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Antiplasmodial and antimalarial evaluation of a Nigerian hepta-herbal *Agbo-iba* decoction: Identification of magic bullets and possible facilitators of drug action

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ABSTRACT

Ethnopharmacological relevance: Malaria remains one of the most important pathogenic infectious diseases. Although Africa suffers the greatest brunt, a sizeable proportion of her population still relies on herbal medicines for reasons of cost as well as the belief etched in the minds of consumers that herbal medicines are safer and more efficacious than Modern medicines. *Agbo-iba*; a concoction of two or more than two plants is commonly used for the management of malaria in Nigeria.

Aim of the study: This study assessed the safety and efficacy of a hepta-herbal *Agbo-iba* (HHA) antimalarial decoction used for the management of malaria in Benin city, Nigeria.

Materials and methods: Assessment was done against malaria parasite in culture as well as *in vivo* in pre-clinical murine model of malaria.

Results: HHA (IC_{50}*Pf*3D7 50 μg/ml) was moderately potent and only one of its constituent plants *Annickia affinis* (IC_{50}*Pf*3D7 1.49 μg/ml) was far more potent, while all others were moderately active to inactive against the parasite *in vitro*. HHA showed good selectivity *in vitro* and was safe at 2 g/kg in mice. However, at 100 mg/kg oral dose, while HHA suppressed parasite growth by 56.76%, the suppression caused by *A.affinis* was only 32.46% in mice malaria suggesting the existence of synergistic partner(s) in the herbal formula. LCMS revealed the presence of quaternary protoberberine alkaloids (QPAs) in *A.affinis* and HHA.

Conclusions: Although QPAs have strong *in vitro* antiplasmodial activity, their *in vivo* antimalarial activity is undermined by being substrates of Permeability glycoprotein (Pgp) efflux pump. Our study suggests that inhibitor(s) of Pgp in HHA could improve the bioavailability of QPAs in mice fed the herbal combo. Further, molecules from other HHA constituent plants may also contribute to the better potency observed for the poly-herbal *in vivo*. These possibilities were validated by the curative antimalarial study at 100 mg/kg, where *A.affinis* was inactive but the HHA suppressed parasite growth by 44.45%.

1. Introduction

The absence of a credible antimalarial vaccine, increasing problem of antimalarial drug resistance as well as the inaccessibility of Artemisinin Combination Therapy (ACTs) to many persons who need them has made herbal remedies an important treatment option (Willcox, 2011; Willcox and Bodeker, 2004). Indeed, it is estimated that about 80% of the African people rely on medicinal plants for the management of their diseases (World Health Organization, 2005). While the WHO recognises the role of Traditional Complementary Medicines (TCM) in keeping populations healthy, it is important that their safety and efficacy be investigated (Willcox, 2011). The neglect of evaluation of safety and efficacy may be the reason why out of the over 2000 plants shortlisted for the management of malaria worldwide, only seven have been approved by the governments of the nations where they were developed for the management of the disease (Willcox, 2011). One of these is Qing hao. Qing hao (*Artemisia annua*) has been in use in the TCM system of China for centuries. Artemisinin is the most potent antimalarial agent in *A. annua*. Since the isolation of artemisinin from *A. annua* in 1971, several NGOs have recommended its use for the management of malaria. One such group, the Anamed (Action for nature and medicine www.ana

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Abbreviations

CC_{50} = 50% cytotoxic concentration (mammalian cells)
cDMEM = Complete Dulbecco's Modified Eagle's Medium
RPMI = Roswell Park Memorial Institute medium
CQ = Chloroquine
DMSO = Dimethyl sulfoxide,
HEK = Human embryonic kidney cell
HHA = Hepta-herbal *Agbo-iba*
HPMC = Hydroxypropyl methyl cellulose
IC_{50} = 50% inhibitory concentration (*P.falciparum*)
ITM = Improved traditional medicine
LCMS = Liquid chromatography mass spectrometry
MST = Mean survival time,
MTT = 3-(4,5-dimethylthiazol-2-l)-2,5-diphenl-2H-tetrazolium bromide
% H = Percent Haematocrit
% P = Percent Parasitaemia
PBS = Phosphate buffered saline
p.i = post-invasion
QPA = Quaternary protoberberine alkaloids
TCM = Traditional Complementary Medicine
TLC = Thin layer chromatography
UBH = University of Benin herbarium
UPLC = Ultra-performance liquid chromatography
SI = Selectivity index

