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Intervention of standardized ethanol leaf extract of *Annickia polycarpa,* (DC.) Setten and Maas ex I.M. Turner. (Annonaceae), in *Plasmodium berghei* infested mice produced anti-malaria action and normalized gross hematological indices

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ABSTRACT

Ethnopharmacological relevance: Malaria is a global public health burden due to large number of annual infections and casualties caused by its hematological complications. The bark of *Annickia polycarpa* is an effective anti-malaria agent in African traditional medicine. However, there is no standardization parameters for *A. polycarpa*. The anti-malaria properties of its leaf are also not known.

Aim of the study: To standardize the ethanol leaf extract of *A. polycarpa* (APLE) and investigate its anti-malaria properties and the effect of its treatment on hematological indices in *Plasmodium berghei* infected mice in the Rane's test.

Materials and methods: Malaria was induced by inoculating female ICR mice with 1.0×10^7 *P. berghei*-infected RBCs in 0.2 mL (i.p.) of blood. Treatment was commenced 3 days later with APLE 50, 200, 400 mg/kg p.o., Quinine 30 mg/kg i.m. (Standard drug) or sterile water (Negative control) once daily per group for 4 successive days. Anti-malarial activity and gross malaria indices such as hyperparasitemia, mean change in body weight and mean survival time (MST) were determined for each group. Changes in white blood cells (WBCs), red blood cells (RBCs), platelets (PLT) counts, hemoglobin (HGB) concentration, hematocrit (HCT) and mean corpuscular volume (MCV) were also measured in the healthy mice before infection as baseline and on day 3 and 8 after inoculation using complete blood count. Standardization was achieved by UHPLC-MS chemical fingerprint analysis and quantitative phytochemical tests.

Results: APLE, standardized to its total alkaloids, phenolics and saponin contents, produced significant ($P < 0.05$) dose-dependent clearance of mean hyperparasitemia of 22.78 ± 0.93% with the minimum parasitemia level of 2.01 ± 0.25% achieved at 400 mg/kg p.o. on day 8. Quinine 30 mg/kg i.m. achieved a minimum parasitemia level of 6.15 ± 0.92%. Moreover, APLE (50–400 mg/kg p.o.) evoked very significant anti-malaria activity of 89.22–95.50%. Anti-malaria activity of Quinine 30 mg/kg i.m. was 86.22%. APLE also inverse dose-dependently promotes weight gain with the effect being significant ($P < 0.05$) at 50 mg/kg p.o. Moreover, APLE dose-dependently increased the MST of malaria infested mice with 100% survival at 400 mg/kg p.o. Quinine 30 mg/kg i.m. also produce 100% survival rate but did not promote ($P > 0.05$) weight gain. Hematological studies revealed the development of leukocytopenia, erythrocytosis, microcytic anemia and thrombocytopenia in the malaria infected mice which were reverted with the treatment of APLE 50–400 mg/kg p.o. or Quinine 30 mg/kg i.m. but persisted in the negative control. The UHPLC-MS fingerprint analysis of APLE led to identification of one oxoaporphine and two aporphine alkaloids (**1**–**3**). Alkaloids **1** and **3** are being reported in this plant for the first time.

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Conclusion: These results indicate that APLE possessed significant anti-malaria, immunomodulatory, erythropoietic and hematinic actions against malaria infection. APLE also has the ability to revoke deleterious physiological alteration produced by malaria and hence, promote clinical cure. These properties of APLE are due to its constituents especially, aporphine and oxoaporphine alkaloids.

Abbreviations

APLE	*A. polycarpa* leaf ethanol extract
MST	Mean survival time
ICR	Institute of Cancer Research
UHPLC-MS	Ultra High Pressure Liquid Chromatography- Mass Spectrometry
WBCs	White blood cells
RBCs	Red blood cells
iRBC	Infected Red blood cells
uRBC	unfected Red blood cells
HGB	Hemoglobin
HCT	Hematocrit
MCV	Mean capsular volume
WHO	World Health Organization
NMIMR	Noguchi Memorial Institute for Medical Research
FORIG	Forestry Research Institute of Ghana
CPMR	Centre for Plant Medicine Research
UG-IACUC	University of Ghana Institutional Animal Care and Used Committee
LD_{50}	Mean lethal dose
OECD	Organization for Economic Co-operation and Development
MP	Mega pixel

1. Introduction

Malaria is one of the deadliest diseases which kill many people mostly due to its deleterious alteration of hematological indices especially, hemoglobin and RBCs which results in severe anemia, multiple organ damage and death. The World Health Organization (WHO) reported an estimated 228,000,000 cases of malaria globally in 2018 out of which 213,000,000 (93%) cases occurred in the WHO African Region (WHO, 2019). The disease also killed about 405 000 people globally in the same year with 94% of the casualties occurring in the WHO Africa Region (WHO, 2019). Malaria is caused by different species of *Plasmodium* parasite. However, the most transmittable and fatal among them is *P. falciparum* which is responsible for nearly all malaria infections and related deaths in Africa (Collins, 2012; WHO, 2019). Malaria infections and its related deaths have been widely reduced over the past few decades by employing insecticide-treated mosquito nets, insecticides and pharmaceutical drugs like artemisinin and its derivatives. The first-line treatment for malaria in Ghana is artemisinin-based combination therapy, however the development of resistance by the *Plasmodium* parasite towards the first line treatment medicines could pose a serious threat to global fight against malaria (Ashley et al., 2014). This has necessitated the search for new anti-malaria medications.

Medicinal plants have provided crude preparations and isolates for treatment of various diseases as well as parasitic infections. For instance, quinine and artemisinin which were isolated from *Cinchona officinalis* and *Artemisia annua* respectively are used to treat severe malaria (Rates, 2001; Li and Vederas, 2009). The African landscape is abundantly occupied with numerous species of medicinal plants whose therapeutic possibilities have not been discovered (Iwu, 2014). *Annickia polycarpa* is a characteristic example of such a plant in the Annonaceae family. The bark of the plant is used in African communities to treat diseases such as malaria, fever, pyrexia, stomach ulcer, bacterial infections, injuries, eye infections and leprous wounds (Irvine, 1961; Ajali, 2000; Atindehou et al., 2004; Govindasamy et al., 2007). Previous studies demonstrated that the stem bark of *A. polycarpa* possessed analgesic, anti-inflammatory, anti-diabetic, anti-oxidant, anti-bacterial, anti-trypanosome and anti-plasmodial activities (Ajali, 2000; Atindehou et al., 2004; Bolou et al., 2011; Kumatia et al., 2016; Lartey, 2016). The anti-malaria activity of the ethanol stem bark extract of *A. polycarpa* has also been evaluated (Anosa et al., 2014). However, the anti-malarial property of the leaf is not known.

Furthermore, most medicinal plant raw materials are not standardized. This leads to variable chemical composition and biological activities of different batches of the same plant and its medicinal products. This problem can be resolved by using standardization tools such as quantitative phytochemical tests and chemical fingerprint analysis to generate standard parameters on the chemical constituents of the plant part to serve as reference standards for subsequent batches of the raw medicinal plant part.

The aim of this study therefore, is to develop standardization parameters for *A. polycarpa* leaf using quantification of its phytochemical constituents and Ultra High Performance Liquid Chromatography (UHPLC) – Mass spectrometry (MS) chemical fingerprint analysis and to investigate the anti-malaria properties of the ethanol leaf extract of *A. polycarpa* (APLE) in *P. berghei* infected mice in the Rane's test. And to evaluate the effect of treatment of APLE on hematological indices of the *P. berghei* infected mice in order to partially determine the anti-malaria mechanism of action of APLE.

