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Research article

Assessment of antimalarial medicinal plants used in Nigerian ethnomedicine reveals antimalarial potential of *Cucurbita pepo* leaf extract

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ABSTRACT

Medicinal plants are often used to treat malaria in different parts of Nigeria and exploiting these can unravel new therapeutic leads. This study evaluated the antiplasmodial potential of selected plants used to treat malaria in Nsukka, Enugu state, Nigeria. Leaves of three different plants (*Cucurbita pepo, Hibiscus rosa-sinensis and Pennisetum purpureum*) were collected for screening and two extracts *viz*., 70%v/v ethanol and dichloromethane/methanol (1:1 v/v), were prepared for each. An acute toxicity test was done in mice and cytotoxicity was assessed using human hepatoma cell line (HUH). The extracts were screened against chloroquine-sensitive *P. falciparum* (*Pf*3D7) *in vitro*, and chloroquine-resistant *P. berghei* ANKA *in vivo* using a 4 day-suppressive test in mice. *Cucurbita pepo* ethanol extract was further tested for hemolytic effect on human erythrocytes and in established infection in mice. Parameters assessed were post-treatment parasitemia, hematological indices, organ (brain, kidney, liver, and spleen) weights, and survival. The extracts were non-cytotoxic up to a test dose of 100 μg/ml and 2000 mg/kg fed - mice did not show acute or delayed toxicity. *Cucurbita pepo* ethanol extract (*Cp*E) displayed excellent *in vitro* antiplasmodial activity with IC_{50} of 3.05 μg/ml. At an oral dose of 500 mg/kg, mice were observed to display significant ($p < 0.01$) ~51% suppression of parasitemia. The extract did not produce any significant hemolytic effect up to a test concentration of 1 mg/ml. In established infection, a dose of 300 mg/kg significantly ($p < 0.01$) protected mice from anemia caused by low hematocrit. The extract produced significant ($p < 0.05$) elevation in red blood cells and platelet counts, and an increase in hemoglobin was evident at 100 and 300 mg/kg. Further, *Cp*E in a dose-dependent manner, reversed liver and spleen weight increase seen in untreated, infected mice. These findings show *C. pepo* as a potential candidate for further studies to identify its bioactive principle(s) and possible mechanism(s) of antimalarial action.

1. Introduction

Malaria is the most devastating and life-threatening disease after diarrhea and pneumonia in Africa and is the third leading cause of death in children under 5 years of age worldwide (WHO, 2020). Apart from children, pregnant women are also a sub-population at high risk of the disease. Most malaria cases and deaths occur in sub-Saharan Africa (WHO, 2019). It has been estimated that globally there were 229 million cases of malaria in 2019 compared to 228 million cases in 2018 (WHO, 2020). The estimated number of malaria deaths stood at 409,000 in 2019, compared with 405,000 deaths in 2018 (WHO, 2019, 2020). In 2019, Nigeria accounted for nearly a quarter of the global malaria burden (WHO, 2020). Since 2020, malaria control gains in high burden countries have begun to plateau as the gaps in accessing control tools widened with the COVID-19 pandemic (WHO, 2020). These gaps in control can increase malaria incidence and the risk of severe disease. Malaria is caused by *Plasmodium* species especially *Plasmodium falciparum*, which is predominant in the tropics and is implicated in severe, complicated malaria. Common clinical signs of malaria include fever, chills, nausea, and flu-like illness. In some cases, there are major complications associated with malaria infection such as cerebral malaria and severe anemia, often accompanying splenomegaly, headache, hepatomegaly, and

