice were weighed and randomly assigned into eight groups (I–VIII) of six animals each, with equal numbers of males and females per group. Mice were inoculated intraperitoneally with 0.2 ml of diluted infected blood. Four hours post-inoculation, treatment was initiated and continued once daily for four consecutive days (Day 0–3). Groups I–VI received oral doses of the plant extracts at 500 mg/kg, prepared in 0.1% Tween 80 in distilled water. Group VII was administered artemisinin at 5 mg/kg daily, while Group VIII received only the vehicle and served as the untreated, infected control.
On Day 4, thin blood smears were collected from the tail vein, fixed in methanol, and stained with 10% Giemsa. Parasitemia was determined by counting infected erythrocytes in five fields of 100 red blood cells each under oil immersion at ×100 magnification. Percentage parasitemia was calculated as:

$$\%\ parasitemia = \frac{Number\ of\ parasitized\ RBCs}{Total\ number\ of\ RBCs} \times 100 \quad (1)$$

The percentage of inhibition caused by the test extracts was measured using:

$$\%\ Suppression = \frac{\%\ Parasitemia\ (control) - \%\ Parasitemia\ (treated)}{\%\ Parasitemia\ control} \times 100 \quad (2)$$

Extracts were considered to have moderate antiplasmodial activity if they produced a parasitemia suppression of ≥50% at 500 mg/kg per day [16].

Screening of C. pepo ethanol extract in established infection (curative antimalarial study)

Experimental design
The curative potential of Cucurbita pepo ethanol extract was evaluated in mice using a standard curative model [14]. Seventy mice were first weighed, randomized, and inoculated on the third day following the establishment of parasitemia in donor animals. The mice were divided into two main groups (A and B), each further subdivided into five subgroups (A1–A5, B1–B5) consisting of seven mice per subgroup. An additional group of seven uninfected mice treated with distilled water served as the normal control.
C. pepo extract was prepared in distilled water and administered orally at doses of 100, 300, and 900 mg/kg once daily to subgroups A1–A3 and B1–B3. Positive control groups (A4 and B4) received artemisinin orally at 5 mg/kg per day, while infected mice receiving only distilled water (A5 and B5) served as untreated controls. Treatments were continued for five consecutive days, from day 3 to day 7 post-infection.
Parasitemia was assessed on day 8 by preparing thin blood smears stained with 10% Giemsa solution. Five fields per smear, each containing 100 red blood cells, were examined under a microscope, and the percentage inhibition of parasite growth (% I) was calculated using the formula:

$$\%\ I = [(\text{parasite count of malaria control} - \text{parasite count of extract group})/\text{parasite count of malaria control}] \times 100 \quad (3)$$

Mice in group A were monitored for survival, whereas those in group B were euthanized on day 8 via chloroform inhalation for organ collection (liver, spleen, kidney, brain) and subsequent hematological analysis.

Hematological parameters, relative organ weight index
At the conclusion of the experiment, mice were sacrificed, and major organs—including liver, kidneys, brain, and spleen—were excised, weighed, and evaluated relative to body weight. Blood samples obtained from the tail vein were collected in EDTA tubes and analyzed with an automated hematology system (YNH7021, Wincom Company Ltd., Hunan, China) to determine key blood parameters such as hemoglobin concentration, red and white blood cell counts, platelet numbers, and differential leukocyte profiles.

Hemolysis assay of C. pepo extract
The potential hemolytic effect of Cucurbita pepo ethanol extract on erythrocytes was assessed using an in vitro assay as described by Ajdačić *et al*. (2016) [17]. Fresh A+ blood was centrifuged at 1000 × g for 5 minutes, and the plasma was discarded. The packed red blood cells were washed three times with three volumes of phosphate-

buffered saline (PBS, pH 7.4) and recentrifuged. Serial dilutions of the extract (0.0016–1 mg/ml) were prepared in PBS. The assay was conducted at 2% hematocrit, with 0.1% Triton X-100 serving as a positive control for complete hemolysis and 1% DMSO as a vehicle control. Following addition of the extract to the erythrocyte suspension, samples were incubated at 37 °C for 4 hours. Tubes were then centrifuged at 1000 × g for 10 minutes, and the absorbance of the supernatant was measured at 540 nm. Hemolysis was expressed as a percentage relative to the Triton X-100 control.