med.org) has distributed seeds of the genetically modified artemisinin-rich varieties of the plant {*Artemisia annua* anamed (A3)} to over 240 of their partner organizations in developing countries for cultivation and use for the management of malaria (Willcox, 2011). The research team identified a dual function gene involved in both the formation of plant trichomes and in the synthesis of terpenes, such as artemisinin (Matías-Hernández et al., 2017). This *AaMYB1* gene promotes trichome formation in the leaves and artemisinin synthesis inside these trichomes. The importance of dual functionality of *AaMYB1 led* Soraya Pelaz, a CRAG researcher and senior author of the study to say, "*By manipulating this gene, we have managed to grow plants which contain much more artemisinin than their wild-type counterparts*". However, to preserve the life span of Artemisinin (which is the core component of Artemisinin-based Combination Therapies; the current frontline antimalarial), the WHO no longer supports the use of non-pharmaceutical forms of *A. annua* for treating the disease (World Health Organization, 2019). This therefore necessitates the scientific assessment of also other herbal formulations which could serve as alternatives.

Agbo-iba is a concoction of two or more than two medicinal plants. In the absence of a pharmacopeia, proper regulation and quality check, the contents of *Agbo-ibas* used for the management of malaria mainly in the western part of Nigeria differ from herbalist to herbalist. It is said to be as old as the Yoruba speaking group of the country whose history can be traced back to the 17th century. In Yoruba language (One of the three major languages spoken in Nigeria), *Agbo* means 'Medicine' while *iba* means 'fever'. Simply put, *Agbo-iba* means medicine against fever. As there was no proper documentation and knowledge was passed on from parents to children by word of mouth, the actual composition of the "original Agbo-iba" is unknown. Further, in a survey carried out by Conrad et al. (2013), several plants were noted as additions to the collections for making *Agbo-iba*, some of which have been previously recorded for the management of other diseases. Thus, several "*Agbo-ibas*" are currently used by different herbal practitioners in different parts of Nigeria for the management of malaria (Adebayo and Krettli, 2011; Iyamah et al., 2017; Nwabuisi, 2002; Okunji et al., 2012; Ukaga et al., 2006). As noted for traditional medicines used in other countries (Willcox and Bodeker, 2004), it is not known which *Agbo-iba* formula or preparation is the most effective in Nigeria. In the current study, a comprehensive molecular-biological assessment of a hepta-herbal *Agbo-iba* (HHA) commonly used for the management of malaria in Benin city, Nigeria is reported. The observation of the probable presence of magic bullet molecules in one plant and the facilitators of their oral bioavailability and improved pharmacokinetics in the other plants makes this study fascinating.

2. Materials and Methods

2.1. Collection & authentication of plant samples

HHA's constituent plants were collected with the help of Mrs Bosede Oluwafemi Benjamin, an expert herbal healer from their natural habitats around Benin city, Edo-State, Nigeria in February 2015. Temperature at the time of collection was 33 °C. They were then submitted to Dr Henry Akinnibosun of the Department of Plant Biology and Biotechnology, Faculty of Life sciences, University of Benin, Benin city, Nigeria for authentication (Table 1). Voucher specimens of all these plants were deposited in the school's herbarium. Further, the names of all the plants were checked with http://www.theplantlist.org on 22nd August 2017. The records derive from World Checklist of Selected Plant Families (WCSP) (data supplied on 2012-03-23).

2.2. Preparation of decoctions of HHA and constituent plants

HHA was prepared in the laboratory adopting the procedure given by the herbalist (see section 2.1 for details). However, in the absence of defined ratio of constituent plants *in Agbo-ibas,* there are gross batch-to-batch inconsistencies in different batches of *Agbo-iba*. The combined dry weight of all of the 7 plants given to us by the herbalist was calculated as 50.3 g. Given that the proportion of each HHA plant constituent may vary from one consumer to another at the point of purchase from the herbalist, we decided to make an equal by weight 1:1:1:1:1:1:1 mixture. Hence, 7 g each of the seven plant parts were air-dried and pulverized (air drying was done in shade and pulverization was done using a blender). The pulverized samples had particle sizes ranging from 0.5 cm to 2 cm. The sample of Pulverized HHA constituent plant parts weighing 49 g was placed in a 2 L beaker. To this, 1L of autoclaved water was added to avoid risk from any form of contamination since extracts were to be tested against the malaria parasite in culture. The beaker was covered using a glass petri dish which acted as a condenser to minimize evaporation and placed in a 5 L beaker containing water. This beaker in beaker assembly was in turn placed on a hotplate after which sample was boiled at 100 °C for 1 h (Supplementary Fig. 1). Thereafter it was cooled and filtered through a 0.45-μm filter, frozen and lyophilized. Conditions for freezing and lyophilization entailed placing sample solutions in freeze drying vessels after which they were properly frozen by slow rotation in a freezing bath chamber. When the sample had frozen to a thin film spread over a large surface area, the vessel was connected to a freeze drier set at temperature of −70 °C and vacuum of 0.120 mbar and dried over night to a constant weight. The dried extract was stored at −20 °C until use. For individual HHA plants decoctions, 7g/L of each of all seven plant parts were prepared separately by transferring 700 mg of each to individual 500 ml beakers containing 100 ml autoclaved water and covered with a glass petri dish. Each beaker was then placed in individual 1 L beakers containing water and heated at 100 °C for 1 h on a

Table 1
HHA constituent plants: names, plant parts & Voucher specimen numbers.