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hemoglobinuria with renal failure. Particularly susceptible to infection are those with low immunity, especially young children. Drug resistance continues to be a major setback for all old drugs and new emerging drugs including drug combinations. This has been a bane for malaria chemotherapy especially in malaria-endemic rural regions. To combat resistance, new therapeutics with novel mechanisms will be required. Two of the most successful antimalarial compounds namely quinine and artemisinin were derived respectively from *Cinchona officinalis* and *Artemisia annua* (Lima et al., 2015). Subsequently, medicinal plants have remained an important source for the discovery of new antimalarials. In the context of Africa, particularly in Nigeria where malaria burden is highest, the practice of malaria treatment with medicinal plants is common. The country is endowed with herbs employed for different ethno pharmacological uses. There is a need to explore these natural resources for the discovery of new antimalarials. Odoh et al. (2018) documented medicinal plants used in the treatment of malaria and fever in Nsukka Local Government Area of Nigeria. Fifty different plant species from thirty botanical families were recorded based on an ethnobotanical survey. The plants include *Azadirachta indica*, *Carica papaya*, *Cymbopogon citratus*, *Mangifera indica* and *Psidium guajava*. *Cucurbita pepo* L. (Cucurbiteae), *Hibiscus rosa-sinensis* L. (Malvaceae), and *Pennisetum purpureum* Schumach (Poaceae) had citation frequencies of 0.85, 0.57 and 1.42 % respectively (Odoh et al., 2018), but further literature survey revealed no previous scientific report on antiplasmodial activity of these three plants. This informed the basis for the selection of these plants for screening in the present study, to ascertain their antimalarial potential. *H. rosa-sinensis* originated from India and is a perennial evergreen shrub, growing 1–3 m with tap root system (Sivaraman and Saju, 2021). A methanol extract of *H. rosa-sinensis* leaves inhibited the growth *E. coli, P. aeruginosa*, and *Salmonella species* and potentiated antibiotic efficacy against resistant clinical bacterial isolates (Maraskolhe et al., 2020). *P. purpureum* commonly called elephant or Napier grass is a robust, perennial plant that originated from Africa. A root decoction of *P. purpureum* is used in the treatment of blennorrhoea, the sap expressed from young shoots is mixed with a small amount of salt and instilled in the eye for treatment of cataracts (Jansen and Burkill, 1999). *C. pepo* is a specie in the *Cucurbita* genus also referred to as cucurbits, exemplified by pumpkins, gourds, squashes, zucchini, and melons. *Cucurbita pepo* extracts reportedly possess anti-inflammatory and anti-carcinogenic properties (Yadav et al., 2010). This study was aimed at investigating the antimalarial potentials of extracts of *H. rosa-sinensis*, *P. purpureum*, and *C. pepo* used by the indigenes of Nsukka for the management of malaria.

2. Materials and methods

2.1. Selection, collection, and identification of plant materials

The plants studied are shown in Figure 1. Fresh leaves of *H. rosa-sinensis* and *C. pepo* were collected from Orba, Enugu State (6.8541° N, 7.4625° E) and identified by Mr. A. Ozioko of the International Centre for Ethnomedicines and Drug Development (InterCEDD) Nsukka, Enugu state. Voucher specimens [InterCEDD/19112 (*H. rosa-sinensis)*, InterCEDD/27648 (*C. pepo*)] were prepared and deposited in InterCEDD herbarium. *P. purpureum* was collected from Niger state (9.2179° N, 7.1898° E) by Mr. I. Muazzam of the plant taxonomy unit, Department of Medicinal Plant Research and Traditional Medicine, National Institute for Pharmaceutical Research and Development (NIPRD) Nigeria. A voucher specimen [NIPRD/H/7199] was prepared and deposited in NIPRD herbarium.

2.2. Extraction of plant materials

Leaves of the plants were air-dried under shade and pulverized using an electric hammer mill. Two extracts per plant material (six extracts in

Figure 1. Antimalarial plants investigated in the study, clockwise from top left: *Cucurbita pepo*, *Hibiscus rosa-sinensis,* and *Pennisetum purpureum.*

total) were prepared by cold maceration. First, a 50 g quantity of each plant material was extracted with dichloromethane/methanol (1:1v/v, 700 ml). Then separately, 50 g of plant material was extracted with 70% v/v ethanol (700 ml). The mixtures were extracted for 48 h with intermittent shaking, after which they were filtered using a fine pore filter cloth and then Whatman filter paper (10 μm). The filtrates were evaporated to dryness on a water bath maintained at 55 °C, and their respective yields determined. The extracts were stored in air-tight containers in a refrigerator at 4 °C.

2.3. HPLC fingerprint of C. pepo extract

A HPLC system (Shimadzu Corporation, Kyoto Japan) consisting of a column (150 × 4.6 mm) with VP-ODS (5μm) as stationary phase was used. The chromatographic conditions were; mobile phase comprising solvent A: 0.2% v/v formic acid in water, and solvent B: 20% acetonitrile; mode: isocratic; flow rate 0.6 ml/min; injection volume of 20 μL of 20 mg/ml aqueous solution of the extract. Ultraviolet detection of constituents was done at 254 nm wavelength. Standard compounds (rutin, morin, gallic acid, caffeic acid, and ferulic acid) (Fluka, Germany) prepared as 50 μg/ml methanol solutions were analyzed separately under the same conditions as the extract. Chromatograph operating conditions were programmed as follows: solvent A: 80%, solvent B: 20%, column oven temperature of 40 °C, and total run time of 20 min (Adamu et al., 2018).