Statistical analysis

All in vitro experiments were carried out in triplicate. The concentration of extract required to inhibit 50% of P. falciparum Pf3D7 growth (IC50) was calculated using non-linear regression in IC Estimator version 1.2 (http://www.antimalarial-icestimator.net/MethodIntro.htm; Free Software Foundation, Boston, MA, USA). For in vivo studies, data were log-transformed prior to statistical analysis and subsequently back-transformed. Results from animal experiments were presented as mean ± standard error of the mean (SEM). Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test, with differences considered significant at $p < 0.05$.

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Results and Discussion

HPLC profile of C. pepo ethanol extract

The HPLC analysis of Cucurbita pepo extract showed a total of nine distinct peaks, with retention times spanning from 3.004 to 23.457 minutes **(Figure 2)**.

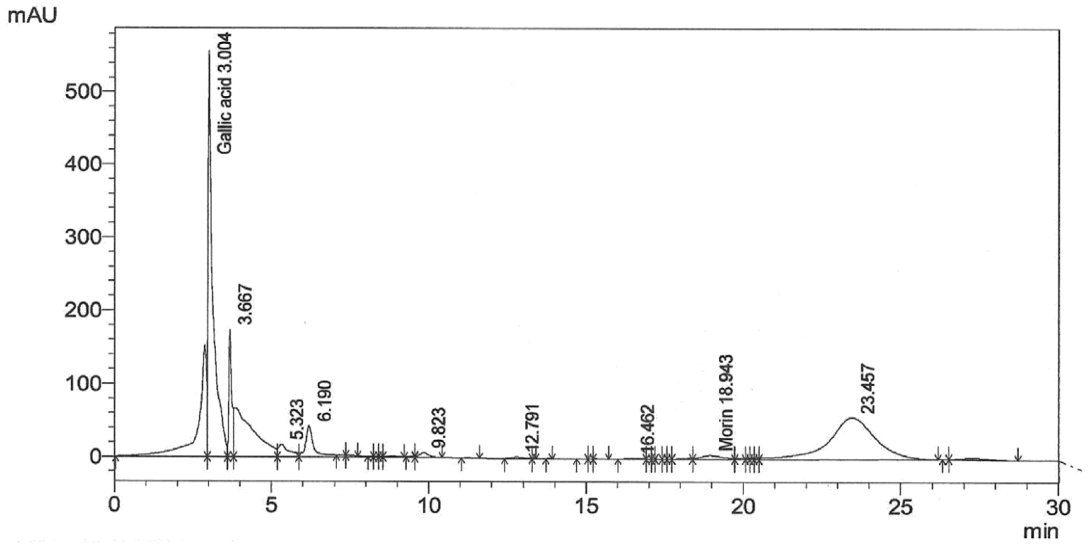

Figure 2. HPLC analysis of the ethanolic leaf extract of Cucurbita pepo revealed nine chromatographic peaks.

The earliest peak, appearing at 3.004 minutes, was identified as gallic acid and represented approximately 39.64% of the extract. A later peak at 18.943 minutes corresponded to morin, comprising 1.65% of the total peak area.

Quantification of phenolic content of C. pepo ethanol extract

The total phenolic content of the Cucurbita pepo ethanol extract, determined using a gallic acid standard calibration curve, was calculated as 0.1837 mg gallic acid equivalents (GAE) per mg of extract.

Extract yields, in vitro antiplasmodial activity, and cytotoxicity

The leaf ethanol extracts generally provided higher recoveries compared to their dichloromethane/methanol (DCM/MeOH) counterparts **(Table 1)**. Among the DCM/MeOH extracts, Pennisetum purpureum and Cucurbita pepo exhibited pronounced antiplasmodial effects, with IC50 values below 5 µg/ml. Moderate to notable activity

was observed for the 70% ethanol extracts of P. purpureum and C. pepo, as well as for both solvent fractions of Hibiscus rosa-sinensis, consistent with previously reported activity classifications [11, 12] **(Table 1)**. No cytotoxicity was detected in any of the extracts at the tested concentrations.

Table 1. Overview of extraction efficiency, in vitro antimalarial activity, safety in mammalian cells, and selectivity indices of tested plant extracts.

Plant Species (Family)	Extraction Solvent	Extract Yield (%)	IC_{50} against *P. falciparum* 3D7 (µg/mL)	CC_{50} on HUH cells (µg/mL)	Selectivity Index (S.I.)
Cucurbita pepo (Cucurbitaceae)	70% Ethanol	8.55	3.05 ± 0.37	>100	>32.79
	DCM/Methanol	4.18	>10	>100	Not Known
Hibiscus rosa-sinensis (Malvaceae)	70% Ethanol	13.1	25.4 ± 2.67	>100	>3.94
	DCM/Methanol	6.75	15.01 ± 2.3	>100	>6.66
Pennisetum purpureum (Poaceae)	70% Ethanol	7.69	6.16 ± 1.37	>100	>16.23
	DCM/Methanol	2.94	1.75 ± 0.01	>100	>57.14

NK = Not known.