S/N	Botanical name/Family name	Common Name	Local name (in Yoruba language, Nigeria)	Traditional indications	Plant part used for HHA Preparation	Voucher Specimen Number
1	*Azadirachta indica* A. Juss. (Meliaceae)	Neem tree	*Dongoyaro*	Fever and malaria	Leaf	UBH – A286
2	*Cymbopogon citratus* (DC.) Stapf (Gramineae)	Lemon grass	*Kooko-oba*	Fever and malaria	Leaf	UBH – C451
3	*Alstonia boonei* De Wild. (Apocynaceae)	Cheese wood, pattern wood or stool wood	*Ahun*	Fever and malaria	Stembark	UBH – A343
4	*Anthocleista djalonensis* A. Chev. (Loganiaceae)	Cabbage tree	*Sapo*	Fever and malaria	Stembark	UBH – A428
5	*Nauclea pobeguinii* (Hua ex Pobég.) Merr. (Rubiaceae)	Pin cushion tree	*Egbesi*	Malaria	Rootbark	UBH – N180
6	*Annickia affinis* (Exell) Versteegh & Sosef (Annonaceae)	African yellow wood	*Awopa*	Fever and malaria	Stembark	UBH – A511
7	*Sorghum bicolor* (L.) Moench (Poaceae)	Sorghum; Great millet	*Poroporo okababa*	Anemia	Leaf	UBH – S468

Key: UBH = University of Benin, Nigeria Herbarium.

Table 2
Antiplasmodial activity, cytotoxicity and hemolytic activity of decoctions of HHA and its constituent plants.

Plant name	IC_{50} *Pf*3D7 (μg/ml)	CC_{50} HEK 293T (μg/ml)	S.I (CC_{50} HEK/ IC_{50} *Pf*3D7)	IC_{50} RBC Lysis (μg/ml)
A. indica	>100	>200	>2	nd
A. boonei	>100	>200	>2	nd
A. djalonensis	>100	>200	>2	nd
C.citratus	>100	>200	>2	nd
N. pobeguinii	>100	>200	>2	nd
A. affinis	1.49 ± 0.19	>200	>134.23	nd
S. bicolor	50 ± 7.43	>200	>4	nd
HHA	50 ± 4.22	>200	>4	388 ± 25.76

nd = Not determined.

hotplate. The cooled extracts of each of the seven samples was filtered through 0.45 μm filter, frozen and lyophilized. The aim of doing this was to know the contribution of each of the seven plants to HHA's activity against the malaria parasite *in vitro*.

2.3 In vitro parasite cultivation, maintenance and antiplasmodial assay

Parasites were cultivated *in vitro* by the prescribed method (Trager and Jensen, 1976) with minor modifications. Cultures were maintained in fresh O^+ human erythrocytes at 4% H in Roswell Park Memorial Institute (RPMI) medium supplemented with 0.2% sodium bicarbonate, 0.5% Albumax I, 45 μg/ml hypoxanthine, and 50 μg/ml gentamicin) at 37 °C under reduced O_2 using a mixed gas system of 5% O_2, 5% CO_2, and 90% N_2. For the antiplasmodial assay, parasite culture was synchronized at ring stage with 5% sorbitol. The synchronized culture was aliquoted to test samples containing 96-well plate at 2% Hematocrit (H) and 1% Parasitemia (P). Every test sample concentration (0, 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 μg/ml) was tested in triplicates. After 48 h of incubation at 37 °C under reduced O_2, smears were made and % parasitemia (% P) computed by microscopic evaluation of Giemsa stained smears. At least 3000 cells were counted for determination of % P in untreated control vs test sample treated cultures. Parasitized and non-parasitized cells were counted by using *Plasmodium* auto count 0.1 software developed by Ma et al. (2010) while 50% inhibitory concentration (IC_{50}) was determined by analysis of dose–response curves using non-linear regression in the IC Estimator-version 1.2 software (http://www.antimalarial-icestimator.net/MethodIntro.htm) (Free Software Foundation, Boston, MA, USA). Chloroquine (CQ) (40 μM) was used as zero growth positive control whereas untreated cells served as 100% growth control in all experiments.

