Journal of Ethnopharmacology 266 (2021) 113427

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Antrocaryon micraster (A. Chev. And Guillaumin) stem bark extract demonstrated anti-malaria action and normalized hematological indices in *Plasmodium berghei* infested mice in the Rane's test

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ARTICLE INFO

Keywords:
Malaria
Anemia
Leucopenia
Thrombocytopenia
Saponins
Phenolic compounds
Anacardiaceae
Chemical fingerprint analysis

ABSTRACT

Ethnopharmacological relevance: Malaria is caused by infection with some species of *Plasmodium* parasite which leads to adverse alterations in physical and hematological features of infected persons and ultimately results in death. *Antrocaryon micraster* is used to treat malaria in Ghanaian traditional medicine. However, there is no scientific validation of its anti-malaria properties. The plant does not also have any chemical fingerprint or standardization parameters.

Aim of the study: This study sought to evaluate the anti-malaria activity of standardized *A. micraster* stem bark extract (AMSBE) and its effect on mean survival time (MST) and body weight reduction of *Plasmodium berghei* infested mice. And to study the effect of treatment of AMSBE on hematological indices of the *P. berghei* infested mice in order to partly elucidate its anti-malarial mechanism of action.

Materials and methods: Malaria was induced in female ICR mice by infecting them with 0.2 mL of blood (i.p.) containing 1.0×10^7 *P. berghei*-infested RBCs from a donor mouse and leaving them without treatment for 3 days. AMSBE or Lonart (standard control) was then orally administered at 50, 200 and 400 mg/kg or 10 mg/kg once daily for 4 consecutive days. The untreated control received sterile water. Malaria parasitemia reduction, anti-malarial activity, mean change in body weight and MST of the parasitized mice were evaluated. Furthermore, changes in white blood cells (WBCs), red blood cells (RBCs), platelets count, hemoglobin (HGB), hematocrit (HCT) and mean corpuscular volume (MCV) were also determined in the healthy animals before infection as baseline and on days 3, 5 and 8 after infection by employing complete blood count. Standardization of AMSBE was achieved by quantification of its constituents and chemical fingerprint analysis using UHPLC-MS.

Results: Administration of AMSBE, standardized to 41.51% saponins and 234.960 ± 0.026 mg/g of GAE phenolics, produced significant ($P < 0.05$) reduction of parasitemia development, maximum anti-malaria activity of 46.01% (comparable to 32.53% produced by Lonart) and significantly ($P < 0.05$) increased body weight and MST of *P. berghei* infected mice compared to the untreated control. Moreover, there were significant ($P > 0.05$) elevation in WBCs, RBCs, HGB, HCT and platelets in the parasitized-AMSBE (especially at 400 mg/kg p.o.) treated mice compared to their baseline values. Whereas, the non-treated parasitized control recorded significant reduction ($P < 0.05$) in all the above-mentioned parameters compared to its baseline values. The UHPLC-MS fingerprint of AMSBE revealed four compounds with their retention times, percentage composition in their chromatograms and m/z of the molecular ions and fragments in the spectra.

Conclusions: These results show that *A. micraster* stem bark possessed significant anti-malaria effect and also has the ability to abolish body weight loss, leucopenia, anemia and thrombocytopenia in *P. berghei* infected mice leading to prolonged life span. The UHPLC-MS fingerprint developed for AMSBE can be used for rapid authentication and standardization of *A. micraster* specimens and herbal preparations produced from its hydroethanolic stem bark extract to ensure consistent biological activity. The results justify *A. micraster*'s use as anti-malaria agent.

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https://doi.org/10.1016/j.jep.2020.113427
Received 18 August 2020; Received in revised form 26 September 2020; Accepted 26 September 2020
Available online 3 October 2020
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Abbreviations

AMSBE	*A. micraster* stem bark extract
MST	Mean survival time
ICR	Institute of Cancer Research
UHPLC	Ultra high pressure liquid chromatography
MS	Mass spectroscopy/Mass spectrometer
RT	Retention time
WBCs	White blood cells
RBCs	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
LC-MS	Liquid chromatography-mass spectrometry
OECD	Organization for Economic Co-operation and Development
SEM	Standard error of mean
ANOVA	Analysis of variance
NPY	Neuropeptide Y

1. Introduction

Malaria poses a major threat to public health, especially in developing countries, dues to the very large number of people who get infected annually and the high mortality rate associated with the disease, despite all the progress made in treatment and prevention of the disease. Malaria is triggered by infection of the body by *Plasmodium* parasites through the bites of infested female *Anopheles* mosquitoes (WHO, 2018). Among the over 100 different types of *Plasmodium* species that produce disease in animals, *P. falciparum* and *P. vivax* are the most common species that cause human malaria (Koning-Ward et al., 2016). The World Health Organization (WHO) estimated that about 228 million malaria cases which led to 405,000 deaths occurred globally in 2018. Moreover, more than 90% of the global malaria burden and deaths were recorded in Africa and 70% of malaria cases reported globally were detected in children under the age of 5 years (WHO, 2019). Enormous efforts have been made by the WHO and the global scientific community to eradicate malaria in the past few decades. Nevertheless, these efforts did not yield the needed results due to the advent and spread of insecticide-resistant mosquitoes, drug-resistant *Plasmodium* parasites and inability to develop a successful malaria vaccine (Abdulrazak et al., 2015).

Majority of the population in developing countries use plants and crude plant extracts as drugs to treat diseases. Newton and his research team reported that more than 1200 species of plants in over 160 families are employed in traditional medicines globally as treatment for malaria. The anti-malaria activity of some of these plants have been scientifically validated in *in vitro* and/or in vivo assays (Newton et al., 2006). However, the scientific basis for the use of most of the plants as anti-malaria agents still remains unknown. *Antrocaryon micraster* is a deciduous timber tree species in the Anacardiaceae family that grows up to 35–50 m high. The bole of the tree is straight and cylindrical and can grow as wide as 1.5 m (Ayarkwa, 2011). In African traditional medicine, the fruit *A. micraster* is employed against cough, chest pain, stomach ache and toothache. Furthermore, the stem bark is employed as a concoction and enema against threatened abortion and impotence (Ayarkwa, 2011). The leaf, stem or root barks are also ground with seeds of *Xylopia aethiopica* and *Aframomum melegueta* to treat chicken pox (Addo-Fordjour et al., 2013). The stem bark or fruit of *A. micraster* are made into a poultice that is drunk or applied to the body to relieve pain and arthritis (Ayarkwa, 2012). Furthermore, a survey carried out by the corresponding author revealed that the seed of *A. micraster* which is known by the Akans as 'Aprokuma ba' is sold in many markets of Ghana as remedy for headache, inflammation, stomachache and boils. Ethnopharmacological survey conducted in some localities in Ghana showed that decoctions of the leaf, stem or root barks of the plant are used to treat malaria fever (Vigbedor et al., 2008) which is indicative of its anti-malaria potentials. Despite the numerous uses of the plant to treat various diseases in African traditional medicine, only in vivo antioxidant and anti-inflammatory activities had been evaluated for the stem bark (Essel et al., 2017). The anti-malaria activity of the plant had not been evaluated despite, its used to treat malarial.