Acute toxicity

Administration of the extracts at an oral dose of 2000 mg/kg did not induce any signs of acute toxicity in the mice. Treatment with Cucurbita pepo extract caused a transient, mild sedation within the first 30 minutes, which resolved within an hour. Throughout the 14-day observation period, no behavioral abnormalities, clinical symptoms, or mortality were observed in any of the treated animals.

Suppressive antimalarial activity

(Table 2) shows that among the eight extracts tested, only the ethanol extract of Cucurbita pepo (CpE) demonstrated moderate antiplasmodial activity. When administered orally at 500 mg/kg body weight once daily for four days, CpE achieved a significant parasitemia suppression of 50.95% ($p < 0.01$). In comparison, the 70% ethanol extract of Pennisetum purpureum reduced parasitemia by 19.4%, whereas the DCM/MeOH extract of C. pepo and the ethanol extract of Hibiscus rosa-sinensis showed minimal activity, suppressing parasitemia by only 6.89% and 9.33%, respectively.

Table 2. In vivo 4-day suppressive antimalarial activity∗.

Group / Treatment	Solvent	% Parasitemia on Day 4 Post-Infection	Parasitemia Suppression (%)
Untreated Control	–	5.78 ± 0.65	0
Cucurbita pepo	Ethanol	2.83 ± 0.43 **	50.95
	DCM/Methanol	5.38 ± 0.65	6.89
Hibiscus rosa-sinensis	Ethanol	5.24 ± 0.90	9.33
	DCM/Methanol	6.14 ± 0.68	0
Pennisetum purpureum	Ethanol	4.66 ± 0.60	19.41
	DCM/Methanol	7.30 ± 0.80	0
Artemisinin	–	1.39 ± 0.36 ***	75.90

∗∗$p < 0.01$, ∗∗∗$p < 0.001$.

∗Testing was done at 500 mg/kg b.wt. for all extracts while 5 mg/kg b.wt. artemisinin was administered to positive control animals.

#% Parasitemia represents the mean of groups of six mice each.

Antimalarial activity in established infection

Effect on parasitemia and survival

Administration of Cucurbita pepo ethanol extract (CpE) at 300 mg/kg/day resulted in a significant reduction in parasitemia compared to the distilled water-treated control group ($p < 0.01$) **(Table 3)**. The antimalarial effect at this dose was comparable to that of artemisinin administered at 5 mg/kg. Although mice treated with 300 mg/kg CpE exhibited a longer mean survival time, the increase was not statistically significant ($p > 0.05$). The observed effects of the extract did not follow a dose-dependent pattern, and the higher parasitemia and associated mortality observed at 900 mg/kg suggest reduced efficacy at this elevated dose **(Table 3)**.

Table 3. Effect of *C. pepo* extract on established infection in mice.

Group / Treatment	Dose (mg/kg)	Parasitemia (%)	Parasitemia Inhibition (%)	Mean Survival Time (days)
Malaria Control	–	Day 3: 0.97 ± 0.20Day 7: 9.5 ± 1.28	–	10.3 ± 1.27
Cucurbita pepo	100	Day 3: 0.50 ± 0.09Day 7: 9.9 ± 1.13	Not Inhibited (NI)	9.2 ± 1.35
	300	Day 3: 1.0 ± 0.15Day 7: 5.9 ± 1.36 **	37.9	11.7 ± 1.30
	900	Day 3: 0.8 ± 0.09Day 7: 8.4 ± 1.17	11.58	8.5 ± 1.17
Artemisinin	5	Day 3: 1.06 ± 0.16Day 7: 4.6 ± 1.36 ***	51.58	11.5 ± 1.17

$^{**}p < 0.01$, $^{***}p < 0.001$ relative to malaria control. NI = No Inhibition.

Effect of ethanol extract of C. pepo extract on hematological parameters

The impact of Cucurbita pepo ethanol extract (CpE) on selected hematological parameters is presented in **(Table 4)**. Administration of CpE at 100 and 300 mg/kg significantly increased red blood cell counts ($p < 0.05$) compared to the infected, untreated control. Infection led to a reduction in hemoglobin levels, which were notably restored by CpE at these same doses. Platelet counts, also suppressed by infection, were significantly elevated in mice treated with CpE or artemisinin ($p < 0.05$), whereas the 900 mg/kg dose of CpE did not improve platelet numbers. Neutrophil levels, diminished in infected animals, showed a modest, non-significant increase ($p > 0.05$) in groups receiving CpE or artemisinin.