2. Materials and methods

2.1. Materials and chemicals

Quinine hydrochloride was purchase from Intravenous Infusions Limited (Koforidua, Ghana). Normal saline was also purchased from Pharmanova Limited (Accra, Ghana). Chloroquine sensitive *P. berghei* ANKA strain was obtained from Noguchi Memorial Institute for Medical Research, NMIMR, (Legon, Ghana). Ethanol used for extraction was food grade.

2.2. Collection and extraction of A. polycarpa leaf

A. polycarpa was located and authenticated at coordinates of N: 06.68282 W: 001.34357 in the Bobiri Forest Reserve (Kumasi, Ghana) by Mr. Jonathan Dabo, a botanist at the Forest Research Institute of Ghana (FORIG) Kumasi. The leaf was collected and a voucher specimen (FORIG 0012) deposited at the herbarium of FORIG. The leaf was then dried in a room for 8 days and pulverized into course powder. It (200 g) was macerated with 96% ethanol (3 L x 2) at room temperature for 4 days and filtered. The resultant extract was evaporated to dryness in a rotary evaporator (Eyeler N1110, Tokyo-Japan) to afford a solid substance labelled APLE which was kept in an air-tight container at 4 °C.

2.3. Phytochemical screening tests

APLE (1.0 g) was re-constituted in 50 mL of 96% ethanol and analyzed for the presence or absence of phytochemicals such as saponins, phenolic compounds, flavonoids, reducing sugars, cyanogenic glycosides, alkaloids, triterpenes, phytosterols, anthracinosides and polyuronides according to methods described previously (Trease and

Evans, 1989).

2.4. Quantification of alkaloid content of APLE

A modified version of the method described by Harborne (1973) was used to determine the percentage total alkaloid present in APLE. APLE (1.00 g) was dissolved in 200 mL of 10% acetic acid ethanol solution in a beaker and concentrated to one-fourth of the initial volume on water bath. Fifteen (15) drops of concentrated ammonium hydroxide were added dropwise to the extract to precipitate the alkaloids. The method was modified at this point by adding 80 mL of distilled water to the mixture and transferring it into a separating funnel. This allowed the precipitate to settle within 15 min. The precipitate was filtered out with filter paper and washed with 20 mL of 0.1 M ammonium hydroxide solution after the supernatant was discarded. The residue was later died in an oven at 80 °C for 1 h. It was finally weighed with analytical balance (AND; HR-202I, Japan). The percentage total alkaloid content was calculated as shown below.

$$Alkaloid = \frac{Weight\ of\ alkaloid}{Weight\ of\ sample} \text{ x } 100\%$$

2.5. Quantification of total phenolic content of APLE

Folin-Ciocalteau procedure was used to quantify the total phenolic content of APLE (Singleton et al., 1999). In summary, 100 μL of 5% Folin-Ciocalteau reagent was added to 20 μL of 0-5 mg/mL of APLE in increasing order of concentration. After which 80 μL of 7.5% bisodium carbonate was added to the mixture in a well plate and thoroughly mixed prior to incubation in the dark at room temperature for 1 h. The sample's absorbance was measured at 765 nm with a micro titer plate reader (Infinite M200Pro, Tecan, Austria). The test was carried out in triplicates. A standard calibration curve was also produced using Gallic acid under similar experimental conditions. The total phenolic content of the extract was calculated as microgram of Gallic acid equivalent (GAE) per milligram of dry extract from the calibration curve.

2.6. Quantification of total saponins content of APLE

The quantity of saponins present in the APLE was determined as described previously (Ejikeme et al., 2014) with slight modification. APLE (0.5 g) was dissolved in 100 mL of 20% aqueous ethanol and concentrated to about 40 mL in ceramic evaporating dish over water bath. The resultant mixture (20 mL) was partitioned twice with diethyl ether (20 mL) in a separation funnel. The diethyl ether fraction was discarded and the aqueous fraction partition twice with n-butanol (60 mL) containing 5% sodium chloride (10 mL). The sodium chloride portion was castoff and the n-butanol fraction poured into a ceramic dish, evaporated to dryness and dried to a constant weight in an oven at 80 °C. The percentage saponins was calculated as follows:

$$Saponin = \frac{Weight\ of\ saponin}{Weight\ of\ sample} \text{ x } 100\%$$

2.7. UHPLC-MS chemical fingerprint analysis of APLE

APLE (25 mg) was dissolved in HPLC grade methanol (25 mL) and homogenized into solution in a round bottom flask by shaking on Vortex mixer (Thermolyne, Mix-Plus) for 5 min. Chemical fingerprint analysis was performed on UHPLC (UHPLC, Agilent Infinity 1290) machine equipped with quaternary pump and a temperature column control compartment (G1327). An aliquot (2 mL) of the APLE solution in GC vial was placed in the autosampler chamber (G4226A autosampler). The temperature of the analytical column of the UHPLC (Phenomenex Kenetex 2.6 u XB-C18.100 × 2.10 mm) was 30 °C. A guard column (Agilent Eclipse C18, 2.1 × 5 mm, 1.8 μm) was connected to the analytical column to prevent it from being contaminated. The mobile phase for the chromatographic separation was made up of 0.1% formic acid in HPLC grade water (solvent A) and 0.1% formic acid in HPLC grade methanol (solvent B). The UHPLC was operated under a constant flow of 30% of solvent A to 70% of solvent B for a period of 0–10 min. The injection volume of the sample was 1 μL and the flow rate was 0.2 mL/min. Mass spectrometer (Agilent Tripple Quadrupole MS) was coupled with the UHPLC as a detector to analyze the eluted compounds. The electron source ionization (ESI) (Positive mode) was employed for fragmentation in the MS chamber at 300 °C and current flow of 0.21 μA, scan cycles of 4.91/S and electron energy of 350 V. Nitrogen (collision gas) flowed at 13 L/min. The nebulizer pressure and the electron energy of the capillary were at 30 psi and 5000 V respectively. The scan was performed at m/z 100–500.

2.8. Animals and ethics statement

The animals used in this study were female ICR mice (21–35 g). They were allowed free access to powered feed and sterilized water under 12 h light/dark cycle with a temperature of 24 ± 2 °C and humidity of 60–70% in the Animal Experimentation Unit of Centre for Plant Medicine Research (CPMR), Mampong-Akwapim, Ghana. Ethical clearance for the study was obtained from the University of Ghana Institutional Animal Care and Used Committee (UG-IACUC) with the clearance number UG-IACUC 013/18–19.

2.9. Oral acute toxicity test

The median lethal dose (LD_{50}) of APLE was determined using the acute toxicity test as per the convention of the Organization for Economic Co-operation and Development (OECD, 2001), with some modifications. APLE at a dose of 5000 mg/kg was reconstituted with distilled water and administered orally to female ICR mice of body weight 21–28 g ($N = 6$). The mice were then monitored for symptoms of toxicity like neurological, autonomic, behavioral changes and/or death at 2 h interval for 24 h. The animals were monitored for extra 13 days.

2.10. Maintenance of the parasite in the laboratory

P. berghei (Chloroquine sensitive ANKA strain) was injected into female ICR mice at NMIMR (Legon) and taken to a laboratory at the Animal Experimentation Unit of CPMR. The parasites were then preserved by sequential passage into naive ICR mice after every 7 days for 3 times.