2.4. Hemolysis study

Red blood cells (RBCs) were washed twice with 1X Phosphate buffered saline (PBS) (1600 rpm, 37 °C, 5 min). Thereafter, a 2% H was made in PBS. RBC suspension (96 μl) was aliquoted to wells of microtiter plate using multi pipette. To this, 4 μl HHA (at 0–500 μg/ml) was added to a final well volume of 100 μl followed by incubation (2 h, at 37 °C). Sample (0, 31.25, 62.5, 125, 250 and 500 μg/ml) was tested in triplicates. Microfuge tubes containing the samples were then centrifuged (1600 rpm, 37 °C, 10 min) and 90 μl supernatant transferred to fresh wells from which hemoglobin release was quantified at 415 nm using a microtiter plate reader (VersaMax ELISA Microplate Reader; wavelength range of 340–850 nm; 96 well plate format; monochromator). A standard calibration curve of % haemoglobin release was plotted against test sample concentration. Triton X-100 (20% v/v) was used as control for 100% hemolysis.

2.5. Measurement of in vitro cytotoxicity against mammalian cell line

The mammalian cell viability assay (Mosmann, 1983) was used to determine the cytotoxic effects of test samples. Human Embryonic Kidney (HEK) cells {obtained from American Type Culture Collection (ATCC)} were cultured in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 5% foetal bovine serum (FBS) until full confluence was achieved. Trypsinized cells (10^4 cells/100 μl) were thereafter seeded into 96-well plates in triplicates. The plates were incubated (12 h at 37 °C) after which spent medium (90 μl) was replaced with fresh cDMEM (86 μl) followed by the addition of 4 μl test samples and a 24-hr incubation at 37 °C in a 5% CO_2 incubator. Every sample concentration (0, 25, 50 and 100 and 200 μg/ml) was tested in triplicates. Dimethyl sulfoxide (DMSO) (10% v/v) added at zero time served as zero growth control while untreated cells at 36 h of incubation served as full growth control. Microculture tetrazolium (MTT) (5 mg/ml) was prepared in 1X PBS from which 20 μl (100 μg) was added to each well. The 96-well plate was then wrapped in aluminium foil and incubated (3 h, @ 37 °C). At the end of the incubation period, 120 μl corresponding to the amount of MTT (20 μl) plus 100 μl of test sample in DMSO/cDMEM was aspirated out. Then, the stop agent DMSO (200 μl) was added to each well so as to dissolve the formazan crystals formed. Formation of formazan, an index of cell growth was measured at 570 nm using a microplate reader (Versa Max). CC_{50} values were computed by analysis of dose–response curves using non-linear regression in the IC Estimator-version 1.2 software (http://www.antimalarial-icestimator.net/MethodIntro.htm) (Free Software Foundation, Boston, MA, USA). Selectivity index (SI) was calculated as CC_{50}HEK/IC_{50}Parasite.

2.6. Fluorescence study

Parasitized Red blood cells (2% H, 4% P) were transferred to microfuge tubes containing 1 ml cRPMI. To this, the test samples or positive control drugs were added to attain the required concentration (100 μg/ml). At the end of the 1 h incubation, tubes were centrifuged (1800 rpm @ 27 °C, 5 min) to remove supernatant after which the pellet was re-suspended in 500 μl of 1X PBS, wet mounts were made, and images captured with a fluorescence microscope (Nikon 50i).

2.7. TLC metabolite fingerprint

Samples (50 μg crude extracts; 2.5 μg pure compounds) dissolved in water or methanol (i.e. water extracts were dissolved in water while all other extracts were dissolved in methanol) were loaded on Merck kieselgel 60 PF254 TLC silica plates (0.2 mm thickness, 10 cm length) and allowed to dry at room temperature after which the plate was placed in a pre-saturated chromatographic tank containing the appropriate developing solvents. The chamber was closed, and the solvent allowed to run till about 90% of the plate length. Plate was thereafter taken out, solvent front marked, allowed to dry and visualized under long UV.

The developing solvents used were.