Also, active ingredients/phytochemicals in medicinal plants vary based on several conditions such as geographical location of the plant, soil type, climatic conditions, seasonal and genetic variations, time of harvest, part of the plant material being used, age of the plant, post-harvest handling of the raw plant material and processing methods used to produce the finished product. These variations mostly lead to great differences in quality and efficacy of various batches of the same plant species and their finished herbal preparations. It is therefore important to quantify the amount of reported active ingredients in raw plant materials and herbal medicines to serve as standard for subsequent batches of the plant so as to ensure consistency and reproducibility in its biological activities. Since the same plant extract standardized to the same quantity of phytochemical constituents will produce similar measurable biological activities. The WHO also introduced chemical fingerprinting of herbal medicine as a means of chemical quality evaluation (Chang et al., 2008; Tang et al., 2010; Wu et al., 2011). This also ensures correct identification of the plant/plant material and consistency in quality of raw medicinal plants materials and herbal medicines by comparing the chromatograms of the authentic samples to future specimens. Among the many chromatographic methods used for chemical fingerprinting such as TLC, GC, HPLC, LC and HPTLC, the WHO has adopted the HPLC fingerprinting technique as the most acceptable procedure (WHO, 1991). Moreover, with the advancement in the functions of modern analytical instruments, detector such as U.V. or MS are being coupled to an UHPLC for chemical fingerprint analysis. The advantage of the later method over the former is that the chemical identity of any known compound in the herbal medicine/preparation can be deduced from their U.V or MS spectral whiles their concentration and fingerprints can also be evaluated from their LC chromatograms. Hence, the biological activities and/or toxicity of a given herbal medicine can be related to its specific constituent(s) in addition to quality assurance. In spite of the advantages of standardization and chemical fingerprint analysis of medicinal plants and herbal medicines, *A. micraster* had neither been standardized nor undergone chemical fingerprint analysis.

The aim of this study therefore, is to standardize the stem bark extract of *A. micraster* using LC-MS chemical fingerprint analysis and quantification of its phytochemical constituents. And to further evaluate the anti-malaria properties of the standardized extract and its effects on hematological parameters in *P. berghei* infested mice in the Rane's test in order to partly elucidate the mechanism of anti-malaria action of *A. micraster*.

2. Methods and methods

2.1. Chemicals

Lonart tablets (Combination of artemether 80 mg and lumefantrine 480 mg) was purchase from Bliss GVS Pharma Limited (Mumbai, India). Normal saline was obtained from Pharmanova Limited (Accra, Ghana). Food grade ethanol was used for extraction. Chloroquine sensitive *P. berghei* ANKA strain was obtained from Noguchi Memorial Institute for Medical Research (NMIMR), Legon, Ghana.

2.2. Collection and extraction of plant material

A. micraster was identified at Asubima Forest Reserve at a location with N: 06 44 05.3 W: 002 48 51.4 coordinates at Asumura in the Ahafo Region of Ghana by Mr. Jonathan Dabo, a botanist at the Forestry Research Institute of Ghana (FORIG) Kumasi. The stem bark of the tree was collected in September 2019 and voucher specimen (FORIG 0014) was deposited at the herbarium of FORIG. The plant material was sun dried for 8 days and pulverized into course powdered. The powder (200 g) was macerated with 2 L of ethanol (70%) at room temperature for 4 days and filtered. The marc was re-extracted under similar conditions and filtered. The two extracts were pulled together and concentrated in a rotary evaporator (Eyeler N1110, Tokyo-Japan) to remove the ethanol. The resultant aqueous portion was freeze dried to afford powder coded AMSBE which was stored in air-tight container at 4 °C until needed.

2.3. Animals and ethical considerations

Female ICR mice (25–38 g) used in this study were supplied by the Animal Experimentation Unit of the Centre for Plant Medicine Research (CPMR), Mampong-Akwapim, Ghana. The mice were kept in standard aluminum cages in a controlled room of 12 h light/dark cycle at 24 ± 2 °C, humidity of 60–70% and allowed free access to sterile water and powdered feed. Ethical clearance for the study with approval number UG-IACUC 013/18–19 was obtained from the University of Ghana Institutional Animal Care and Used Committee (UG-IACUC).

2.4. Phytochemical screening of the extract

The extract of *A. micraster* stem bark (50 mL) was taken and screened for the presence or absence of phytochemical constituents such as alkaloids, triterpenes, saponins, phenolic compounds, anthracinosides, polyuronides, flavonoids, reducing sugars, cyanogenic glycosides, phytosterols according to methods described previously (Trease and Evans, 1989).

2.5. Determination of total phenolic in AMSBE

Folin-Ciocalteau method was used to determine the total phenolic content of AMSBE (Singleton et al., 1999). Briefly, 100 μL of 5% Folin-Ciocalteau reagent was mixed with 20 μL of 0–5 mg/mL of AMSBE in increasing order of concentration. Bisodium carbonate solution (80 μL) of 7.5% concentration was added to the mixture in a well plate and thoroughly mixed before it was incubated in the dark at room temperature for 1 h. The absorbance of the sample was determined at 765 nm with a micro titer plate reader (Infinite M200Pro, Tecan, Austria). The test was performed in triplicates. A standard calibration curve was also produced using Gallic acid under the same experimental conditions. The total phenolic contents of AMSBE, expressed as microgram of Gallic acid equivalent (GAE) per milligram of dry sample, was then calculated from the standard calibration curve.

2.6. Determination of total saponins in AMSBE

The quantity of saponins present in the AMSBE was determined using the method described by (Ejikeme et al., 2014) with slight modification. The extract (5.0 g) was dissolved in 100 mL of 20% aqueous ethanol. The mixture was transferred into a ceramic evaporating dish and concentrated to about 40 mL by heating over water bath for 4 h. The concentrated aqueous extract obtained (20 mL) was partitioned twice with diethyl ether (20 mL) in a separation funnel. The diethyl ether fraction was discarded and the aqueous fraction partitioned twice with n-butanol (60 mL) containing 5% sodium chloride solution (10 mL). The sodium chloride portion was discarded and the n-butanol fraction transferred into a ceramic dish and evaporated over water bath to dryness and thereafter dried to a constant weight in an oven. The percentage saponin content of the extract was calculated as indicated below.