2.4. Quantification of phenolics in C. pepo extract

A standard gallic acid plot was prepared using serial dilutions of gallic acid (0.0016–1 mg/ml). To 1 ml of each dilution, 2.5 ml of 10% Folin Dennis reagent was added and the mixture was allowed to stand for 2 min at room temperature. After 2 min, 2 ml of sodium carbonate solution (75 g/L) was added and the resulting mixture was maintained at 50 °C for 15 min in a water bath, then cooled in an ice-cold bath for 3 min. The absorbance was read spectrophotometrically at 760 nm (Cary 60 UV-Vis spectrophotometer, Agilent, Santa Clara, USA). Serial dilutions (1 – 0.25 mg/ml) of *C. pepo* extract were prepared and treated as described for gallic acid. The solutions were made in triplicates and absorbance read at 760 nm. The phenolic content was extrapolated from the standard gallic acid plot ($y = 1.474x - 0.001782$, $R^2 = 0.9978$).

2.5. Malaria parasite

Chloroquine (CQ)-resistant rodent parasite (*P. berghei* ANKA) was sourced from the Institute for Medical Research and Training, University of Ibadan, Nigeria. The parasite was maintained by continuous reinfection (intraperitoneal) in healthy mice. Chloroquine - sensitive *Pf*3D7 (MRA 102) was obtained from MR4 and maintained in the International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi.

2.6. In vitro cultivation of Plasmodium falciparum

Chloroquine sensitive *P. falciparum* (*Pf*3D7) was obtained from MR4 and maintained in the Malaria Drug Discovery lab, ICGEB, New Delhi as described by Trager and Jenson with slight modification (Trager and Jensen, 1976). The parasites were maintained and supplemented at 4 % hematocrit using complete medium (16.2 g/L RPMI 1640, 0.2 % sodium bicarbonate, 0.5 % Albumax I, 50 mg/L hypoxanthine, and 10 mg/L gentamicin) in fresh O^+ erythrocytes. The parasite culture was incubated at 37 °C under 5 % O_2, 5 % CO_2, and 95 % N_2.

2.6.1. Preparation of extract stock solutions

DMSO was used to prepare 25 mg/ml stock solutions of each extract. Further dilution was made by adding 10 μL of each stock solution to 90 μL of parasite culture medium. This was serially diluted in culture medium to obtain the final working dilutions. A 4 μL volume of working dilutions of each extract was added to the 96-well microplate for the assay. Chloroquine was used as control and a stock solution prepared in sterilized distilled water.

2.6.2. Antiplasmodial assay of extracts against P. falciparum

A SYBR-green I fluorescence-based assay was used to examine the antiplasmodial activities of the extracts (Smilkstein et al., 2004). Parasite culture was synchronized using 5% sorbitol and then incubated at 2 % hematocrit and 1 % parasitemia in a 96-well plate. Extract test concentrations ranged from 0.78-100 μg/ml, DMSO (0.4%) was used as negative control and CQ (40 nM) as positive control. The plates were incubated under culture conditions and after 48 h, 100 μl of SYBR Green I solution in lysis buffer [0.2 μl of 10,000 × of SYBR Green I per ml of lysis buffer (20 mM Tris buffer pH 7.5, 5mM EDTA, 0.008% saponin and 0.08% v/v Triton X-100)] was added to each well. The mixture was incubated for 1 h in the dark at 37 °C. Fluorescence was measured at 485 nm excitation and 530 nm emission wavelengths. Extracts with $IC_{50} < 5$ μg/ml, 6–15 μg/ml, and 16–30 μg/ml were classified as highly active, promising, and moderately active respectively. Extracts with IC_{50} of 31–50 μg/ml were classified as having low activity, and as inactive extracts, if the IC_{50} was >50 μg/ml (Kraft et al., 2003; Pink et al., 2005).

2.7. Cytotoxicity test

Human Hepatoma (HUH) - derived cells were grown in Dulbecco's Modified Eagles Medium (DMEM) that contains 5–10 % fetal bovine serum (Gibco) and dispensed into a 96-well plate containing 10^4 cells/100 μL/well according to the method described by Mosmann (1983) with slight modification. The plate was incubated at 37 °C and 5% CO_2 for 24 h and the test samples added and incubated for another 24 h under normal condition. MTT-PBS solution (20μL) (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide dissolved in phosphate buffer solution (5 mg/ml) was incorporated into the 96-well plate and incubated for 3 h. The medium was removed and 200μL of DMSO/well added to dissolve crystal formed from MTT-formazan and absorbance due to cleavage of MTT ring by living cells read at 570 nm on multi-well plate reader. The assay was done in triplicates. CC_{50} values were determined from concentration-response curves. For negative and positive controls, 0.4 % and 10 % DMSO were used, respectively.

2.8. Animals

Swiss albino mice of both sexes weighing between 15-25 g were acclimatized for two weeks to laboratory conditions in the animal facility center of the Department of Pharmacology and Toxicology, NIPRD, Abuja. The mice were housed in plastic cages in a ventilated room at a temperature of 25 °C, fed with standard rodent chow, and allowed free access to potable water.