(i) Non-polar/Basic- Benzene: Ethanol: Ammonium hydroxide (BEA) (18:2:0.2 v/v/v)
(ii) Intermediate polar/acidic- Chloroform: Ethyl acetate: Formic acid (CEF) (10:8:2 v/v/v) and n-Butanol: Glacial acetic acid: Water (BGW) (14:3:4 v/v/v)
(iii) Polar/neutral- Ethyl acetate: Methanol: Water (EMW) (10:1.35:1 v/v/v)

2.8. LCMS/MS of samples

Samples (HHA and *A. affinis* decoctions) (12.5 mg/ml) were dissolved in water and filtered through a 0.22-μm syringe filter into autosampler vials. The analysis was thereafter performed using a Waters AQUITY-QSM Time of Flight mass spectrometer (Waters cooperation, India) with an electrospray ionization (ESI) source whose inlet was interfaced with a nanoAcquity UPLC configured for separation. Separations of samples were performed on an Acquity UPLC BEH C18 column (1.7 μM) maintained at 35 °C at a flow rate of 0.35 ml/min for a total run time of 24 min. Mass range was set at 100–800 m/z. Elution gradient used was 0.1% formic acid in water (A) and methanol (B) with analysis done in the positive mode. Gradient condition of 10–20% B (1–5 min), 20–80% B (5–7.5 min), 80–90% B (7.5–20 min) and 90%–10% B (20–25 min) was used. MassBank (https://www.massbank.jp/) database was used for the interpretation of results. Additionally, following isolation and chromatographic purification, m/z peak 352 was identified as Palmatine by proton NMR (Erhunse et al. unpublished data), while the other protoberberine alkaloids were identified by comparing with literature data.

2.9. Soxhlet extraction

The seven different air-dried plant parts that make up the herbal combo were pulverized separately with the aid of a blender. Thereafter, 30 g each of pulverized samples were thoroughly mixed to give a combined weight of 210 g. From this, 145 g was taken and subjected to polarity dependent extraction in a Soxhlet apparatus. The following solvents at their respective boiling points {Petroleum ether (40 °C–60 °C), Chloroform (61.15 °C), Ethyl acetate (77.1 °C), Acetone (56 °C) and Methanol (64.7 °C)} were used for 24 h (20 cycles for each solvent extract). While the residual marc obtained upon extraction with the preceding solvent was subjected to extraction with the next solvent in the series, the supernatant-extracts obtained from each extraction were concentrated using a rota-vapor following which they were frozen and lyophilized as described in section 2.2. The marc obtained after methanol extraction was dried in open air and divided into two equal halves. While one half was extracted with sterile cold water for 24 h, the other half was boiled at 100 °C on a laboratory hot plate for 1 h. Both extracts were frozen and lyophilized. Soxhlet extraction of *A.affinis* was done following a similar procedure by using 145 g of a pulverized stem bark of *A.affinis*.

2.10. Phytochemical screening

Methanol stock solutions (1 mg/ml) of samples were prepared and screened for phytochemicals using standard protocols (Harborne, 1973; Sofowora, 1984; Trease and Evans, 1978).

2.11. Acute toxicity study of HHA and potent fractions in mice

The limit test dose up and down procedure of the Organisation for Economic Cooperation and Development guideline 423 (OECD, 2001) was adopted for the acute toxicity testing of extracts. Three female nonparous Balb/C mice (6–8 weeks old) were randomly placed in a group and fasted for 6 h with water *ad libitum* after which they were fed 2 g/kg b.wt. of test extracts. After administering the extracts, animals were observed for signs of toxicity including mortality for a period of 14 days.

2.12. In vivo Antimalarial study of HHA and potent fractions in Balb/C mice infected with P. Berghei ANKA

2.12.1. Prophylactic study

The ability of HHA and potent fractions to protect against malaria was tested in a prophylactic study (Peters, 1965). Thirty-six male inbred Balb/C mice (6–8 wks old) weighing 20–22 g were obtained from the animal house facility of ICGEB, New Delhi and randomly distributed into six groups of six mice each. They were fed test extracts (100 mg/kg b. wt.) daily for a total duration of 3 days. Positive and negative controls were fed chloroquine (25 mg/kg b.wt.) and the vehicle (2% HPMC: 2% Tween 80) (1:1 v/v)} respectively. Thereafter mice were infected with 1 x 10^7 *P.berghei* ANKA intraperitoneally. After 3 days (72 h) of parasite inoculation i.e. on the 6th day since the beginning of prophylactic treatment, Giemsa stained smears were prepared from the lateral tail vein of each mouse and % P determined. Mean Survival time (MST) was computed after 30 days of monitoring for mice survival.

2.12.2. Peter's 4-day Suppressive study

The test extracts were triturated with vehicle {2% Hydroxypropyl methyl cellulose (HPMC): 2% Tween 80 (1:1 v/v)}. Thirty-six male Balb/C mice (6–8 weeks old weighing 20–22 g) were randomly distributed into six groups of six mice each and intraperitoneally inoculated with 1X10^7 *P. berghei* ANKA infected RBCs and orally fed with 100 mg/kg b.wt. extracts 3 h later. Positive control received 25 mg/kg b. wt. chloroquine while the negative control received the vehicle alone. Oral feedings were done daily for a total duration of 4 days. On the 4th day after treatment with extracts, smears were made form the lateral tail vein of all animals in the different groups, fixed in methanol and stained with Giemsa. These were thereafter subjected to microscopic determination of % P using a Nikon 50i microscope. Animals were kept for a total duration of 30 days to compute MST (Knight and Peters, 1980).