2.11. Parasite inoculation and anti-plasmodial effect of APLE in the 7 days curative test

A donor mouse blood with parasitemia of 20–30% was diluted with normal saline for each 0.2 mL to contained 1.0×10^7 *P. berghei*-infected RBCs. Female ICR mice (N = 25) were each injected with 0.2 mL (i.p.) of the diluted blood and then allowed to stay for 72 h without treatment for parasitemia to establish (Fidock et al., 2004). Baseline parasitemia level was determined for each mouse after the 72 h. The mice were then randomly selected into 5 Groups ($N = 5$). Group I (Negative control) received sterile water. Group II received 0.2 mL of 30 mg/kg Quinine (i.m.) per mouse as reference drug. Each mouse in Group III, IV or V also received 0.2 mL of APLE 50, 200 or 400 mg/kg p.o. respectively for 4 consecutive days.

2.12. Parasite monitoring

The parasitemia levels in the blood of the *P. berghei*-infected mice were monitored using methods described previously (Arrey et al., 2014). Blood samples from each mouse was taken onto a microscope slide by tail-bleeding on day 3, 5 and 8 post infection. Thin film was prepared from each sample, dried, fixed in methanol for 15 min and stained with

10% Giemsa stain for 25 min. Excess stain was washed off and the slides dried. The film was then immersed in oil and viewed at × 100 magnification using the Olympus light microscope (Olympus C × 21; Tokyo, Japan). The level of parasitemia was determined by counting the number of infected-RBCs (iRBCs) and uninfected-RBCs (uRBC) per randomly selected fields. Parasitemia and anti-malaria activity were calculated as follow:

$$Parasitemia = \frac{\text{iRBCs } counted}{Total \text{ RBCs } counted} \text{ x } 100\%$$

$$Anti-malaria\ activity = \frac{Parasitemia\ in\ untread\ control - parasitemia\ in\ \text{TG}}{Parasitemia\ in\ untread\ control} \text{ x } 100\%$$

where TG = APLE or Quinine treatment group.

2.13. Change in body weight

$$Change\ in\ body\ weight = \frac{Weight\ of\ animal\ at\ \text{Dt} - Weight\ of\ animal\ at\ \text{Do}}{Weight\ of\ animal\ at\ \text{Do}} \text{ x } 100\%$$

Body weights of the animals were measured on day 0 (Do) to serve as baseline before initiation of inoculation. The weight of the animals was thereafter measured on each other day (Dt) till day 8. The effect of treatment on body weight of parasitized mice was determined as percentage change in body weight as indicated below.

2.14. Mean survival time (MST)

The number of mice which died in each group and the dates of deaths were recorded during the experimental period and for further 22 days after termination of the experiment on the 8th day. The MST was evaluated over the 30 days period as shown below.

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

The data obtained was presented as percentage survival rate using the Kaplan-Meier estimator.

Table 1
Phytochemical constituents present or absent in APLE.

Constituent	Result
Alkaloids	Detected
Phenolic compounds	Detected
Saponins	Detected
Phytosterols	Detected
Free reducing sugars	Detected
Flavonoids	Not detected
Triterpenes	Not detected
Anthracenosides	Not detected
Cyanogenic glycoside	Not detected
Polyuronides	Not detected

2.15. Hematological analysis

Blood sample was taken from each mouse using tail bleeding procedure on days 0 (Baseline, when the mice were healthy), 3 and 8 after parasite inoculation into Eppendorf tubes precoated with anti-coagulant (Na-EDTA). The blood samples, 50–80 μL, were diluted in 420 μL normal saline. Complete blood count analysis of the diluted blood was performed using hem analyzer (Abacus 380; Budapest, Hungary) to evaluate hematological indices. The results were multiplied with the dilution factor to account for the dilution of the blood.

2.16. Micrographs

Micrographs of selected fields showing the levels of iRBCs and uRBCs were obtained with a 16.0 MP camera of Samsung galaxy A30 mobile phone.

2.17. Statistical analysis

Data was analyzed using Graph Pad Prism version 6 and presented as mean ± SEM. Variations were determined by comparative analysis using one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test to determine where the variation lies. Variations were considered statistically significant when $P < 0.05$.

3. Results

3.1. Yield of extract

Rotary evaporation of the leaf (200 g) extract of *A. polycarpa* to dryness produced 31.56 g of a dark green hygroscopic solid (APLE) equivalent to a yield of 15.78% w/w.

3.2. Phytochemical screening

The results of the phytochemical screening are summarized in Table 1 below. The results show that the extract contains 5 out of the 10 classes of phytochemical constituents analyzed.

Table 2
Quantity of phenolic compounds, saponins and alkaloids present in APLE.

Phytochemical constituent	Calibration curve equation	Quantity present in APLE
Phenolic compounds	y = 0.3986x + 0.003	59.467 ± 0.0003 mg/g of GAE
Saponins	_	51.65%
Alkaloids	_	33.06%

3.3. Quantity of alkaloids, phenolic compounds and saponins present in APLE

The results of the quantitative determination of the above-mentioned constituents in APLE are shown below in Table 2. The straight-line equation produced for the standard calibration curve of Gallic acid which was used to calculate the total phenolics content of APLE is also presented. The correlation coefficient (R^2) of the standard calibration curve was 0.999.

3.4. UHPLC-MS chemical fingerprint of APLE

The results of the UHPLC-MS chemical fingerprint analysis of APLE are shown in Fig. 1, Table 3 and Table 4 below. The characteristic features of the UHPLC chromatogram fingerprint of the extract are described in Table 3. The mass spectra fragmentation pattern of the APLE compounds indicating their mass to charge ratio (m/z) are also described in Table 4.

3.5. Identification of compounds in APLE

The structures of the compounds identified in APLE using UHPLC-MS chemical fingerprint analysis are shown below in Fig. 2.

3.5.1. Identification of glaucine (1)

Glaucine (**1**) (Fig. 2) eluted out of the column first (RT = 1.052 min) and was the most abundant chemical constituent in APLE with peak height of 100% and peak area sum of 95.9% (Fig. 1; Table 3). The mass spectrum of **1** showed molecular ion peak of m/z at 356.2. This is indicative of $[M + H]^+$ parent molecular ion of glaucine (Singh et al., 2017) with the molecular formula of $C_{21}H_{25}NO_4$. The peaks at m/z 357.3, 342.2, 328.2, 314.2 represent $[M+2H]^+$ and the removal of CH_3, $[M + H - 15]^+$, CO, $[M + H - 28]^+$ and CH_3, $[M + H - 43]^+$ fragment ions from glaucine respectively. Therefore, **1** was identified as glaucine (Molecular mass = 355.18 g/mol). This is the first time this compound has been identified in *A. polycarpa*.

3.5.2. Identification of lysicamine (2)

Lysicamine (**2**) (Fig. 2.) was the second compound to elute from the column (RT = 3.436 min). It forms only 2.27% of the peak area sum although, it is slightly higher than **3** (Fig. 1; Table 3). The mass spectrum of **2** showed m/z at 293.2. This corresponds to the molecular formula $C_{18}H_{13}NO_3$ which is indicative of $[M+2H]^+$ parent ion of lysicamine, an oxoaporphine alkaloid, with molecular weight of 291.3 g/mol. A previous study which characterized lysicamine isolated from the leaf of *Phoebe grandi* shows that the mass spectrum of **2** displayed a molecular ion peak at m/z 292.0963 which corresponded to $[M+H]^+$ (Omar et al., 2013). Hence, **2** was identify as lysicamine which was earlier reported in this plant (Jossang et al., 1977).