2.8.1. Ethical approval

Experiments on animals were approved by NIPRD Animal Care and Ethics Committee (NIPRD/05:03:05/007) prior to conducting the study. The experiments were carried out following National Institutes of Health Guide for the Care and Use of Laboratory Animals as detailed in NIH publication No. 85-23 (NIH, 2011), and according to NIPRD's standard operating procedures (NIPRD/05.03.0.5–1).

2.9. Acute toxicity

The acute oral toxicity of the plant extracts was investigated using the Organization for Economic Co-operation and Development protocol, guideline 425 (OECD, 2008). Twenty-four female mice were used for acute toxicity testing of the six extracts. The mice were fasted overnight with free access to water after weighing, coding and randomization. The mice were divided into 6 groups of 4 mice each. with one mouse per group serving as control and recieved the vehicle without extract. The

ethanol extracts were prepared in distilled water while DCM/MeOH extracts were reconstituted in coconut oil. Each group was given a dose of 2000 mg/kg body weight of each extract accordingly and observed continuously for behavioral and autonomic profiles for 2 h and any signs of toxicity or mortality up to 14 days.

2.10. In vivo *antimalarial study*

2.10.1. *Parasite inoculation*

Eight days after parasite inoculation of a healthy mouse, a Giemsa-stained thin blood film was prepared from its tail vein blood for the assessment of parasitemia. The donor mouse was anesthetized, and blood was collected by cardiac puncture. The withdrawn blood sample was diluted with physiological saline such that 0.2 ml contained approximately 10^7 parasitized red blood cells based on the parasitemia level of the donor (Fidock et al., 2004). Each experimental mouse was inoculated intraperitoneally with 0.2 ml of the diluted infected blood.

2.10.2. In vivo *suppressive test of extracts*

The 4-day suppressive test model was used to measure the schizonticidal activity of the extracts against *P. berghei* infected mice (Peters et al., 1975). In brief, 48 female and male mice were used in this study. The mice were weighed and randomized by weight into eight groups (I–VIII) of six each (three male, three female). A 0.2 ml volume of dilute infected blood was used to innoculate test mice by intraperitoneal injection. After 4 h, animals were orally given the extracts once daily (prepared using 0.1% tween 80 in distilled water) for 4 consecutive days starting from Day 0 at a dose of 500 mg/kg for each extract for Groups I–VI. Group VII was given 5 mg/kg of artemisinin daily, while Group VIII was given 5 ml of vehicle and served as untreated, infected control. Treatment was done for 4 days (day 0–3). Thin blood smears were prepared from the tail vein blood of the mice on day 4 and fixed in methanol, then stained using 10% Giemsa stain, counting parasite per 100 red blood cells in five fields under a microscope with immersion oil and ×100 objective.

Percentage of parasitemia was determined by:

$$\%\, parasitemia = \frac{Number\ of\ parasitized\ RBCs}{Total\ number\ of\ RBCs} \times 100$$

And suppression (%) produced by test extracts was determined by:

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

Based on the percentage suppression produced, antiplasmodial activity was classified as moderate for extracts that suppressed parasitemia by equal to or greater than 50% at a dose of 500 mg/kg body weight per day (Nardos and Makonnen, 2017).

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

2.11.1. *Experimental design*

The curative antimalarial activity of *C. pepo* ethanol extract was carried out using a curative test model in mice (Fidock et al., 2004). The experimental mice were weighed, randomized, and inoculated on the third day after establishing parasitemia in host mice. A total of 70 mice were inoculated and divided into groups A and B, and each further divided into 5 sub-groups (A1 - A5; B1 - B5) of 7 mice each. An addition group of 7 mice served as distilled water - treated, uninfected normal control. *C. pepo* extract was dissolved in distilled water and doses of 100, 300, and 900 mg/kg (tests groups) was fed orally once a day to the mice in groups A1 - A3 and B1 - B3. The positive control groups (A4 and B4) received once daily, artemisinin (Sigma Aldrich, Germany) orally at 5 mg/kg while infected, distilled water - treated groups (A5, B5) were administered distilled water (10 ml/kg) once daily. Treatment was done for 5 days, from day 3 to day 7. Parasite load was determined on day 8 using thin smears film stained with 10 % Giemsa solution. A total of 5 fields containing 100 red blood cells per field were counted under the microscope. The percentage of inhibition of the parasite growth (% I) of each dose extract was determined according to the following formula:

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

Mice in group A were kept for monitoring survival, while Group B mice were euthanized by chloroform inhalation on day 8 for collection of organs (liver, spleen, kidney, brain) and hematological assessment.