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

2.7. UHPLC-MS chemical fingerprint analysis of AMSBE

AMSBE (25 mg) was dissolved in 25 mL of HPLC grade methanol in a round bottom flask. A homogeneous solution was obtained by shaking the flask on Vortex mixer (Thermolyne, Maxi Mix-Plus) for 5 min. To perform the liquid chromatographic separation, an aliquot (2 mL) of the homogeneous methanol solution was transferred into a GC vial and place in the autosampler chamber (G4226A autosampler) of an Ultra High Performance Liquid Chromatography (UHPLC, Agilent Infinity 1290) machine equipped with quaternary pump and a temperature column control compartment (G1327). The analytical column of the UHPLC (Phenomenex Kenetex 2.6 u XB-C18. 100 × 2.10 mm) was set at 30 °C and connected to a guard column (Agilent Eclipse C18, 2.1 × 5 mm, 1.8 μm) to prevent contamination of the analytical column. The mobile phase of the UHPLC constituted two solvents systems namely 0.1% formic acid in HPLC grade water (A) and 0.1% formic acid in HPLC grade methanol (B). The UHPLC was run at a constant flow of 30% of A: 70% of B at a run time of 0–10 min. The methanol solution of AMSBE was injected at a volume of 1 μL and the flow rate was 0.2 mL/min. Mass spectrometer (Agilent Tripple Quadrupole Mass Spectrometer) was coupled to the UHPLC as a detector to analyze the eluted compounds. The electron source ionization (Positive mode) was used for fragmentation in the mass spectrometer chamber set at 300 °C and current flow of 0.21 μA, scan cycles of 4.91/S and electron energy of 350 V. The collision gas (Nitrogen) had a flow rate of 13 L/min. The nebulizer pressure was 30 psi and the electron energy of the capillary was 5000 V. The scan was performed at a range of m/z 100–500.

2.8. Acute toxicity test

Acute toxicity or safety test of AMSBE was evaluated orally in mice as per previously described method pre (OECD, 2001) with slight modifications. The acute toxicity assay was to also help estimate the LD_{50} of the extract and to determine the experimental doses which would not kill the animals during the study. A single oral dose of 5000 mg/kg of AMSBE was reconstituted in distilled water and orally administered to female ICR mice (25–29 g; N = 6) per body weight. The animals were observed at 2 h interval for 24 h for signs of toxicity such as autonomic, neurological and/or behavioral changes and mortality after administration of the extract. The mice were again observed for 13 extra days.

2.9. Maintenance of the parasite in the laboratory

Chloroquine sensitive *P. berghei* ANKA strain were transferred into mice at NMIMR and transported to a laboratory at the Animal Experimentation Unit of the CPMR. The parasites were then maintained alive by serial transfer into naive female ICR mice after every 7 days for 3 times.

2.10. Evaluation of anti-malaria effect of AMSBE in the Rane's test

Efficacy of the extract as anti-malaria agent was evaluated in the Rane's test (the 7 days curative test) against *P. berghei* in mice according to Fidock et al. (2004). Blood from donor mice with rising parasitemia of 20–30% was diluted in physiological saline (Pharmanova Limited) such that each 0.2 mL contained 1.0×10^7 *P. berghei*-infected RBCs. Female ICR mice (N = 30) were each given 0.2 mL (i.p.) of the diluted blood and allowed to stay for 72 h without treatment for parasitemia to establish. Parasitemia level of each mouse was determined after the 72 h of inoculation to serve as baseline parasitemia level before commencement of treatment. The animals were then randomly selected into 5 Groups (N = 6) and given various treatments for 4 consecutive days. Group I (Untreated negative control) received sterile water; Group II received 0.2 mL of Lonart (10 mg/kg p.o.) per mouse as reference drug. Furthermore, each mouse in Group III, IV and V received 0.2 mL of AMSBE 50, 200 or 400 mg/kg p.o. Respectively.

2.11. Parasite monitoring

Parasitemia levels in the blood of the *P. berghei*-infected mice were monitored using methods described by Arrey et al. (2014). Briefly, thin film was prepared from blood sample taken from each animal onto a microscope slide by tail-bleeding on day 3, 5 and 8 after infection. The film of the blood was dried, fixed in methanol for 15 min and stained in 10% Giemsa stain for 25 min. Excess stain was washed off and the slide dried. The film was then immersed in oil and viewed at x100 magnification using the Olympus light microscope (Olympus CX21; Tokyo, Japan). The level of parasitemia was determined by counting the number of parasitized RBCs and normal RBCs per randomly selected fields. Percentage malaria parasitemia and anti-malaria activity (taken as percentage parasite clearance) were calculated as shown below.

$$\text{Percentage malaria parasitemia} = \frac{\text{Parasitized RBCs counted}}{\text{Total RBCs counted}} \text{ x } 100\ \%$$

$$\text{Anti-malaria activity} = \frac{\text{Parasitemia in untreated control} - \text{parasitemia in treatment group}}{\text{Parasitemia in untreated control}} \text{ x } 100\ \%$$

2.12. Hematological analysis

Prior to infection of the mice with *P. berghei,* blood sample was taken from each animal by tail bleeding on days 0 (Baseline) and thereafter, on day 3, 5 and 8 after the inoculation into Eppendorf tubes precoated with anti-coagulant (Na-EDTA). The blood samples, 50–80 μL, were diluted in 420 μL normal saline. Hematological parameters were determined by running a complete blood count analysis of the diluted blood using hem analyzer (Abacus 380; Budapest, Hungary). The dilution of the blood was accounted for by multiplying through the results with the dilution factor.

2.13. Change in body weight

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 of the experiment. The effect of treatment on body weight reduction or increase of parasitized mice was determined as mean change in body weight as indicated below.

$$\text{Mean change in body weight} = \frac{\text{Weight of animal at Dt} - \text{Weight of animal at Do}}{\text{Total number of mice in a group}}$$

2.14. Mean survival time (MST)

The number and date of death of each animal from each group was recorded during the 8 days experimental period and for further 22 days. The mean survival time (MST) was evaluated over the 30 days period as shown below.

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

2.15. Micrographs

Micrographs of selected fields showing the levels of parasitized RBCs and unparasitized RBCs were taken with a 16.0 MP camera of Samsung galaxy A30 mobile phone.

2.16. 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 $P < 0.05$.

Table 1
Phytochemical screening results of AMSBE.

Phytochemical constituent	AMSBE
Saponins	Present
Phenolic compounds	Present
Free reducing sugars	Present
Alkaloids	Absent
Flavonoids	Absent
Triterpenes	Absent
Antracenosides	Absent
Polyuronoids	Absent
Phytosterols	Absent
Cyanogenic glycosides	Absent

3. Results

3.1. Yield of crude extract

The 200 g of the stem bark of *A. micraster* extracted afforded 41.29 g of non-absorptive dark brown powder which translated into a yield of 20.65% w/w.

3.2. Phytochemical tests

The results of the Phytochemical tests are shown below in Table 1. The results show that the extract contained saponins, phenolic compounds and free reducing sugars. The rest were absent.

Table 2
Quantity of phenolic compounds and saponins present in AMSBE.