3.5.3. Identification of dehydrocorydaline (3)

Dehydrocorydaline (**3**) (Fig. 2.) was the last compound to elute out of the column (RT = 5.995 min) and the smallest constituent in APLE (Area sum = 1.93%). The mass spectrum of **3** displaced a molecular ion peak at m/z = 367.3 for $[M+H]^+$ which corresponds to the molecular formula $C_{22}H_{24}NO_4^+$ (Molecular mass = 366.4 g/mol). A previous study identified dehydrocorydaline with $[M]^+$ peak at m/z = 366.0 for its molecular ion (Guan et al., 2017). This is the first report of identification of dehydrocorydaline in *A. polycarpa*.

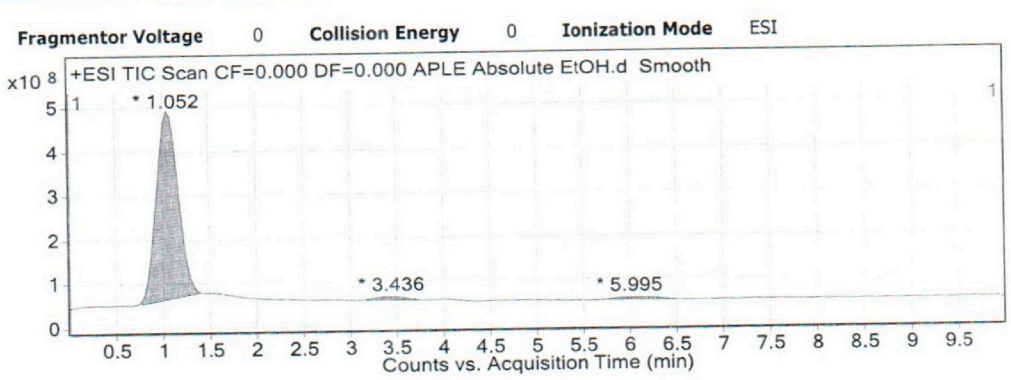

Fig. 1. UHPLC fingerprint chromatogram of APLE showing the peaks of compounds present.

Table 3
Characteristic features of UHPLC chromatographic fingerprint of compounds in APLE.

Com-pound	RT/min	Height	Area %	Height %	Area	Area Sum %	Symme-try	Width
1	1.052	426032539.7	100	100	6919190162.0	95.9	1.2	0.631
2	3.436	6796277.38	2.01	1.6	139306344.5	1.93	0.92	0.561
3	5.995	5033354.63	2.26	1.18	156680279.4	2.17	1.83	0.806

Table 4
Mass spectrum fingerprints of compounds in APLE.

Compound	Mass to charge ratio (m/z)									
1	104.2	118.2	180.2	328.2	338.1	342.2	343.2	352.3	356.2	357.3
2	111.1	117.1	122.2	124.2	157.1	203.2	293.2	427.5	428.5	449.5
3	111.1	122.2	157.1	293.2	341.2	367.3	394.4	427.5	428.5	449.5

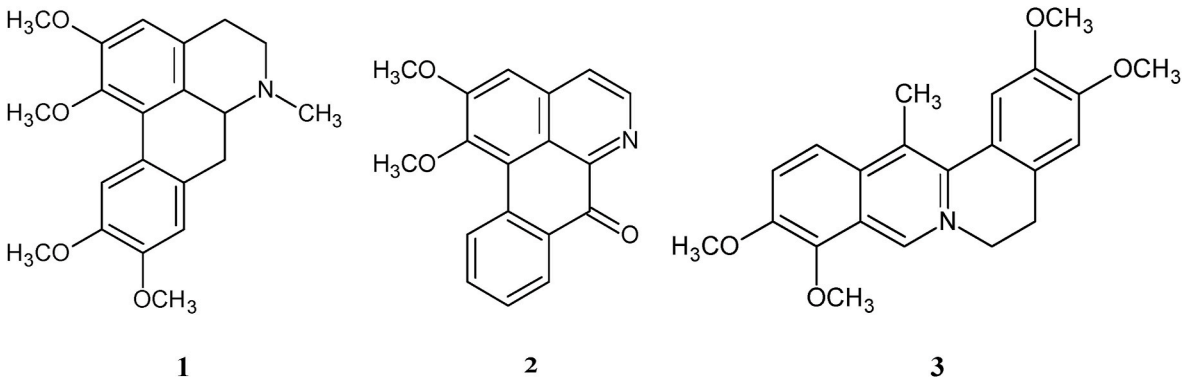

Fig. 2. Compounds identified in APLE using UHPLC-MS chemical fingerprint analysis.

3.6. Acute toxicity test of APLE

Neither of the mice died nor showed any sign of toxicity such as autonomic, neurological and/or behavioral changes at the dose of 5000 mg/kg p.o. after the 14 days observational period.

3.7. Effect of APLE on hyperparasitemia development in mice

Average parasitemia of the *P. berghei*-infected mice was 22.78 ± 0.93% on day 3 post infection. It increased to 44.62 ± 5.94% at day 8 in the untreated controls. However, the parasite levels in the APLE 50–400 mg/kg p.o. or Quinine 30 mg/kg i.m. treated mice reduced significantly ($P < 0.05$) to 4.74 ± 1.15, 2.64 ± 0.40, 2.01 ± 0.25 or 6.15 ± 0.92% respectively on day 8 (Fig. 3A). The area under the curves normalized in the negative control (Fig. 3B) was also profoundly reduced in APLE 200–400 mg/kg p.o. and Quinine treatment groups. However, the P values were statistically insignificant ($P > 0.05$) due to the high S.E.M. values of the untreated control.

3.8. Anti-malaria activity of APLE in P. berghei infested mice

The anti-malaria activity of the various treatment groups is indicated in Table 5 below. APLE produced dose-dependent anti-malaria activity especially on day 8. It could also be observed that the anti-malaria activity of APLE was directly proportional to the duration of treatment.

3.9. Effect of APLE or quinine on mean survival time (MST) of P. berghei-infected mice

Fig. 4 shows the results of the MST of the animals analyzed using Kaplan-Meier method. It could be seen from Fig. 4 that only 25.00% of the negative control mice survived on the 30th day after infection. However, 66.70% of the infected mice treated with APLE 50 mg/kg p.o. survived from the 15th-30th day. In the APLE 200 mg/kg p.o. treated group, 83.30% of the mice survived from the 6th-30th day. Finally, 100% of the infected mice treated with APLE 400 mg/kg p.o. or Quinine 30 mg/kg i.m. survived from the 3rd-30th day.

3.10. Effect of APLE or quinine on body weight of P. berghei-infected mice

The result for this section is shown below in Table 6. APLE 50 mg/kg p.o. evoked significant increase in body weight ($P < 0.05$) of mice on day 8. Although, APLE 200–400 mg/kg p.o. or Quinine (30 mg/kg i.m.) did not protect the mice against weight loss ($P > 0.05$) on day 5 or 8.

3.11. Effect of APLE or quinine on hematological indices of P. berghei infested mice

The results of changes in hematological indices of *P. berghei* infested mice treated with APLE or Quinine are shown below in Fig. 5 (A - F).