2.12.3. Curative study

The ability of HHA and potent fractions to cure established infections was assessed in a curative study as described by Ryley and Peters (1970). Briefly, thirty-six female Balb/C mice (6–8 weeks old) were inoculated with 1 X10^7 *P. berghei* ANKA intraperitoneally and distributed into six groups of six mice each. Seventy-two hr post infection, mice were fed 100 mg/kg b.wt. of test extracts. Positive control received 25 mg/kg b. wt. chloroquine while the negative control received the vehicle alone.

Extract administration was continued for the next 5 days. On the 8th day post infection, Giemsa stained smears were made to determine the % P. MST was monitored for a total duration of 30 days.

2.13. Ethical statement

Animal experiments were carried out in strict accordance with the recommendations of National Research Council of the National Academies Guide for the Care and Use of Laboratory Animals (National Research Council of the National Academies, 2011) with Institutional Animal Ethics Committee (IAEC) approval number ICGEB/IAEC/2017/01/MDD-1.

2.13. Statistical analysis

In vitro antiplasmodial activity, cytotoxicity and hemolysis assays were done in triplicates. IC_{50} and CC_{50} values were determined using the antimalarial IC Estimator-version 1.2 software (http://www.antimalarial-icestimator.net/MethodIntro.htm). All results have been expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to analyse the difference between means. P values < 0.05 were taken to be statistically significant.

3. Results

3.1. Biological assessment of decoctions of HHA and constituent plants: Antiplasmodial activity, cytotoxicity and hemolysis

The process for making HHA was mimicked in the lab by making a decoction of the constituent plants together as described by the herbalist as well as singly. As shown (Table 2), while HHA showed an antiplasmodial IC_{50} of 50 μg/ml, decoction with water done on each of the seven constituent plants individually showed the most promising activity with IC_{50} 1.49 μg/ml for *A. affinis*. The next in order of promise was *Sorghum bicolor* (IC_{50} 50 μg/ml) while all the other five plant samples had IC_{50} values > 100 μg/ml (Table 2).

HHA (IC_{50} 50 μg/ml) caused a moderate inhibition of parasite growth (Fig. 1A–C). Further, a weak green fluorescence was observed upon incubating trophozoite stage parasite with 100 μg/ml of the extract for 1 h (Fig. 1D).

SYBRgreen 1 assay is routinely used in our laboratory for the determination of IC_{50} of samples against the parasite. Although Fluorescence based SYBRgreen 1 assay adds to convenience for high throughput screens, it is prone to give false negative results with truly antiplasmodial but auto fluorescent samples and false positive results with samples which have quenchers of fluorescence but no antiplasmodial activity (Sharma et al., 2016). Therefore, results of SYBRgreen assay must be validated by microscopy of Giemsa stained smears. Against the weak staining of the parasite with HHA (Fig. 1D) it was interesting to see that upon incubation with each of the seven constituent plant extracts individually, it was *A. affinis* (Fig. 3D) alone which was contributing to the autofluorescence of HHA. As a result of this, the gold standard Giemsa-microscopy was relied upon for determining the true antiplasmodial activity of extracts.

Saponins can make the plasma membranes of cells permeable allowing in and out transport of both small and macromolecules (Baumann et al., 2000). *Plasmodium* parasites inhabit the RBC during their erythrocytic stages. Hence many plant extracts that contain saponins can cause RBC lysis leading to false positive antiplasmodial activity (Sparg et al., 2004). However, the 8-fold difference between the *Pf*3D7 IC_{50} of HHA (50 ± 4.22 μg/ml) and for RBC lysis (388 ± 25.76 μg/ml) (Table 2) suggests the hepta-herbal formulation to be a true antiplasmodial agent.