Phytochemical constituent	Calibration curve equation	Quantity present in AMSBE
Phenolic compounds	$y = 0.3986x + 0.003$	234.960 ± 0.026 mg/g of GAE
Saponins	_	41.51%

3.3. Quantity of phenolic compounds and saponins present in AMSBE

The results of the quantification of the phytochemical constituents present in AMSBE are shown below in Table 2. The straight-line equation generated from the standard calibration curve of Gallic acid which was used to calculate the total phenolics in the extract is also indicate in the Table. The correlation coefficient (R^2) obtained from the standard calibration curve of Gallic acid was 0.999.

3.4. UHPLC-MS fingerprint of AMSBE

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

3.5. Acute toxicity test and determination of doses for the anti-malaria activity

Oral administration of 5000 mg/kg p.o. of AMSBE at a single dose to female ICR mice did not produce any explicit clinical symptom of toxicity, trauma or alteration of physical features and/or behavior of mice during the 14 days of observation. Additionally, none of the mice died during the observation period. Based on the results of the acute toxicity test, reasonable doses of 50, 200 and 400 mg/kg p.o. of AMSBE which were below the 5000 mg/kg p.o. were selected for the anti-malaria activity test.

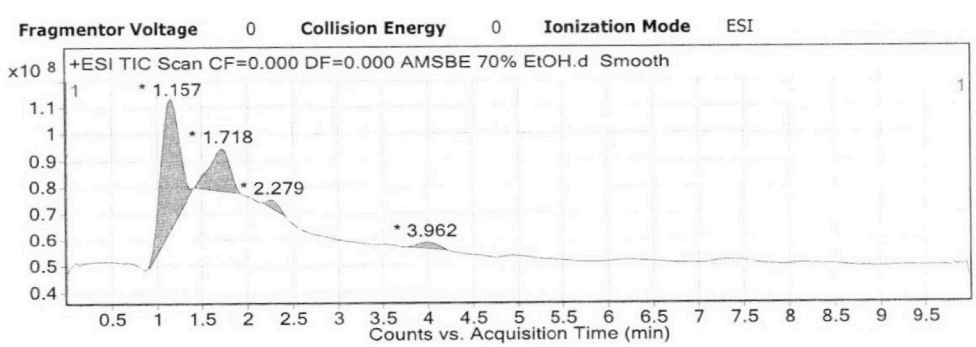

Fig. 1. Fingerprint of UHPLC chromatogram of AMSBE showing the peaks of compounds present.

Table 3
Characteristic features of UHPLC chromatogram fingerprint of compounds in AMSBE.

Compound	RT/min	Height	Height %	Area	Area Sum %	Symmetry	Width
1	1.157	49632751.43	100.0	710703396.10	69.25	1.54	0.491
2	1.718	16129249.86	32.5	250091658.00	24.37	0.57	0.491
3	2.279	3148460.69	6.34	27790467.52	2.71	0.76	0.245
4	3.962	2350241.11	4.74	37765117.04	3.68	0.93	0.456

Table 4
Mass spectra fragmentation fingerprints of compounds in AMSBE using UHPLC-MS.

Compound	Mass to charge ratio (m/z)									
1	104.2	118.2	124.2	219.1	233.1	249.1	291.2	465.4	467.3	485.3
2	111.1	124.2	141.1	157.1	159.1	199.2	427.4	469.3	487.3	488.3
3	111.1	124.2	141.1	157.1	159.2	199.1	427.5	449.5	471.3	488.4
4	111.1	122.1	124.2	149.1	157.1	279.2	301.2	427.5	428.4	449.4

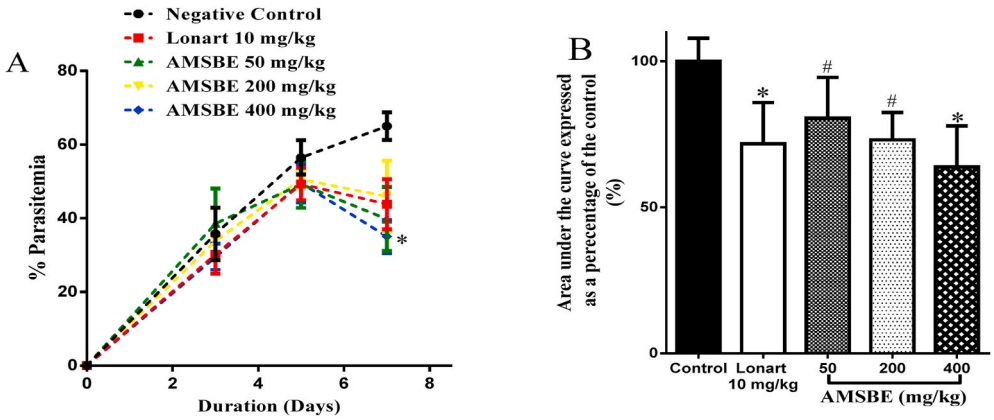

Fig. 2. Effect of AMBSE on parasitemia development in *P. berghei* infested mice on time-course curve (A) and reduction of overall percentage parasitemia development expressed as percentage area under the curve of the control (B). *P < 0.05 or #P > 0.05 compared to the Negative control.

3.6. Level of parasitemia

The effect of AMSBE on parasitemia levels in *P. berghei* infested mice are shown below in Fig. 2 on the time-course curve (A) and as area under the curve expressed as percentage of the non-treated negative control group (B).

It has been observed that the parasitemia levels in the non-treated negative control group increased from the day of infection to day 7 of the experiment. Whereas, the parasitemia levels of Lonart (10 mg/kg p.o.) and AMSBE (50–400 mg/kg p.o.) treated groups declined on day 5–7. AMBSE produced a dose-dependent reduction of parasitemia with significant ($P < 0.05$) effect at 400 mg/kg p.o. on the time-course curve and area under the curve (Fig. 2 A and B). The effect of the standard anti-malarial medicine Lonart (10 mg/kg p.o.) was also statistically significant ($P < 0.05$) in area under the curve (Fig. 2 B).

3.7. Anti-malaria activity

Anti-malaria activity of the extract and the controls calculated as percentage parasitemia clearance is given in Table 5 below. The results show that AMSBE (50–400 mg/kg p.o.) produced dose-dependent anti-malaria activity of 10.49–12.83% on day 5 (2 consecutive days of treatment) comparable to the anti-malaria activity (12.76%) of Lonart (10 mg/kg p.o.). When treatment was prolonged to day 8 (4 consecutive days of treatment), AMSBE again produced dose-dependent anti-malaria activity which considerably increased to about 2–4 folds of that of day 5 (Table 5). The anti-malaria activity of AMSBE at 400 mg/kg p.o. (46.10%) was higher than 32.53% anti-malaria activity produced by the standard drug, Lonart at 10 mg/kg p.o.

Table 5
Anti-malaria activity of ASMBE or Lonart in mice on day 5 and 8 post *P. berghei* infection.