3.11.1. White blood cells (WBCs)

The results show that inoculation of mice with *P. berghei* resulted in consistent reduction of WBCs in the untreated negative controls from day 0 through to day 8. The reduction in WBC counts was very

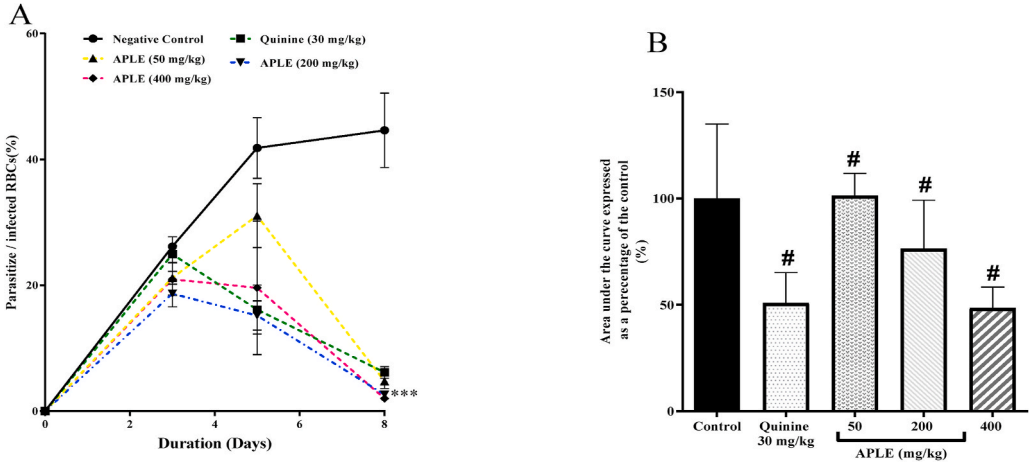

Fig. 3. Effect of APLE or Quinine on parasitemia levels of *P. berghei*-infected mice on time course curves (A) and area under the curves normalized in the negative control (B). Results are presented as mean ± S.E.M ($N = 5$). ***$P < 0.001$; $^{\#}P > 0.05$ compared to the negative control.

Table 5
Anti-malaria activity of APLE/Quinine in *P. berghei* infested mice.

Dose	Anti-malaria activity (%)	
	Day 5	Day 8
Negative Control	00.00	00.00
Quinine (30 mg/kg)	61.45	86.22
APLE (50 mg/kg)	25.73	89.37
APLE (200 mg/kg)	53.14	94.08
APLE (400 mg/kg)	64.69	95.50

Results are presented as percentage mean ($N = 5$).

significant ($P < 0.01$) on days 8 for the untreated negative controls. The reduction in WBC counts in the APLE or Quinine treated mice compared to their day 0 values were recorded on day 3 when the mice were infected without treatment (for 72 h) (Fig. 5 A). However, treatment of the infested mice with APLE or Quinine lead to significant ($P > 0.05$) elevation of WBC counts compare to their day 0 values (Fig. 5 A).

3.11.2. Red blood cells (RBCs)

The results show that there was significant ($P < 0.05$) reduction in RBC counts of *P. berghei* infected mice on day 3 in the negative untreated group and all other treatment groups when the mice were inoculated after 72 h without treatment compared to day 0 RBC counts. The significant ($P < 0.01$) reduction in RBC counts in the untreated negative mice persisted to day 8. However, treatment of the infested mice with APLE or Quinine significantly ($P > 0.05$) restored the RBC counts to their day 0 values (Fig. 5B).

3.11.3. Hemoglobin (HGB)

The result for the HGB concentration followed a similar trend as that of the RBC counts. Inoculation of mice with *P. berghei* produced significant ($P < 0.05$) reduction in HGB concentration on day 3 in all the experimental groups after 72 h without treatment compared to day 0 HGB concentrations and continued to day 8 in the untreated controls (Fig. 5 C). Conversely, treatment of the infested mice with APLE or Quinine significantly ($P > 0.05$) elevated the HGB concentration in the mice compare to the day 0 values (Fig. 5 C).

3.11.4. Hematocrit (HCT)

The results (Fig. 5 D) show that HCT levels of the healthy mice were high (day 0). However, substantial ($P < 0.05$–0.001) reduction in HCT were observed 72 h after they were infected with *P. berghei* (day 3). It was also observed that the HCT levels of mice which were treated with APLE (50–400 mg/kg p.o.) or Quinine (30 mg/kg i.m.) were

Table 6
Mean change in body weight of *P. berghei* infected mice treated with APLE or Quinine.

Dose	Change in Body Weight (g)	
	Day 5	Day 8
Negative Control	-1.67 ± 0.26	-1.50 ± 0.32
Quinine (30 mg/kg)	$2.00 \pm 0.00^{\#}$	$2.00 \pm 0.63^{\#}$
APLE (50 mg/kg)	$-0.60 \pm 0.51^{\#}$	$-0.75 \pm 0.42^{*}$
APLE (200 mg/kg)	$-1.00 \pm 1.05^{\#}$	$-2.20 \pm 1.02^{\#}$
APLE (400 mg/kg)	$-1.80 \pm 1.16^{\#}$	$-3.25 \pm 1.52^{\#}$

Results are presented as mean ± SEM. $^{*}P < 0.05$ or $^{\#}P > 0.05$ compared to negative control.

significantly restored ($P > 0.05$) to their day 0 values on day 8 whereas the HCT of the untreated control mice declined further ($P < 0.001$) on day 8 (Fig. 5 D).

3.11.5. Mean capsular volume (MCV)

MCV levels (Fig. 5E) of the healthy mice were high on day 0. Nevertheless, there were significant ($P < 0.05$–0.001) reduction in MCV values 72 h after infection (day 3) except for APLE at 50 mg/kg p.o. However, treatment of the infested mice with APLE (50–400 mg/kg p.o.) or Quinine (30 mg/kg i.m.) from day 3 to day 7 significantly ($P > 0.05$) restored the MCVs compared to day 0 values on day 8. Whereas that of untreated controls further declined ($P < 0.001$) to day 8 (Fig. 5E).

3.11.6. Platelets (PLT)

The results (Fig. 5F) showed that PLT levels of the healthy mice were high (day 0). Conversely, when the mice were infected with *P. berghei* after 72 h, substantial ($P < 0.05$–0.001) reduction in PLT were observed (day 3). The reduction in PLT levels was significantly ($P > 0.05$) reversed when the mice were treated with APLE (50–400 mg/kg p.o.) or Quinine (30 mg/kg i.m.) once a day from day 3 to day 7 to their day 0 values on day 8. PLT of the untreated control mice declined further ($P < 0.001$) on day 8 (Fig. 5F).

3.12. Effect of treatment with APLE on micrographs of RBCs

Fig. 6 A – E below shows the effect of treatment with APLE or Quinine on RBCs of *P. berghei* infested mice. The micrographs show that the untreated negative controls (A) produced higher number of parasitized-RBCs (1) at the termination of the Rane's test than the Quinine 30 mg/kg i.m. (B) or APLE 50–400 mg/kg p.o. (C, D and E) treatment groups respectively.

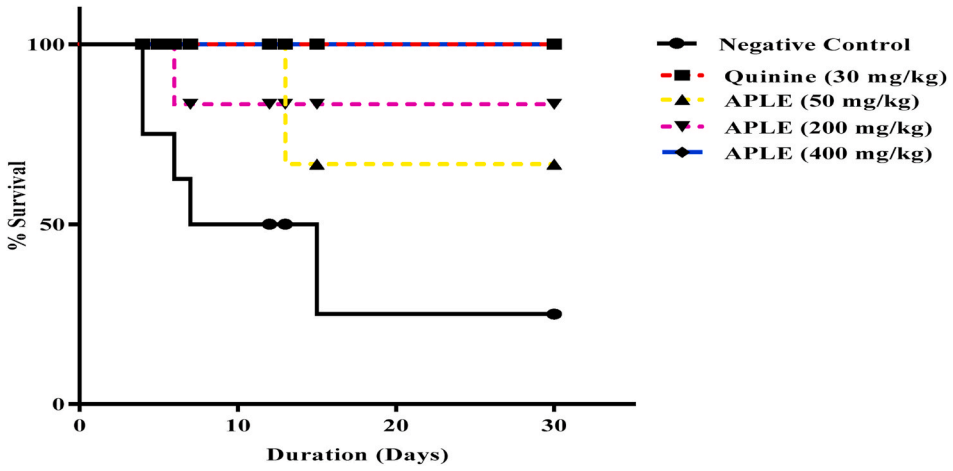

Fig. 4. Effect of APLE or Quinine on the survival rate of *P. berghei*-infected mice up to 30 days post infection. Each point represents percentage number of mice alive on a specific number of days. Data was analyzed using the Kaplan-Meier method.