2.11.2. *Hematological parameters, relative organ weight index*

The mice were euthanized, and organs (liver, kidneys, brain, and spleen) were harvested and weighed. Relative weight index of each organ per body weight was determined. Blood was collected from the tail vein of the mice into EDTA tubes for evaluation of hematological parameters using an automated hematology analyzer YNH7021 (Wincom Company Ltd. Hunan, China). Parameters evaluated included hemoglobin concentration, red blood cell, and platelet, neutrophil, and lymphocyte counts.

2.12. *Hemolysis assay of C. pepo extract*

Cucurbita pepo ethanol extract was evaluated for toxicity to erythrocytes using *in vitro* hemolysis assay as previously described (Ajdačić et al., 2016). Briefly, fresh A^+ blood was centrifuged at 1000 ×g for 5 min and the supernatant discarded. The packed cells were washed thrice with three volumes of phosphate-buffered saline (PBS, pH 7.4) and centrifuged. Serial dilutions of *C. pepo* extract was prepared in PBS, with concentrations ranging from 0.0016- 1 mg/ml. The assay was performed at 2% hematocrit, and 0.1% Triton X-100 was used as a positive control to represent 100% hemolysis. DMSO (1%) was prepared as a control for vehicle-induced hemolysis. After adding the extract solution and 2% hematocrit, incubation was carried at 37 °C for 4 h, afterwards, the tubes were centrifuged at 1000 ×g for 10 min. The supernatants were collected and absorbance read at 540 nm. Results were expressed as a percentage relative to 100% hemolysis produced by Triton X-100 as a positive control.

2.13. *Statistical analysis*

All *in vitro* assays were performed in triplicates. Concentrations producing 50% inhibitory effect (IC_{50}) against *Pf*3D7 were obtained by non-linear regression using the IC Estimator-version 1.2 software (http://www.antimalarial-icestimator.net/MethodIntro.htm), free software foundation, Boston, MA, USA. Data from *in vivo* antimalarial assay were log-transformed before statistical analysis and back-transformed afterward. Data obtained from *in vivo* assays were expressed as mean ± standard error of the mean (SEM), with one-way analysis of variance (ANOVA) with Dunnett's *post hoc* test used for statistical comparisons. Significant difference was accepted for p values less than 0.05 ($p < 0.05$).

3. Results

3.1. *HPLC profile of C. pepo ethanol extract*

The HPLC fingerprint of *C pepo* extract revealed nine peaks with retention times ranging between 3.004 to 23.457 min (Figure 2).

The first peak (retention time: 3.004 min) corresponded to that of the reference compound, gallic acid, with a relative abundance of 39.64% in the extract. The fingerprint also showed the presence of a peak corresponding to morin, detected at 18.943 min and a relative abundance of 1.65%.

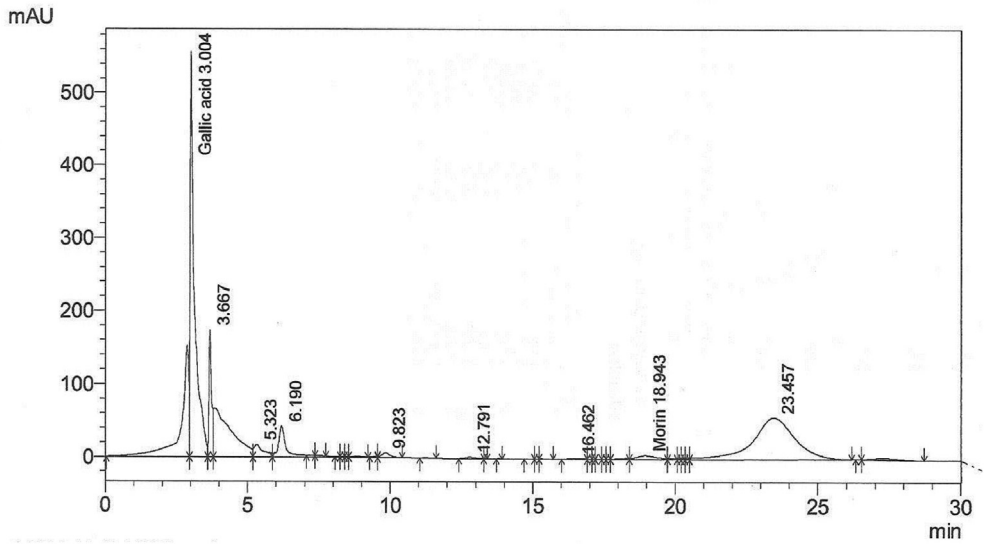

Figure 2. RPHPLC Chromatogram of ethanoic leaf extract of *Cucurbita pepo*.

3.2. Quantification of phenolic content of C. pepo ethanol extract

The total phenolic content of *C. pepo* ethanol extract estimated from the standard gallic acid calibration plot was found to be 0.1837 gallic acid equivalent (GAE) per mg of extract.