Dose	Anti-malaria activity (%)	
	Day 5	Day 8
Negative Control	00.00	00.00
Lonart (10 mg/kg)	12.76	32.53
ASMBE (50 mg/kg)	10.49	29.36
ASMBE (200 mg/kg)	12.28	38.80
ASMBE (400 mg/kg)	12.83	46.10

Data is presented as percentage mean (N = 6).

3.8. Effect of treatment of AMSBE on mean change in body weight and mean survival time of P. berghei infested mice

The effect of AMSBE on the mean change in body weight and MST of parasitized mice are shown below in Table 6. The results showed that the extract had prevented *P. berghei* infested mice from losing considerable weight compared to the untreated negative control. The weight improvement effect of Lonart and AMSBE (at all doses) was statistically insignificant ($P > 0.05$) on day 5. However, AMBSE at a lower dose of 50 mg/kg p.o, significantly ($P < 0.05$) improved the weight of *P. berghei* infested mice compared to the untreated negative control on day 8 (Table 6).

The weight improvement effect of the AMSBE in *P. berghei* infested mice was inversely dose-dependent. Lonart (10 mg/kg p.o.) produced insignificant ($P > 0.05$) weight improvement effect similar to AMBSE at 400 mg/kg p.o. (Table 3). Furthermore, treatment of *P. berghei* infected mice with AMBSE (50–400 mg/kg p.o.) or Lonart (10 mg/kg p.o.) significantly ($P < 0.001$) increased the MST of the treatment groups compared to the untreated control (Table 6).

3.9. Effect of AMSBE on hematological indices of P. berghei infested mice

The results of the effect of AMSBE on hematological indices of *P. berghei* infested mice are presented below in Fig. 3 (A - F). The results show that infection of mice with *P. berghei* lead to consistent depletion of WBCs in the untreated negative control group. The WBCs depletion became very significant ($P < 0.001$) on days 5–8 compared to the baseline values recorded when the animals where healthy without any

Table 6
Mean change in body weight on 5th and 8th day post infection and Mean Survival Time (MST) calculated after 30 days of *P. berghei* infected mice.

Dose	Change in Body Weight (g)		Mean Survival Time (MST)
	Day 5	Day 8	(Days)
Negative Control	−1.77 ± 0.57	−2.38 ± 0.41	13.00 ± 0.56
Lonart (10 mg/kg)	−0.80 ± 0.76#	−0.60 ± 0.89#	28.00 ± 0.00***
ASMBE (50 mg/kg)	2.00 ± 2.45#	2.63 ± 2.12*	24.50 ± 0.49***
ASMBE (200 mg/kg)	0.80 ± 0.93#	−0.25 ± 0.86#	28.00 ± 0.00***
ASMBE (400 mg/kg)	−0.60 ± 0.62#	−0.64 ± 0.74#	28.00 ± 0.00***

Data presented as mean ± SEM. *P < 0.05; ***P < 0.001; #P > 0.05 compared to the negative control.

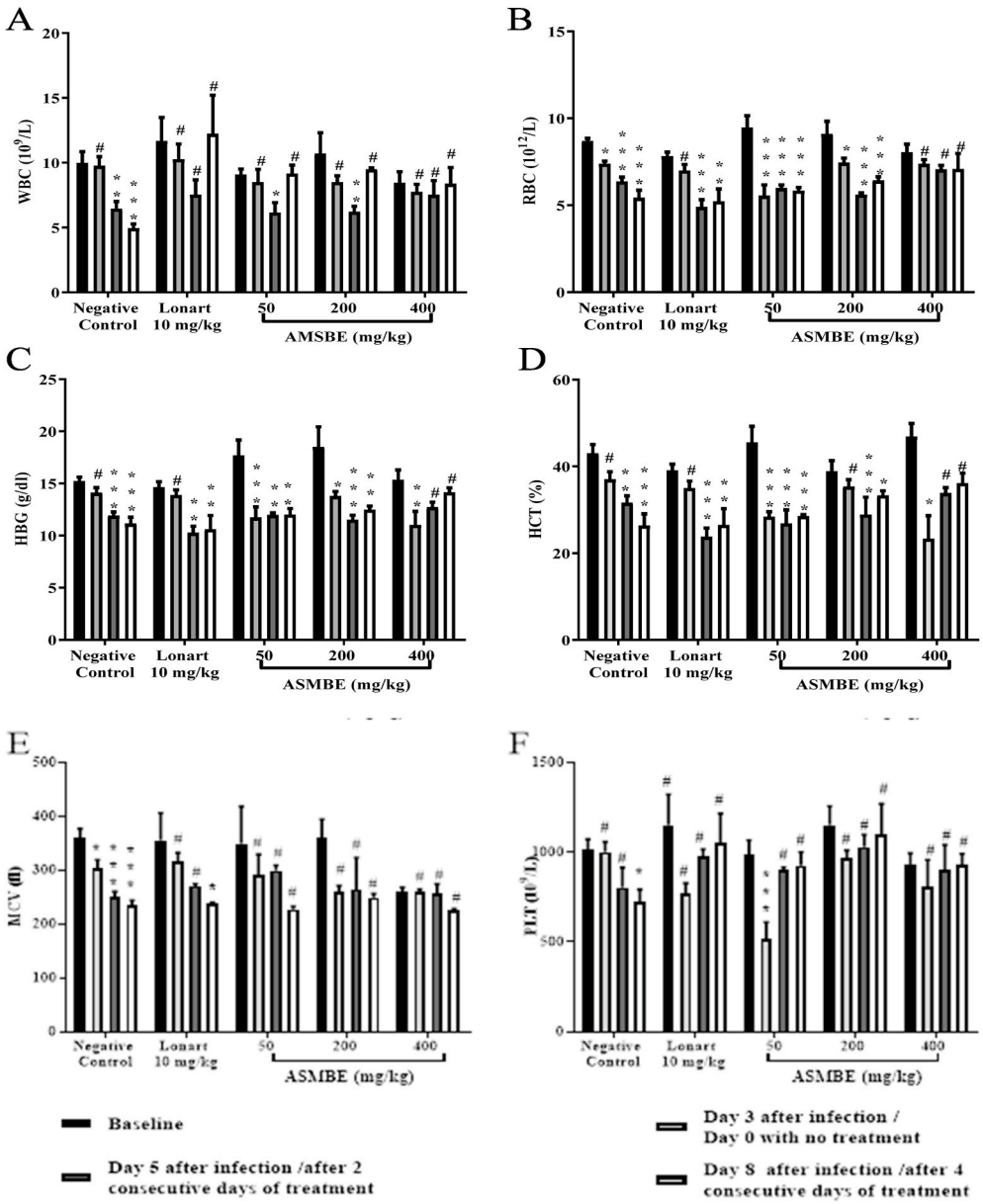

Fig. 3. Effect of AMSBE or Lonart on hematological indices in *P. berghei* infested mice. Values are expressed as Mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 or #P > 0.05 compare to the mean baseline values.

treatment (Fig. 3 A). Similarly, infection in the Lonart and AMSBE treatment groups also depleted the WBC levels consistently until it reached the minimum levels on day 5 after infection (Fig. 3 A).