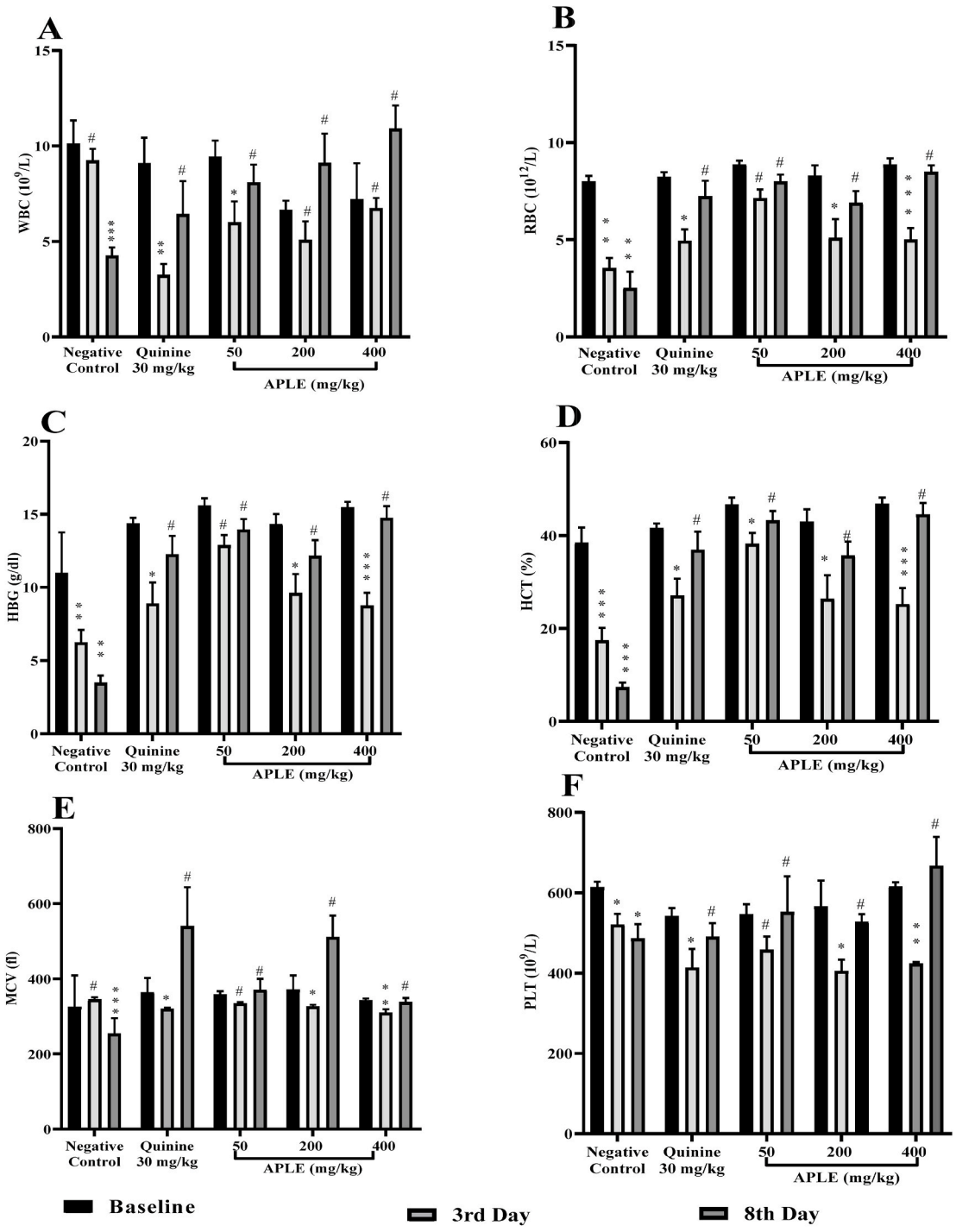

Fig. 5. Effect of APLE (50–400 mg/kg p.o.) or Quinine (30 mg/kg i.m.) on hematological indices of P. berghei infested mice. Values are expressed as mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ or #$P > 0.05$ compare to the mean baseline (day 0) values.

4. Discussions

The result of the acute toxicity test shows that none of the mice died or shows any sign of toxicity. This indicates that the LD_{50} of APLE was above 5000 mg/kg p.o. This also indicates that APLE has a high degree of safety (OECD, 2001). Based on this result, three arbitrary doses (50, 200 and 400 mg/kg p.o.) lower than the LD_{50} value of APLE were selected for the anti-malaria test. Since the doses below the LD_{50} values will neither produce toxicity nor death of the mice during the experimental period.

4.1. Effect of APLE on hyperparasitemia development

Hyperparasitemia is defined as > 2% parasitemia in low malaria endemic localities or >5% parasitemia in high malaria endemic areas (WHO, 2010), even though > 5% parasitemia in less endemic areas and >10% parasitemia in a higher endemic area are mostly employed to describe hyperparasitemia (Wilairatana et al., 2013). The WHO list hyperparasitemia as one of the indications of severe *falciparum* malaria for over thirty years (WHO, 1990). Earlier studies have revealed that hyperparasitemia in malaria patients is associated with anemia, high risk of development of cerebral malaria in young children and high

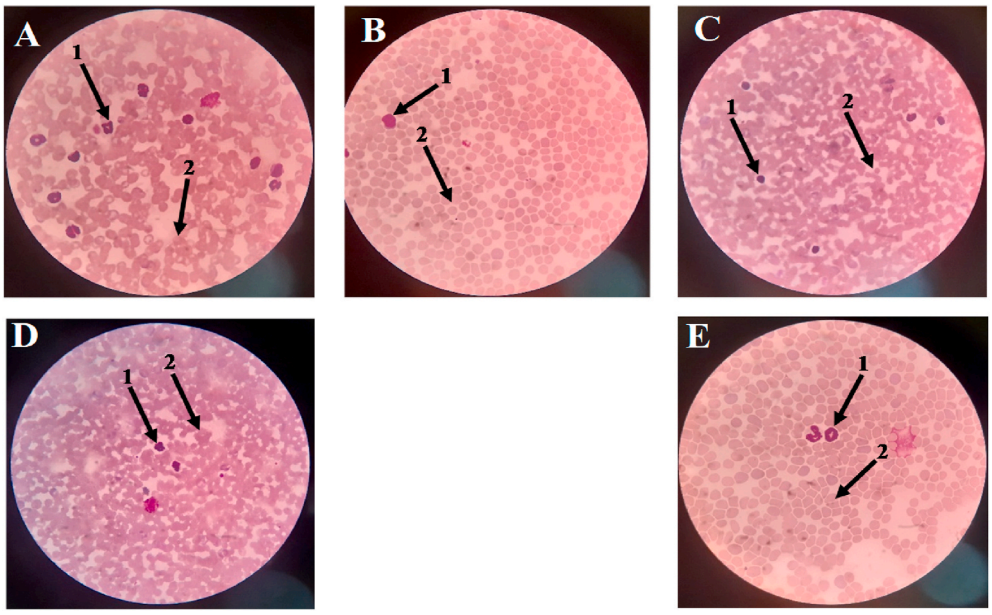

Fig. 6. Micrographs of selected fields showing iRBCs and uRBCs of negative control (A), Quinine 30 mg/kg i.m. (B), APLE 50 (C), 200 (D) and 400 mg/kg p.o. (E) on day 8.1 = iRBC, 2 = uRBC.

death rates (Murthy et al., 2000; Ekvall, 2003; Sowunmi et al., 2011). The results from this study show that APLE at 50–400 mg/kg p.o. significantly reduce an average hyperparasitemia of 22.78 ± 0.93% on day 3 post infection to lower parasitemia range of 4.74 ± 1.15–2.01 ± 0.25 after 4 successive days of treatment. This indicates that APLE produced hyperparasitemia clearance and hence, prevented its associated complications such as anemia, cerebral malaria and death.