3.3. Extract yields, in vitro antiplasmodial activity, and cytotoxicity

The yields from ethanol extracts of the plant leaves were generally higher than the yields of their respective DCM/MeOH extracts (Table 1). The DCM/MeOH extracts of *P. purpureum* and *C. pepo* were highly active (<5 μg/ml) while extracts of *P. purpureum* (70%v/v EtOH), *C. pepo* (70% v/v EtOH) *H. rosa-sinensis* (70%v/v EtOH and DCM/MeOH) exhibited promising activity (Kraft et al. 2003; Pink et al., 2005) (Table 1). None of the extracts produced cytotoxic effects at the tested concentrations.

3.4. Acute toxicity

Oral ingestion of 2000 mg/kg of the extracts did not elicit acute toxicity in treated mice. Treatment with *C. pepo* extract initially produced mild sedation within 30 min, but this was reversed within 1 h. None of the animals presented behavioural changes or clinical signs and symptoms of toxicity. No death was recorded in any of the extract-treated mice (2000 mg/kg, p.o.) during the 14 days observation period.

3.5. Suppressive antimalarial activity

As shown in Table 2, only *Cucurbita pepo* ethanol extract (*Cp*E) exhibited moderate activity among the eight extracts tested. Upon oral feeding at 500 mg/kg body weight daily for four days, the extract produced 50.95 % suppression ($p < 0.01$) of parasitemia (Table 2). *P purpureum* ethanol extract exhibited 19.4% suppression of parasitemia, while *C. pepo* (DCM/MeOH extract) and *H. rosa-sinensis* (ethanol extract) caused <10 % suppression (6.89 and 9.33 % respectively) of parasitemia.

3.6. Antimalarial activity in established infection

3.6.1. Effect on parasitemia and survival

Treatment with 300 mg/kg/day *Cucurbita pepo* extract (*Cp*E) produced a significant ($p < 0.01$) reduction in parasitemia compared to the distilled water-treated control group (Table 3). The effect produced at 300 mg/kg was like the effect elicited by artemisinin at the dose of 5 mg/kg. Mean survival time was prolonged only in mice treated with 300 mg/kg *Cp*E but this increase was statistically insignificant ($p > 0.05$). These changes produced by the extract were observed to be non-dose-dependent. The higher mortality at 900 mg/kg body weight can be attributed to the higher %parasitemia recorded at that dose (Table 3).

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

The effects of *Cp*E on some hematological parameters are summarized in Table 4. At 100 and 300 mg/kg doses of extract, there was a significant ($p < 0.05$) increase in the red blood cell count compared to the untreated, infected control group. Infection caused a reduction in hemoglobin concentration in untreated mice, but treatment with *Cp*E at 100 and 300 mg/kg doses elevated hemoglobin levels. Similarly, a decrease in platelet count was observed in infected mice, but significant ($p < 0.05$) increases were produced in *Cp*E- and artemisinin-treated groups. The extract at 900 mg/kg did not elevate the platelet concentration (Table 4). Neutrophils were reduced in infected mice but increased ($p > 0.05$) in groups treated with *Cp*E or artemisinin.

Table 1. Yields, *in vitro* antiplasmodial activity, mammalian cell cytotoxicity and selectivity indices of extracts.

Species	Solvent	Yield (%)	IC_{50}*Pf*3D7 (μg/ml)	CC_{50}HUH (μg/ml)	Selectivity Index (S.I.)
C. pepo (Cucurbitaceae)	70% Ethanol	8.55	3.05 ± 0.37	>100	>32.79
	DCM/MEOH	4.18	>10	>100	NK
H. rosa-sinensis (Malvaceae)	70% Ethanol	13.1	25.4 ± 2.67	>100	>3.94
	DCM/MEOH	6.75	15.01 ± 2.3	>100	>6.66
P. purpureum (Poaceae)	70%Ethanol	7.69	6.16 ± 1.37	>100	>16.23
	DCM/MEOH	2.94	1.75 ± 0.01	>100	>57.14

NK = Not known.

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

		%Parasitemia# on day 4 p.i.	Parasitemia suppression (%)
Untreated control	Nil	5.78 ± 0.65	0
C. pepo	Ethanol	2.83 ± 0.43**	50.95
	DCM/MeOH	5.38 ± 0.65	6.89
H. rosa-sinensis	Ethanol	5.24 ± 0.90	9.33
	DCM/MeOH	6.14 ± 0.68	0
P. purpureum	Ethanol	4.66 ± 0.6	19.41
	DCM/MeOH	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.