The depletion of the WBCs was statistically significant (P < 0.05) in AMBSE 50–200 mg/kg p.o. treatment groups on day 5. It was however, insignificant (P > 0.05) in the higher dose of AMBSE (400 mg/kg p.o.) and the standard drug, Lonart 10 mg/kg p.o. treatment groups compared to their baseline WBC values respectively. Also, treatment with AMBSE (50–400 mg/kg p.o.) and Lonart (10 mg/kg p.o.) significantly (P > 0.05) restored the WBCs of the diseased mice to their baseline values on day 8 (after 4 consecutive days of each treatment post infection) (Fig. 3 A).

Furthermore, RBC levels had substantially (P < 0.05–0.001) decreased in the untreated control group and in all the treatment groups (including Lonart 10 mg/kg p.o.) compared to the baseline RBCs except for AMSBE at 400 mg/kg p.o. (Fig. 3 B). The decreased in RBC levels had also led to the corresponding decrease (P < 0.05–0.001) in HGB and HCT levels in the respective treatment groups compared to their respective baseline levels (Fig. 3 C and D). However, AMSBE at 400 mg/kg p.o. (the highest dose) has significantly prevented (P > 0.05) *P. berghei* parasites from destroying the RBCs of mice with corresponding increase (P > 0.05) in HGB and HCT levels compared to their baseline values (Fig. 3 C and D). MCV values of the untreated negative control group significantly plummeted (P < 0.05–0.001). Nonetheless, the reduction in MVC levels of AMSBE treatment groups were statistically insignificant (P > 0.05) especially in AMSBE 400 mg/kg p.o. (Fig. 3 E). Finally, *P. berghei* infection of the mice resulted in significant (P < 0.05) reduction in platelet levels in the untreated control group on day 8. In contrast, significant (P > 0.05) increase in platelet levels was observed for the AMSBE and Lonart treatment groups on day 5–8 (Fig. 3 E).

3.10. Effect of treatment with AMSBE on micrographs of RBCs

The result of the effect of treatment with AMSBE on RBCs of *P. berghei* infested mice is shown below in Fig. 4 A – E. It could be observed from the micrographs that the untreated negative controls (A) developed more parasitized-RBCs at the end of the Rane's test than Lonart at 10

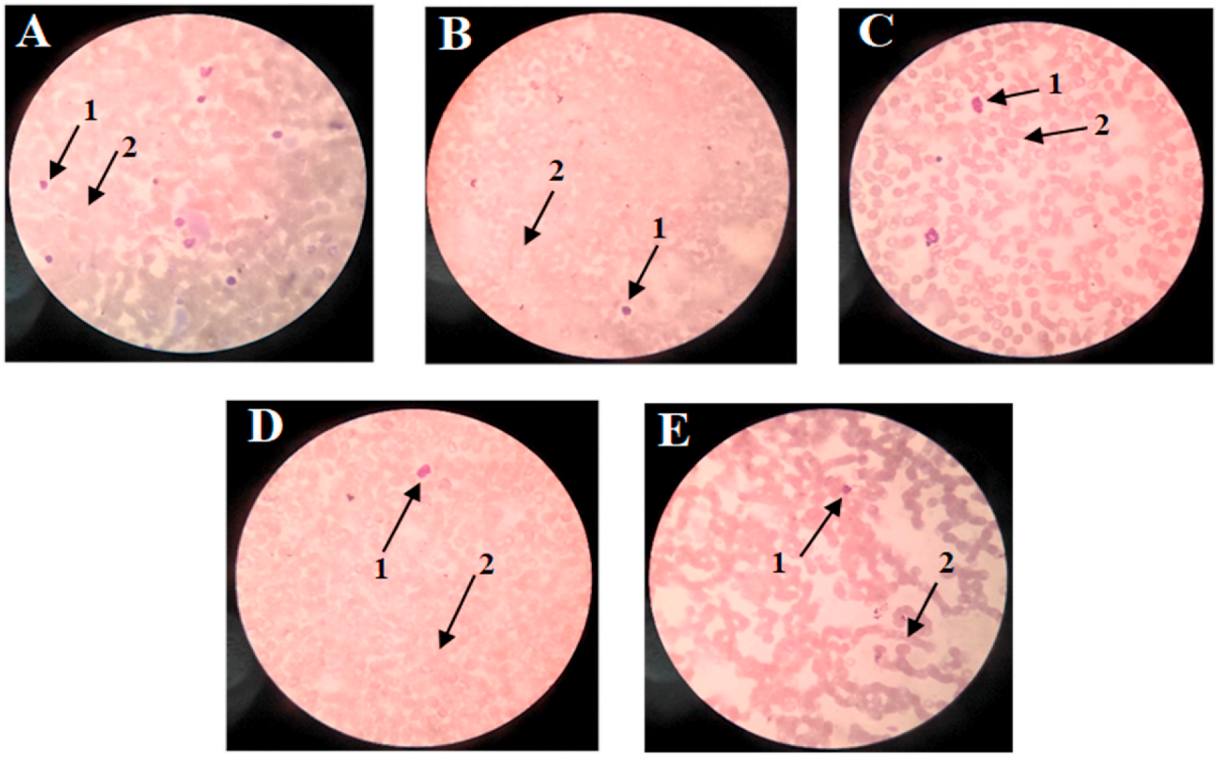

Fig. 4. Micrographs showing selected fields of the levels of *P. berghei* infected and uninfected RBCs of both the Negative control group (A) and the treatment groups, Lonart (B), ASMBE 50 mg/kg (C), 200 mg/kg (D) and 400 mg/kg (E) respectively on the 8th day. 1 = Infected RBC; 2 = Normal RBC.

mg/kg p.o. (B) or AMSBE at 50–400 mg/kg p.o. (C – E) treatment groups respectively.