4.2. Anti-malaria activity of APLE in P. berghei infested mice

It has been reported that in vivo anti-malarial activity of substances could be grouped as moderate, good, or very good if an extract produced anti-malaria activity equal to or greater than 50% at doses of 500, 250, and 100 mg/kg body weight (Adugna et al., 2014). The results show that APLE at 200–400 mg/kg p.o. produced anti-malaria activity of 53.14–64.69% on day 5 which increased to 89.37–95.5% on day 8. Hence, APLE evoked good to moderate anti-malaria effect after 2 consecutive days of malaria treatment and very good anti-malaria effect after 4 consecutive days of once daily treatment. The 95.5% anti-malaria activity evoked by the extract at 400 mg/kg p.o. also indicates almost 100% parasitemia clearance was achieved with the treatment of APLE at 400 mg/kg p.o. over the period. The anti-malaria activity of 86.22% produced by Quinine at 30 mg/kg p.o. was also very good. Furthermore, the 7 days curative test is used to assess the efficacy of chemotherapeutic anti-malaria agents such as Quinine, Chloroquine or Artesunate which work against the erythrocytic schizont stage of the *Plasmodium* parasite in other to abrogate clinical malaria attacks. APLE can therefore be classified as erythrocytic schizonticide anti-malaria substance since it evoked profound anti-malaria effect in the 7 days curative test.

4.3. Effect of treatment of APLE on mean change in body weight of P. berghei infested mice

Weight loss is one of the characteristics of malaria infection in mice (Langhorne et al., 2002). Besides, the continuous presence of *Plasmodium* parasite in the blood of humans leads to chronic malaria with signs like acute malaria bouts combined with weight loss, anemia or other contagions (Attwood, 2011). Weight loss during malaria is attributed to decreased food consumption, loss of appetite and disturbed metabolism which accompany the disease (Ezike et al., 2016). The results show that APLE at the lowest dose of 50 mg/kg p.o. significantly protected mice against weight loss during malaria infection compared to the negative control. However, increasing the dose of APLE resulted in weight loss during malaria infection. This may be due to certain components of the extract whose concentration increases with rising dose leading to weight loss. For instance, saponins which are known to promote weight loss through the inhibition of adipogenesis and/or appetite suppression (Kim et al., 2009; Khan et al., 2015). Quinine 30 mg/kg i.m. did not protect mice against weight loss in this study.

4.4. Mean survival time (MST)

MST is one of the most important parameters used to evaluate the efficacy of anti-malaria agents in preclinical animal models of malaria. It has been reported that *P. berghei* infection of ICR mice is a form of cerebral malaria which produce elevated levels of parasitemia with eventual death, indicative of the harshness of the disease in this model. Similarly, death is the definitive result of the many complication of untreated *P. falciparum* infections in humans (Basir et al., 2012). Moreover, active anti-malaria agents have been reported to suppress the overall pathologic action of *P. berghei* infection in mice leading to prolonged MST (Toma et al., 2015). In this study, the results show that APLE dose-dependently prolonged the MST of treated mice compared to the negative control mice such that APLE at 400 mg/kg p.o. or Quinine 30 mg/kg i.m. treatment groups produced 100% survival of mice compared to 25% survival rate in the negative control group on the 30th day after *P. berghei* infection. This indicates that APLE is able to reduce hyperparasitemia, ameliorate the numerous complications of malaria and produce clinical cure and hence prolonged the MST of malaria infected mice.

4.5. Hematological analysis

4.5.1. White blood cells (WBCs)

Reduction in WBC counts on day 3 in the mice after inoculation with *P. berghei* signifies malaria infection. Decreased WBC count has been reported as common indicator of severe malaria infection mediated by lymphocytopenia as a result of redistribution of lymphocytes from circulation to the spleen and other body organs (Tangpukdee et al., 2008; Tobon-Castano et al., 2015). Neutrophils, the most abundant group of

WBCs in the body, destroys pathogens through phagocytosis by production of reactive oxygen species, anti-microbial agents or creation of neutrophil extracellular traps (Hsieh et al., 2007; Kolaczkowska and Kubes, 2013; Tecchio and Cassatella, 2016). The significant elevation of WBC counts in the APLE or Quinine treatment groups of *P. berghei* infested mice on day 8 compared to the day 0 values indicates that the extracts activated the immune system to produce WBCs, especially neutrophils, which enhanced opsonization of the *Plasmodium* parasites in the body. Therefore, APLE possessed immunomodulatory effect which is responsible for its anti-malaria action.

4.5.2. Red blood cells (RBCs)

Malaria infection leads to decrease production of RBCs by the body's erythropoietic system. Moreover, iRBCs exhibit a reduced structure integrity and altered exterior properties which frequently lead to their clearance from circulation by the spleen (Kotepui et al., 2015). Combination of these processes in malaria leads to significant decrease in RBCs which results in the development of anemia. The results indicate a significant ($P < 0.05$) reduction in RBC counts of *P. berghei* infected mice on day 3 in all the treatment groups. This was however, significantly ($P > 0.05$) restored in the APLE and Quinine treated mice on day 8 to their pre-inoculation RBC counts. This indicates that APLE may possess erythropoietic action.

4.5.3. Hemoglobin (HGB)

HGB is an iron complex protein found in RBCs. HGB transport molecular oxygen from the lungs to the cells for metabolism of glucose to release energy and carries the waste products of metabolism such as carbon dioxide (CO_2) and hydrogen radicals (H^+) from the cells to the lungs where they are expelled out of the body. Destruction of RBCs leads to decrease HGB concentration. Lower than normal RBC counts or HBG concentration in the blood is a disorder called anemia which produce symptoms such as dizziness, fatigue, shortness of breath, weakness, drowsiness (WHO, 2017) and eventual death. In this study, inoculation of mice with *P. berghei* produced significant ($P < 0.05$) reduction in HGB concentration in all treatment groups on day 3 compared to day 0 HGB concentrations. This is indicative of anemia. However, HGB concentration was restored ($P > 0.05$) to pre-inoculation levels in the APLE treatment groups on day 8. This indicates that the extract possessed hematinic effect against malaria-induced anemia.

4.5.4. Hematocrit (HCT)

HCT which is also known as packed cell volume (PVC) is a measured of the percentage of blood which is made up of RBCs. Anemia, which is assessed using HCT or HGB concentration, is an important measurement of efficacy of anti-malarial therapies and success of malaria control programs (Lee et al., 2008). HCT is a measurement of the body's capacity to carry oxygen and absorbed nutrients. A raise in HCT indicates a better transport ability of the RBCs (Isaac et al., 2013). Reduction in HCT signifies anemia (Ugwu et al., 2013; Briggs and Bain, 2017). The ability of APLE to significantly restore HCT in *P. berghei* infected mice to their pre-infection levels confirms that the extract is able to eradicate malaria-induced anemia.