Table 4. Effect of ethanol extract of *C. pepo* extract on the hematological indices in established infection in mice.

Treatment / Dose	RBC ($\times 10^{12}$/L)	Hemoglobin (HGB, g/L)	Platelets (PLT, $\times 10^{9}$/L)	Lymphocytes (%)	Neutrophils (%)
Infected Control	0.12 ± 2.14	19.7 ± 1.68	10.3 ± 3.3	54.33 ± 1.13	12.6 ± 1.62
Normal Control	1.077 ± 2.06	73.8 ± 2.00	137.4 ± 1.7 *	54.83 ± 1.13	15.28 ± 1.35
Cucurbita pepo (100 mg/kg)	1.71 ± 1.08 *	120.50 ± 1.14	131.5 ± 1.3 *	48.53 ± 1.09	26.73 ± 1.23
Cucurbita pepo (300 mg/kg)	2.32 ± 1.08 *	143.9 ± 1.05	155.6 ± 1.1 *	43.4 ± 1.06	36.81 ± 1.07
Cucurbita pepo (900 mg/kg)	0.34 ± 2.24	52.60 ± 1.92	73.28 ± 1.96	51.9 ± 1.16	19.8 ± 1.6
Artemisinin (5 mg/kg)	0.58 ± 1.16	51.64 ± 1.93	114.29 ± 1.7	49.77 ± 1.13	25.53 ± 1.35

Values represent mean ± SEM (n = 5). $^{*}p < 0.05$: Significant difference compared to the malaria control.

Relative organ weight index

Malaria infection resulted in noticeable enlargement of the brain, spleen, liver, and kidneys in the infected control mice. Treatment with Cucurbita pepo ethanol extract (CpE) for five days attenuated this organ enlargement, producing lower relative organ weight indices compared to the untreated infected group. However, these differences remained less pronounced when compared statistically to the uninfected, untreated control group **(Table 5)**.

Table 5. Impact of Cucurbita pepo Extract on Relative Organ Weights in Mice with Established Malaria

Treatment / Dose	Liver (g)	Kidney (g)	Brain (g)	Spleen (g)
Normal Control	4.34 ± 0.301	1.29 ± 0.076	1.22 ± 0.101	0.52 ± 0.045
Malaria Control	6.53 ± 0.199^{d}	1.42 ± 0.12	1.51 ± 0.108	1.28 ± 0.114^{d}
Cucurbita pepo (100 mg/kg)	6.45 ± 0.458^{c}	1.29 ± 0.108	1.56 ± 0.085^{a}	1.05 ± 0.120^{b}
Cucurbita pepo (300 mg/kg)	5.89 ± 0.199^{b}	1.21 ± 0.072	1.51 ± 0.057	1.11 ± 0.113^{c}
Cucurbita pepo (900 mg/kg)	5.64 ± 0.372^{a}	1.23 ± 0.087	1.23 ± 0.12	1.02 ± 0.096^{b}
Artemisinin (5 mg/kg)	5.07 ± 0.253**^{a}	1.22 ± 0.099	1.39 ± 0.063	1.13 ± 0.12^{c}

Values represent mean ± SEM (n = 5). $^{*}p < 0.05$; $^{**}p < 0.01$: Significant difference compared to the malaria control. $^{a}p < 0.05$; $^{b}p < 0.01$; $^{c}p < 0.001$; $^{d}p < 0.0001$: Significant different compared to normal control.

Effect of C. pepo ethanol extract on red blood cells (hemolysis assay)

ucurbita pepo ethanol extract showed negligible hemolytic activity across concentrations of 0.016–1 mg/ml, compared with complete lysis induced by 0.1% Triton X-100 **(Table 6)**. Even at the highest concentration tested (1 mg/ml), hemolysis, as measured by methemoglobin absorbance, remained below 1%.

Table 6. Absence of *in vitro* hemolytic activity in *C. pepo* extract.