Compared to HHA (*Pf*3D7 IC_{50} 50 ± 4.22 μg/ml), *A. affinis* decoction (*Pf*3D7 IC_{50} 1.49 ± 0.19 μg/ml) turned out to be ~34-fold more potent (Fig. 2A–C). At 100 μg/ml, in contrast to the weak fluorescence seen with HHA decoction (Fig. 1D), *A. affinis* decoction showed a much

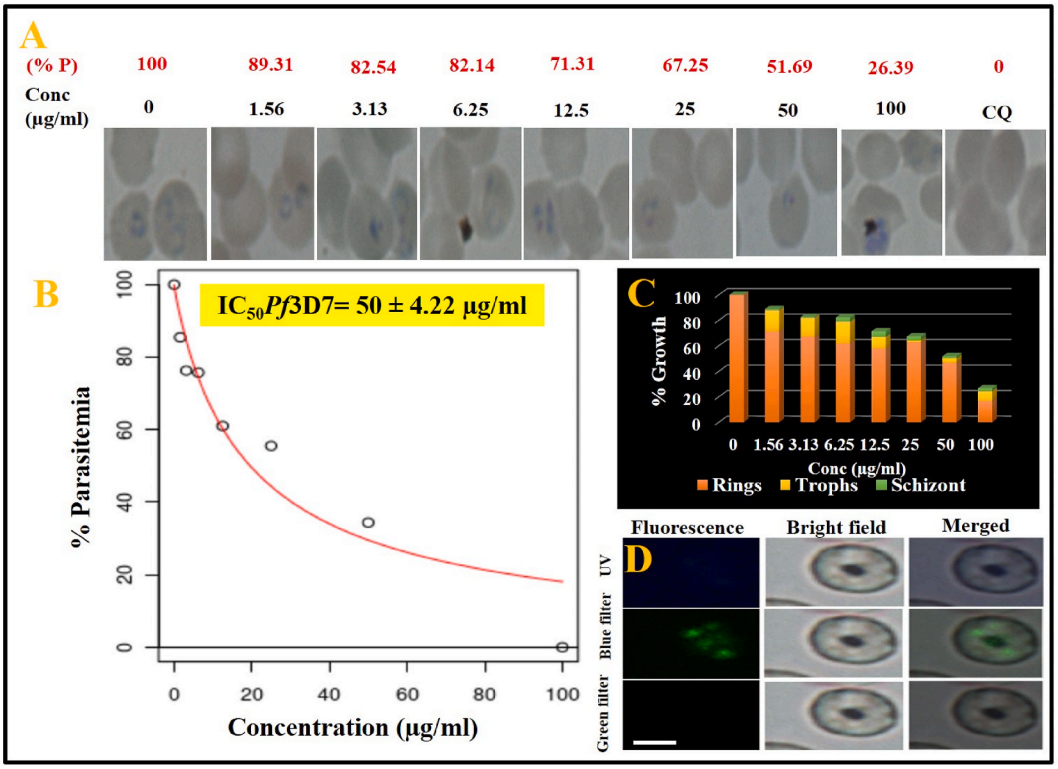

Fig. 1. Antiplasmodial activity, cytotoxicity, and fluorescence study of HHA decoction. A; Inhibition of parasite growth by HHA. B; Parasite growth curve upon incubation with HHA for 48 h C; Parasite stages under drug pressure from HHA. D; Parasite staining by 100 μg/ml of HHA for 1 h @ 37 °C. Scale bar = 2 μm. Microscopy of Giemsa stained smears was used to determine % parasitemia with >3000 cells counted for each concentration studied.