4.5.5. Mean capsular volume (MCV)

MCV describes the size of the RBCs and is measured in femtoliters (fl) or cubic microns (μm^3) (Sarma, 1990). Anemia is classified into three groups based on the size of the RBCs (MCV values). Namely, normocytic (Normal MCV), macrocytic (Increased MCV) or microcytic (Decreased MCV) anemia (Sarma, 1990). Thus, by these definitions, *P. berghei* infection of the ICR mice in this study produced microcytic anemia since there were significant ($P < 0.05$) reduction in the MCV values after 72 h of infection. The significant restoration of MCV values to their pre-infection values in the anemic mice after treatment with APLE for four consecutive days also indicates that APLE is able to treat microcytic anemia.

4.5.6. Platelets (PLT)

Thrombocytopenia (Low PTL levels) is one of the most important hematological characteristics of malaria infection in rodents and man. Development of thrombocytopenia during malaria involves various mechanisms. It has been reported that thrombocytopenia is related to high parasite density as a result of platelets activation by iRBCs which subsequently intensify PTLs reactivity to adenosine diphosphate (ADP) and excretion of higher dense-granule (Prasad et al., 2009; Wickramasinghe and Abdalla, 2000). These modifications might catalyze PTLs amalgamation on the endothelium as in cerebral malaria (Grau et al., 2003). The results from this study shows that mice inoculated with *P. berghei* developed thrombocytopenia within 3 days, since significantly ($P < 0.05$) low PLT levels were recorded in all treatment groups of mice after that period. The disappearance of thrombocytopenia from APLE and Quinine treatment groups after four continuous days of each treatment indicates that the extract and Quinine are effective in clearing high parasite density with consequent clearance of thrombocytopenia. This confirm that APLE is an effective anti-malaria agent.

4.5.7. Effect of treatment with APLE on micrographs of RBCs

The micrographs in Fig. 6 showed that the untreated controls developed more iRBCs at the termination of the anti-malaria test than the APLE (50–400 mg/kg p.o.) or Quinine (30 mg/kg i.m.) treatment groups. This further established that APLE is an effective blood schizonticide type of anti-malaria substance since the extract was able to inhibit the formation of iRBCs in the plasma of *P. berghei* infested mice as compared to the untreated control.

4.5.8. Phytochemical constituents

The phytochemical tests on APLE shows that it contains alkaloids, phenolics, saponins, phytosterols and free reducing sugars. The results from the quantification of the phytochemical constituents in the extract showed that APLE contains 33.06% alkaloids, 51.65% saponins and 59.467 ± 0.0003 mg/g of GAE phenolic compounds. These results can serve as a reference standard for *A. polycarpa* leaf and herbal medicines produce from its ethanol extract to ensure consistency in the chemical constituents of different batches of *A. polycarpa* leaf collected from different location and hence maintain reproducible biological activities of the plant material.

Moreover, chemical fingerprint analysis of the leaf further revealed the presence of three alkaloids in APLE, namely glaucine, lysicamine and dehydrocorydalyline. Glaucine is an aporphine alkaloid which is being reported in *A. polycarpa* for the first time. Glaucine acts as a sedative, anti-tussive, hallucinogen, anti-inflammatory, bronchodilatory, calcium channel blocking and anticancer agent (Cortijo et al., 1999; Dargan et al., 2008; Chiu et al., 2012; Lei et al., 2013; Kang et al., 2015). Although the anti-malaria activity of glaucine is not yet known, other aporphine alkaloids such as anonaine and isoboldine are known to possessed anti-plasmodial/anti-malaria, anti-parasitic and anti-microbial activities with IC_{50} recorded for anonaine against chloroquine sensitive/resistant *P. falciparum* to be 25.9 ± 0.2/19.6 ± 1.1 μM respectively (Mde et al., 1992; Graziose et al., 2011; Levrier et al., 2013). Furthermore, Maleba et al. (2013) showed that, the oxoaporphine alkaloid, lysicamine possessed anti-plasmodial activity against CQ-resistant K1 strain of *P. falciparum* with IC_{50} of 8.23 μM in addition to anti-trypanosomal and anti-leishmanial activities. Dehydrocorydaline nitrate, the salt of dehydrocorydaline the second aporphine alkaloid which is also being reported in this plant for the first time, was also proven to possess anti-plasmodial activity against *P. falciparum* Dd2 strain with growth inhibitory rate IC_{50} of 94.5 μg/mL as well as anti-malarial activity against trophozoite and schizont phases of the *Plasmodium* life cycle with low cytotoxicity (Nonaka et al., 2018). This confirms that APLE is a schizonticide class of anti-malaria agent as discussed in Section 4.2. The high anti-malaria activity demonstrated by APLE in this study was therefore, due to its constituents such glaucine, lysicamine, dehydrocorydaline, phenolic compounds and saponins.

Furthermore, glaucine, lysicamine and dehydrocorydaline can serve as marker compounds for standardization of herbal medicine produced from *A. polycarpa* leaf. Moreover, the chromatogram and the mass spectrum obtain from the chemical fingerprint analysis can serve as reference samples for rapid identification and standardization of future specimens of *A. polycarpa* and its leaf.

Finally, some plant extracts and herbal medicines which contain phytochemicals such as alkaloids, saponins, tannins and glycosides have been demonstrated to possessed significant hematinic activity in humans or elevate the production of erythropoietin, which increase RBC production and triggers the division of stem cells into blood cells in animals (Adusi-Poku et al., 2008; Koffuor et al., 2012; Enechi et al., 2019). Hence, the presence of aporphine and oxoaporphine alkaloids such as glaucine, lysicamine and dehydrocorydaline in addition to phenolics and saponins in APLE may account for its hematopoietic and immunomodulatory actions which resulted in the treatment of malaria-induced microcytic anemia in this study.

5. Conclusion

APLE produced remarkable anti-malaria activity and hyper-parasitemia clearance in *P. berghei* infected mice. The extract also demonstrated erythropoietic and immunomodulatory actions and abrogated the development of microcytic anemia and thrombocytopenia due to its ability to annul deleterious hematological alteration produced by malaria and thereby extend the life expectancy of *P. berghei* infected mice. These fascinating pharmacological effects of APLE were due to its biologically active constituents especially, aporphine and oxoaporphine alkaloids such as glaucine, dehydrocorydalyline and lysicamine. The LD_{50} of APLE was found to be above 5000 mg/kg p.o. These findings suggest that the leaves of *A. polycarpa* could be used to treat malaria. Based on these results, the constituents of APLE will be isolated, characterized and subjected to anti-malaria tests.

Availability of data and materials

The data upon which the conclusions in this work are made is included in the article. The UHPLC-MS fingerprint data can be obtained from the corresponding author upon request.

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Authors' contributions

EKK, RAO and VCM conceived the study and supervised the work. EKK was involved in all stages of the investigations, data analysis, writing, corrections, proof reading and final editing of the manuscript. FA was involved in all stages of the investigations and data analysis. GKB and FA were involved in development of protocols and data acquisition. KOA performed full blood count analysis and examinations using the microscope. JD was involved in conception of the study, identification, harvesting and processing of the plant material.

Consent for publication

All authors have given their consent for publication of this manuscript after they reviewed it.

Declaration of competing interest

The authors declare that they have no competing interest with respect to this work.

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