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

Group	Dose (mg/kg)	Parasitemia (%)		Inhibition (%)	Mean survival time
		Day 3	Day 7		
Malaria Control		0.97 ± 0.2	9.5 ± 1.28	–	10.3 ± 1.27
C. pepo	100	0.5 ± 0.09	9.9 ± 1.13	NI	9.2 ± 1.35
	300	1.0 ± 0.15	5.9 ± 1.36 **	37.9	11.7 ± 1.3
	900	0.8 ± 0.09	8.4 ± 1.17	11.58	8.5 ± 1.17
Artemisinin	5	1.06 ± 0.16	4.6 ± 1.36***	51.58	11.5 ± 1.17

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

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)	HBG (g/L)	PLT (10^9/L)	LYMP (%)	NEUT (%)
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
C. pepo (100 mg/kg)	1.71 ± 1.08*	120.50 ± 1.14	131.5 ± 1.3*	48.53 ± 1.09	26.73 ± 1.23
C. pepo (300 mg/kg)	2.32 ± 1.08*	143.9 ± 1.05	155.6 ± 1.1*	43.4 ± 1.06	36.81 ± 1.07
C. 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.

3.6.3. Relative organ weight index

Induction of malaria led to an enlargement of the organs (brain, spleen, liver and kidney) as was seen in the infected control. Following treatment in *Cp*E-treated groups for 5 days, the increase in relative organ indices was lower and less significant than that of the malaria control group, when statistically compared to the uninfected, untreated control group (Table 5).

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

C. pepo extract did not produce significant hemolysis at concentrations ranging between 0.016 and 1 mg/ml, relative to 100% lysis induced by Triton X-100 (Table 6). At a maximum concentration of 1 mg/ml of extract, hemolysis produced (measured as the absorbance due to methemoglobin generated) was <1%.

Table 5. Effect of *C. pepo* extract on relative weight indices of liver, kidneys, brain and spleen in established infection.

Treatment/Dose	Liver	Kidney	Brain	Spleen
	Mouse organ weight relative to body weight (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]
C. pepo (100 mg/kg)	6.45 ± 0.458[c]	1.29 ± 0.108	1.56 ± 0.085[a]	1.05 ± 0.120[b]
C. pepo (300 mg/kg)	5.89 ± 0.199[b]	1.21 ± 0.072	1.51 ± 0.057	1.11 ± 0.113[c]
C. 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.

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

Treatment	Concentration (mg/ml)	Mean Absorbance due to Methemoglobin	Hemolysis (%)
PBS		0.00603 ± 0.0026	0.18
DMSO (1 %v/v)		0.00607 ± 0.0027	0.18
Extract	1.00	0.0311 ± 0.0032	0.90
(*Cp*E)	0.5	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

4. Discussion

In this study, six extracts from three selected plants, *Cucurbita pepo, Pennisetum purpureum* and *Hibiscus rosa-sinensis* were investigated for antiplasmodial activity using *in vitro* and *in vivo* models of infection. This was based on report of their use to treat malaria by the people of Nsukka Local Government Area, Enugu state of south-eastern Nigeria, (Odoh et al., 2018). Prior to this report, there had been reports of the use of *Hibiscus rosa-sinensis* in Malaysia and the use of *Pennisetum purpureum* by people in Allada, South of Benin Republic, against malaria (Al-Adhroey et al., 2010; Yetein et al., 2013). The plants studied herein are used as medicine, food, forage for animals, and ornamental plants. Their selection for this study was informed by a paucity of previous scientific evidence on antimalarial activity on any of the plants, and the choice of the solvents (70% v/v ethanol and DCM/MeOH) for extraction was to ensure that a wide range of constituents was extracted. *In vitro* cytotoxicity test was performed on the six extracts and it was found that none of the six extracts were cytotoxic. This was further confirmed by the absence of *in vivo* toxicity at 2000 mg/kg doses in mice, and this gives credence to their safety and long history of use by traditional healers for malaria fever. To evaluate herbal products in the development of new drugs, toxicity screening is very crucial to ensure substance safety in humans (WHO, 2013). The essence of cytotoxicity assay is to ensure that a tested substance will not impart deleterious effects to human cells. There should also be a correlation between *in vitro* cytotoxicity and *in vivo* toxicity to ensure the test substance does not pose any potential hazard when consumed. Among the extracts tested, *C. pepo* 70%v/v EtOH extract and *P. purpureum* DCM/MeOH extract produced promising *in vitro* antiplasmodial activity and displayed good selectivity. However, only *C. pepo* ethanol extract produced significant suppression of parasite growth *in vivo*. This phenomenon may imply that antiplasmodial principles of *P. purpureum* may be influenced by poor drug-likeness or pharmacokinetic factors in the mouse model of infection (Deharo and Ginsburg, 2011). Thus, efforts can be made to identify its active components and improve drug-likeness.