Treatment	Concentration (mg/mL)	Mean Absorbance (Methemoglobin)	Hemolysis (%)
PBS	–	0.00603 ± 0.0026	0.18
DMSO (1% v/v)	–	0.00607 ± 0.0027	0.18
Cucurbita pepo Extract (CpE)	1.00	0.0311 ± 0.0032	0.90
	0.50	0.0239 ± 0.0043	0.69
	0.25	0.0105 ± 0.0015	0.31
	0.125	0.0084 ± 0.0015	0.24
	0.0625	0.0067 ± 0.0014	0.19
	0.03125	0.0032 ± 0.0002	0.09
	0.016	0.0026 ± 0.0009	0.03
Triton X-100 (0.1% v/v)	–	3.4463 ± 0.028	100

In this investigation, six extracts derived from three selected plants—Cucurbita pepo, Pennisetum purpureum, and Hibiscus rosa-sinensis—were evaluated for antiplasmodial activity using both in vitro and in vivo models. These plants were chosen based on ethnomedicinal reports of their use for malaria treatment by inhabitants of Nsukka Local Government Area, Enugu State, Nigeria [4]. Previous studies have also documented the use of H. rosa-sinensis in Malaysia and P. purpureum in Allada, southern Benin Republic, for malaria management [18, 19]. Beyond their medicinal application, these plants serve as food, animal forage, and ornamentals. The selection of 70% ethanol and dichloromethane/methanol (DCM/MeOH) for extraction aimed to capture a broad spectrum of phytochemicals, given the limited prior data on their antimalarial properties.

Cytotoxicity assays revealed that none of the extracts exhibited toxicity in vitro, which was further supported by the absence of acute toxicity in mice at 2000 mg/kg doses. This aligns with their traditional use and underscores their apparent safety. Toxicological screening remains an essential step in validating herbal products for potential therapeutic use, ensuring that candidate agents do not harm human cells [20]. Among the extracts tested, the 70% ethanol extract of C. pepo and the DCM/MeOH extract of P. purpureum demonstrated promising in vitro activity with favorable selectivity indices. Notably, only the ethanol extract of C. pepo achieved significant suppression of

parasitemia in vivo, suggesting that the active principles in P. purpureum may have limited bioavailability or suboptimal pharmacokinetic properties in the murine malaria model [21]. Further work to isolate and optimize its active constituents is warranted.

Acute oral toxicity assessment using the OECD limit test confirmed the safety of all extracts, as no mortality occurred at 2000 mg/kg. According to the Globally Harmonized System (GHS), agents with no lethality at 2000–5000 mg/kg are classified as Category 5, indicating low acute toxicity potential [22]. While these findings support safety, additional studies are needed to assess other routes of administration, long-term exposure, and histopathological effects.

The ethanol extract of C. pepo demonstrated moderate activity in the 4-day suppressive test and reduced parasitemia in established infection. Interestingly, increasing the dose beyond 300 mg/kg did not enhance antimalarial efficacy, as the 900 mg/kg dose produced less pronounced effects, suggesting that the optimal therapeutic window may lie below this dose. Despite this, beneficial effects were observed in organ weight normalization and hematological parameters. Severe malaria is associated with sequestration of parasitized erythrocytes in organs such as the liver, spleen, kidneys, and brain, leading to organ enlargement and inflammation [23]. Treatment with C. pepo mitigated splenomegaly and attenuated liver and kidney hypertrophy, highlighting its protective potential.

Malaria infection typically induces anemia, leukocytosis, and thrombocytopenia due to hemoglobin consumption by the parasite and destruction of infected and uninfected erythrocytes [24-26]. In this study, C. pepo extract prevented reductions in hemoglobin, red blood cell, and platelet counts, potentially by lowering parasite burden

and/or stimulating erythropoiesis. Future studies should investigate the specific phytochemicals responsible and the mechanisms through which they modulate hematological parameters.

Safety toward erythrocytes is a key consideration for antimalarial agents, as selective targeting of the parasite without damaging host red blood cells is critical [27, 28]. C. pepo extract exhibited no hemolytic activity, supporting its suitability as a selective antimalarial candidate.

The presence of secondary metabolites such as phenolics, flavonoids, alkaloids, and glycosides in medicinal plants contributes to diverse therapeutic effects. Phenolic compounds—including gallic acid, rutin, catechin, tannins, quercetin, and cinnamic acid—exert antioxidant effects by scavenging reactive oxygen species (ROS), which are implicated in cerebral malaria pathogenesis via neuroinflammation [29]. Notably, gallic acid and ellagic acid have been reported to inhibit P. falciparum 3D7 proliferation in vitro [30]. The HPLC fingerprint of C. pepo and its gallic acid-equivalent phenolic content suggest that polyphenols may contribute substantially to the observed antimalarial activity.

Conclusion

This study demonstrates, for the first time, that the ethanol extract of Cucurbita pepo leaves possesses significant antimalarial activity, supporting its traditional use by healers in southeastern Nigeria. These findings provide a strong rationale for further investigations aimed at isolating and characterizing the bioactive compounds responsible for its antiplasmodial effects.

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