4. Discussion

Plants produces diverse classes of chemical compounds as defensive weapons when they are under attack or stressed. These compounds are not utilized by the plants for their basic metabolism or growth but possessed chemical and biological characteristics which make the plants medicinal. Saponins and phenolic compounds are among some of these plant-based chemicals. Some plants, such as *Momordica charantia, Newbouldia laevis*, *Khaya senegalensis* and *Dichrostachys cinereal*, which contain high amounts of saponins are used in traditional medicine to treat malaria and other diseases. Furthermore, the anti-malarial and/or anti-plasmodial effects of some of these plants, crude saponin extracts and pure saponin isolates such as spiroconazole A have been scientifically proven (Gbeassor et al., 1990; Okunji et al., 1996; Manga et al., 2018; Akanbi et al., 2018; Kweyamba et al., 2019). Moreover, the medicinal plant *Sorindeia juglandifolia* used in African traditional medicine to treat malaria and its isolated phenolic compounds such as 2,3,6-trihydroxy benzoic acid and 2,3,6-trihydroxy methyl benzoate were shown to demonstrate significant anti-malaria activity (Kamkumo et al., 2012). *S. juglandifolia* belongs to the Anacardiaceae plants family just as *A. micraster*. The IC_{50} values of 2,3,6-trihydroxy benzoic acid and 2,3, 6-trihydroxy methyl benzoate against *P. falciparum* W2 were 16.5 and 13.0 μM respectively and against *falcipain* - 2 were 35.4 and 6.1 μM respectively. Furthermore, 2,3,6-trihydroxy benzoic acid also produced in vivo antimalaria activity against *P. berghei* strain B, with average parasitemia suppressive dose and curative dose of 44.9 mg/kg and 42.2 mg/kg respectively (Kamkumo et al., 2012). Therefore, the presence of saponins and phenolic compounds in AMSBE are responsible for its observed anti-malarial effect in this study.

AMSBE was standardized to 41.51% saponins and 234.960 ± 0.026 mg/g of GAE phenolic compounds. Biological activities of medicinal plants are related to the nature and concentration of their chemical constituents. Hence, any batch of *A. micraster* stem back standardized to the quantities of the above-mentioned phytochemicals should lead to reproducibility of antimalaria properties of AMSBE observed in this study.

The UHPLC chromatogram shows the presence of four different peaks which represent four compounds coded 1–4 in AMSBE. Compound 1–4 have a retention time (RT) in increasing order of 1.157, 1.718, 2.279 and 3.962 min respectively. This indicates that they eluted from the column in the order of Compound 1, 2, 3, and 4 respectively. Compound 1 was the most abundant in the extract (Peak area = 69.25%). It was followed by compound 2, 4 and 3 with percentage peak areas of 24.37, 3.68 and 2.71% respectively. The m/z of the fragment ions produced in the mass spectra of the compounds are very similar or the same in some cases. This indicates that the compounds may be closely related plant secondary metabolites which have the possible m/z of the parent molecular ions ranging from 449.4 to 485.3. The name(s) and chemical structure(s) of compound(s) obtained by UHPLC-MS chemical fingerprint analysis can be identified by comparing their m/z to those in literature if the compounds are already known. However, these compounds seem as unknown/new chemical entities. Hence, a search through literature to use their m/z to identify their names and structures did not produce any result. Furthermore, there is no report on isolation and characterization of compounds from *A. micraster* yet. Hence, the structure of these compounds could not be identified in this study. Nonetheless, the RT and other parameters in the chromatogram of the compounds are chemical fingerprint of AMSBE which can be used to identify and authenticate specimens of *A. micraster*. Moreover, the other parameters of the peaks in the UHPLC-MS fingerprint chromatogram of AMSBE such as height, percentage height, area, percentage area sum, symmetry and width can also be used to determine and standardized the composition of these compounds in the plant of any age collected from any geographical location. This will ensure reproducible biological activities of the plant.

Anti-malarial drugs are classified into four groups according to their mechanisms of action against the *Plasmodium* parasite. The blood

schizonticides class are the most important class of anti-malaria drugs used to produce suppressive and clinical cure in malaria treatment. These class of anti-malarias acts on the erythrocytic forms of the parasite in the blood and disrupt the clinical attack of malaria (Tracy and Webster, 2001). The Rane's test is used to evaluate the efficacy of the blood schizonticide class of anti-malarias. In the Rane/7 days test which is also called the curative test, parasitemia is established 72 h post inoculation of *P. berghei* before treatment is commenced. A substance which is able to consistently reduced the established parasitemia 72 h post-inoculation is able to interrupt the erythrocytic schizogony stage of the *Plasmodium* parasite and terminate clinical attacks, and thus produce clinical cure (Ezike et al., 2016). AMSBE produced significant anti-malaria activity (46.10%) in the Rane's test. This indicates that AMSBE is a blood schizonticide type of anti-malaria substance.

Reduction in body weight and anemia were reported as some of the major characteristics of malaria in mice (Adugna et al., 2014). Since the extracts at 50 mg/kg p.o. was able to significantly ($P < 0.05$) improved the weight of *P. berghei* infested mice compared to the untreated negative control group, it shows that the extract is able to protect against weight loss induced by *Plasmodium* infection at lower doses. However, the results also show that this effect of the extract diminishes with increasing dose even though, the weight reduction effect of the extract at higher doses (200–400 mg/kg p.o.) was lesser ($P > 0.05$) than that of the untreated control mice. Saponin containing plant extracts, crude saponin fractions and pure saponins have been shown to demonstrate reduction in body weight (anti-obesity activity) through the inhibition of adipogenesis (Khan et al., 2015; Nguyen et al., 2011), inhibition of pancreatic lipase which leads to reduction in fat absorption (Xu et al., 2005; Marrelli et al., 2016) and suppression of appetite by inhibition of hypothalamic neuropeptide Y (NPY) and serum leptin expression (Kim et al., 2005, 2009). Higher doses (200–400 mg/kg p.o.) of AMSBE resulted in increased saponin content. Therefore, the reduction in body weight of *P. berghei* infested mice treated with AMSBE at 200–400 mg/kg p.o. may be due to increased quantities of saponins which promote adipogenesis or pancreatic lipase inhibitions and/or appetite suppression of the treated animals.

MST is one of the most useful parameters used to evaluate the effectiveness of anti-malaria agents in animals. A substance which possessed effective anti-malaria effects increases the MST whiles animals treated with ineffective antimalaria substances have a significantly shorter MST.

P. berghei ANKA infection of ICR mice is a model of cerebral malaria characterized by elevated level of parasitemia which produce severe anemia and high degree of death after infection within a short period (Basir et al., 2012). Histopathological examination of *P. berghei* ANKA infected ICR mice by Basir et al. (2012) showed that mice in this model of malaria dies as a result of enlarged spleen, enlarged liver, microvascular sequestration of parasitized red blood cells in the brain, liver, lung and kidney injuries in addition to elevated levels of proinflammatory and anti-inflammatory cytokines in the plasma. Results from this study showed that treatment of *P. berghei* infected ICR mice with AMSBE or Lonart lengthens the MST of malaria infested mice to about ≥ 2 folds compared to the untreated control. This indicates that AMSBE is an effective anti-malaria agent and is able to prevent death due to malaria infection by amelioration of injuries caused to major body organs, reduction of parasitemia and/or prevention of anemia. These findings were also confirmed by the potent dose-depended anti-malaria activity shown by AMSBE (46.10% at 400 mg/kg p.o.) and Lonart (32.53% at 10 mg/kg p.o.) respectively. In addition to the significant reduction in parasitemia development in *P. berghei* infection by AMSBE and Lonart.