The safety of an agent can be established by assessing the acute toxicity using the limit test (OECD, 2008). The test is used in situations where there is information indicating that the test material is likely to have toxicity below regulatory limit doses. No mortality was recorded in all the mice that received 2000 mg/kg, p.o. of the extracts. Based on the Globally Harmonized Classification (GHS) system, test agents that produce no lethality at doses of 2000–5000 mg/kg are considered as Category 5 agents, indicating that they do not possess fatally injurious potential after acute oral ingestion (OECD, 2001). Thus, the extracts can be considered safe based on this test model. It should be noted however that this study did not examine other routes of administration, effects of long-term use, or histological evaluation of different organs.

The ethanol extract of *C. pepo* was moderately active in a 4-day antimalarial study and inhibited parasitemia in established malaria infection. However, higher doses of the extract did not translate into better antimalarial activity in established infection. The absence of significant activity of the extract at a dose of 900 mg/kg may imply that the effective dose required to produce maximum efficacy falls below this dose. Nevertheless, there was still evidence of activity seen in organ weight changes and hematological profiles in all extract-treated groups. One of the features of severe malaria is the adherence of infected red blood cells to vascular endothelial cells of organs such as the liver, kidney, spleen, and brain causing blood impairment leading to enlargement and subsequently inflammation and pain (Grau and Craig, 2012). In established infection, the antimalarial potential of *C. pepo* extract was evident by the reversal of splenomegaly, liver, and kidney weight increase.

Malaria usually results in anemia, leukocytosis, thrombocytopenia, which are some major physiological changes, in malaria condition (Saltarelli et al., 2019). Plasmodium parasite feed on host cell hemoglobin to generate amino acid building blocks which are directed for parasite development, thereby leading to reduction in red blood cells, hemoglobin and hematocrit (Klonis et al., 2013). Malaria-induced anemia is one of the commonest causes of mortality and morbidity especially in pregnant women and children under five years of age. In malaria, healthy red blood cells (RBCs) are usually cleared from the circulation alongside parasitized RBCs (Nussenblatt and Semba, 2002). In this study, *C. pepo* extract prevented decreases in hemoglobin, platelet and red blood cells counts. This may be elicited via a reduction of parasite burden leading to decreased hemoglobin depletion, and/or by upregulation of erythroid precursors which boosted erythropoiesis and consequently influenced hematological parameters positively. Further studies will be important to elucidate the specific mechanisms and compounds in the extract which mediate these effects. In addition to possessing compounds that directly target the malaria parasite, the plant may also have bioactive compounds that can activate and boost erythropoiesis. *In vitro* test for hemolysis is important for evaluating cytotoxicity of herbal products in antimalarial drug discovery (Haq et al., 2017). Non-hemolytic activity is an essential property for a candidate antimalarial to possess, as this indicates that the parasite selectively targets *P. falciparum,* which is an intracellular blood parasite that lives in and depends on the red blood cells for its growth and replication. Any medicinal plant with antimalarial activity should be non-toxic, target-specific, and selective for the parasite (Hooft van Huijsduijnen and Wells, 2018). *C. pepo* extract had no untoward effect on red blood cells and selectively targeted malaria parasites, making the extract a suitable candidate for further investigation.

Medicinal plants contain secondary metabolites such as phenolics, alkaloids, and glycosides which possess various therapeutic properties. Phenolic compounds like gallic acid, rutin, catechin, flavonoids, tannins, quercetin, cinnamic acid act as antioxidants that scavenge reactive oxygen species (ROS). ROS are implicated in cerebral malaria as free heme becomes toxic because of ROS and cross the blood brain barrier to cause neuro-inflammation (Pamplona et al., 2007). Ellagic acid and gallic acid have been previously reported to potently inhibit *P. falciparum* 3D7 proliferation *in vitro* (Ndjonka et al., 2012). Hence, the chromatographic fingerprint and the presence of gallic acid -equivalent phenolics in the extract suggest that the antimalarial activity of *C. pepo* may have been conferred by polyphenolic phytoconstituents such as gallic acid.

5. Conclusion

This study provided evidence for the antimalarial activity of the ethanol extract of *C pepo* leaves which gives credence and justification for its use by traditional healers in southeast Nigeria. This is the first time the plant is being reported to possess antimalarial activity. Further studies are thus warranted to isolate and characterize bioactive constituent(s) of *C pepo* leaves.

Declarations

Author contribution statement

Chinelo Ezeani: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Ifeoma Ezenyi: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Nekpen Erhunse: Performed the *in vitro* antiplasmodial & cytotoxicity experiments and analyzed the data therefrom; contributed to writing the paper.

Dinkar Sahal: Supervised the in vitro antiplasmodial and cytotoxicity study; contributed reagents, materials, analysis tools; revised the initial draft of the manuscript.

Theophine Akunne and Charles Okoli: Conceived, designed and supervised the study.

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Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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