WBC counts decreases during malaria infection because most of these cells are recruited to destroy the *Plasmodia* parasites through phagocytosis which results in their destruction. Hence, substantial decrease in WBC counts, a condition known as leucopenia. Leucopenia is one of the commonest deleterious hematological alterations which occurs during malaria-infection (Kotepui et al., 2014). Leucopenia weakens the body's immune system and makes it susceptible to infections. The extract was able to prevent substantial depletion of WBCs in *P. berghei* infested mice on day 5. The extract also significantly restored the WBC levels to their baseline values on day 8. This indicates that AMBSE possessed immunomodulatory effects and is able to prevent the development of leucopenia during malaria attack.

Besides WBC count, other major hematological parameters commonly used clinically to diagnose malaria infections are red blood cell (RBC) count, hemoglobin (HGB) concentration, Mean corpuscular volume (MCV), and platelet (PLT) counts. These parameters are also monitored during malaria treatment in other to determine the effectiveness of a drug against the disease (Enechi et al., 2019). Moreover, aberrations in these hematological indices are key clinical indicators of malaria (Lamikanra et al., 2007). RBCs easily degrade in mice infested with *P. berghei* (Iyawe and Onigbinde, 2009). This results in substantial reduction in HCT, MCV and HGB.

HGB is a biomolecule found in RBCs which absorbs molecular oxygen from the lungs and transports it to animal tissues for oxidation of ingested food to produce energy for body functions. HGB also transport carbon dioxide out of the body tissues (Ugwuene, 2011; Isaac et al., 2013). Reduced production of RBCs and their rapid destruction during malaria greatly reduces HGB concentration of blood which results in severe anemia (Menendez et al., 2000). Severe anemia is a common clinical indicator of malaria and a main source of sickness and death in *Plasmodium* infection due to hypoxemia-induced hypoxia and congestive cardiac failure (Phillips and Pasvol, 1992; English et al., 1996). The indicators of severe anemia in hematological analysis are reduced RBCs, HGB, HCT and MCV levels. The ability of AMSBE to restore RBC counts, HGB, HCT and MCV levels in parasitized-AMSBE-treated mice when compared with their pre-infection values suggests that AMSBE possess erythropoietic action. AMSBE may therefore protect against development of anemia during malaria attack and hence, prevent death due to malaria induced anemia.

During malaria attack, platelets encase *Plasmodium* parasites and become destroyed in the action and get eliminated from circulation, resulting in decreased platelet count thus, making malaria a principal cause of thrombocytopenia in environments where the disease is endemic (Ifeanyichukwu and Esan, 2014). Kotepui and co-workers also reported that thrombocytopenia and leucopenia were the most accurate hematological indices which could be used to predict malaria in populations from malaria endemic environments. They showed that thrombocytopenia and leucopenia have 85 and 17% sensitivity and 85 and 94% specificity in diagnosis of malaria respectively (Kotepui et al., 2014). Thus, the effectiveness of an anti-malarial agent can also be measured by its effectiveness to prevent or reverse the development of thrombocytopenia and leucopenia in *Plasmodium* infection.

The results from this study show that the platelet counts of the parasitized untreated control mice decreased progressively without reversal until day 8 when the platelet count became very low ($P < 0.05$) compared to the pre-infection count. Meanwhile, in the AMSBE and Lonart treated groups, platelet counts were at their lowest levels on day 3 when treatment did not commence. On day 5–8, when the various treatments were given for 2–4 consecutive days respectively, the platelet counts were significantly ($P > 0.05$) elevated to values comparable to their pre-infection counts. This shows that the extract may have the ability to reverse thrombocytopenia, an indication that AMSBE is an effective anti-malarial agent as Lonart.

The micrographs in Fig. 4. Section 3.10. which showed that the untreated negative controls developed more parasitized-RBCs at the end of the test than the treatment groups of Lonart 10 mg/kg p.o. or AMSBE (50–400 mg/kg p.o.). This further confirms that AMSBE is an effective blood schizonticide class of anti-malaria agent. Since the extract was able to inhibit the formation of parasitized- RBCs/erythrocytes in the plasma of *P. berghei* infested mice as compared to the untreated control.

Free radicals such as reactive oxygen species (ROS) are produced from metabolic processes of the *Plasmodium* parasite, host immune

expression and destruction of hemoglobin during malaria infection (Iyawe and Onigbinde, 2009; Enechi et al., 2019). Excessive free radicals in the body leads to oxidative stress which is associated with malaria complications (Sandro et al., 2012). Antioxidant substances disrupts the vicious effects of free radicals by interrupting the initiation, propagation or termination of the oxidative stress processes (Cui et al., 2004). Phenolic compounds act as antioxidants by moaping up free radicals and chelating metal ions which initiate the formation of ROS (Kostyuk et al., 1990) and hence alleviate the negative effects associate with oxidative stress. Also, the antimalaria properties of certain plants have been shown to be associated with their in vivo antioxidant activities by significantly decreasing malondialdehyde (MDA) and lipid peroxidation levels (George et al., 2012; Enechi et al., 2019). The phytochemical analysis of the extract show that ASMBE contains high quantities (234.960 ± 0.026 mg/g of GAE) of phenolic compounds in addition to other constituents. Furthermore, Essel et al. (2017) also showed that *A. micraster* stem bark possessed in vivo antioxidant activity. Hence, the antimalaria properties of AMSBE may also be due to its high phenolic content which might contribute to its antioxidant activity. The anti-malaria activity of some of these anacardiaceous phenolic compounds were discussed above in the first paragraph of section 4.

Finally, the result from the acute toxicity test shows that the LD_{50} of AMSBE is above 5000 mg/kg p.o. in female ICR mice. Hence, AMSBE could be classified as been safe (OECD, 2001) in the short-term usage.

5. Conclusion

The results from this study show that the 70% hydroethanolic stem bark extract of *A. micraster* (AMSBE) possessed significant anti-malaria effect and high level of safety. The study also reveals that the extract has the ability to abolish body weight loss, leucopenia, anemia and thrombocytopenia leading to prolonged life span in *P. berghei* infected mice. The result also showed that AMSBE could be classified as a blood schizonticide type of anti-malaria agent. These results lend credence to the use of *A. micraster* stem bark as anti-malaria agent in Ghanaian traditional medicine. The stem bark of *A. micraster* could therefore be explored for novel anti-malaria compounds.

Availability of data materials

Data upon which conclusions from this study were made are contained within the article.

Author contributions

EKK and RAO conceptualized, planned and supervised the research, EKK performed the statistical analysis of the data and wrote the manuscript. FA, PB and EE conducted the research work. In addition, FA also analyzed the data. JD collected and processed the plant materials. All authors have read and approved the manuscript.

Funding

This work did not receive any specific funding but was supported by the authors.

Declaration of competing interest

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

Acknowledgement

The authors wish to express their profound gratitude to the staffs of the Animal Experimentation and Clinical Laboratory Units of CPMR, Mampong-Akwapim, Ghana, for their technical support.

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