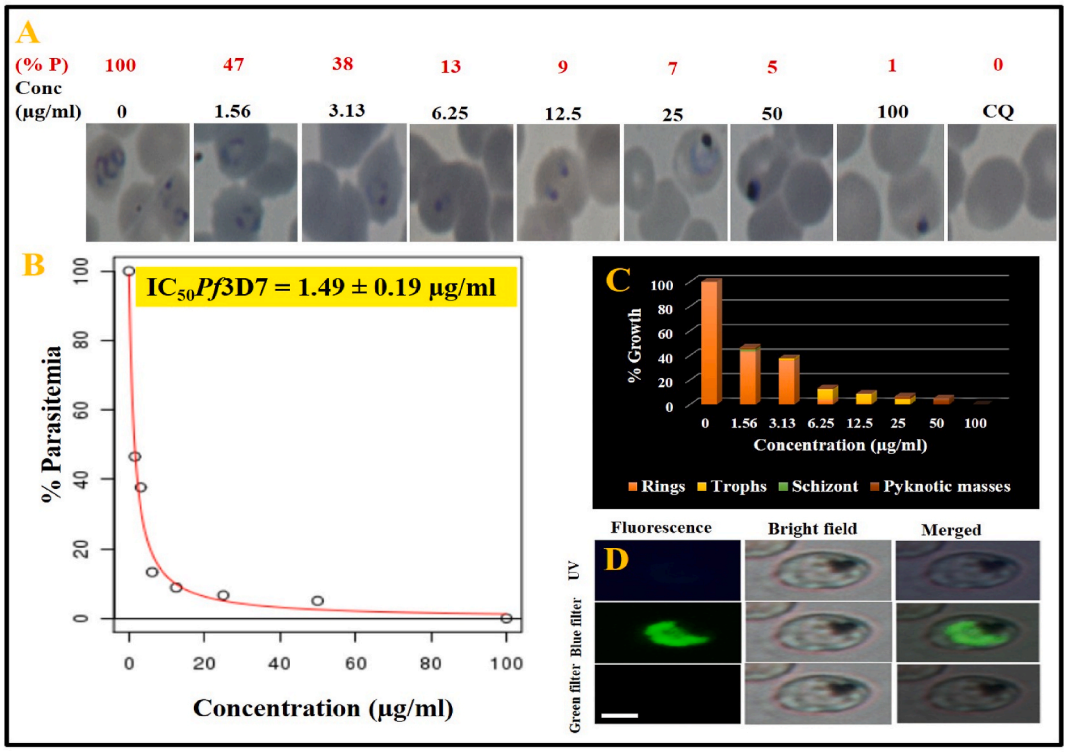

Fig. 2. Antiplasmodial activity, cytotoxicity and fluorescence study of *A. affinis* decoction. A; Inhibition of parasite growth by different concentrations of extract. B; Parasite growth curve after treatment by extract. C; Parasite stages under drug pressure. D; Parasite staining by 100 µg/ml of extract treated for 1 h @ 37 °C. Scale bar = 2 µm.

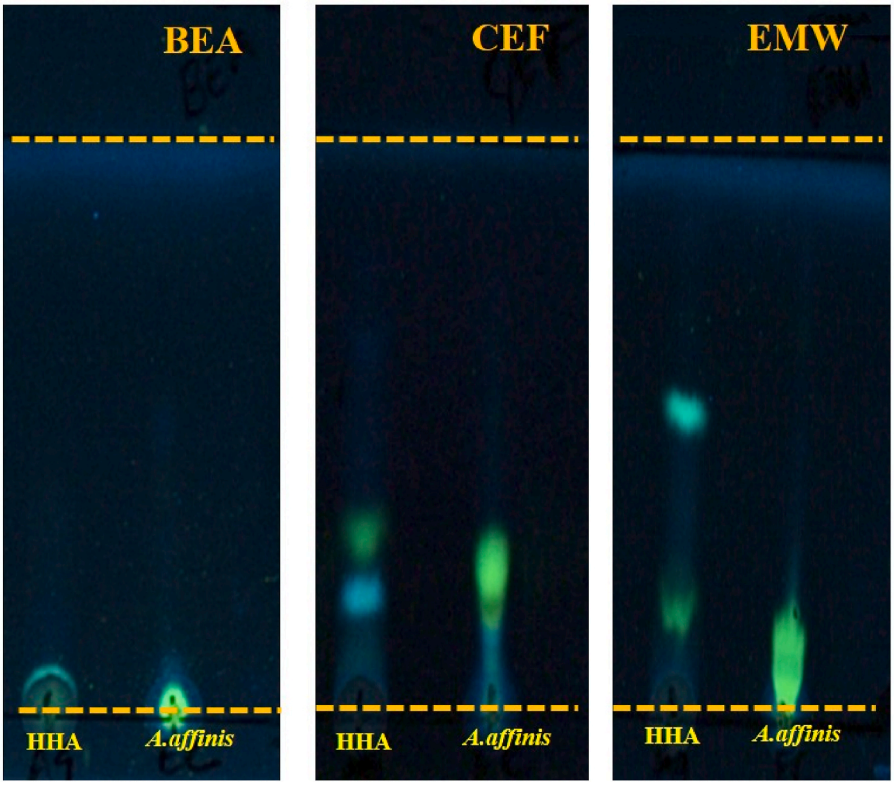

Fig. 3. TLC metabolite fingerprint of decoctions of HHA and *A. affinis* as visualized under long UV. Solvent systems used: Benzene: Ethanol: Ammonium hydroxide (BEA) (18:2:0.2 v/v/v); Chloroform: Ethyl acetate: Formic acid (CEF) (10:8:2 v/v/v); Ethyl acetate: Methanol: Water (EMW) (10:1.35:1 v/v/v). Amount loaded = 50 µg.

stronger fluorescent staining of parasite (Fig. 2D).

TLC metabolite fingerprint of decoctions of both *A. affinis* and HHA was done using different solvent systems and visualized under long UV (Fig. 3). The intermediate polar/acidic solvent system Chloroform: Ethyl acetate: Formic acid (CEF) (10:8:2) was found to be the best in separating the components of both decoctions.

HHA was then analysed by LC-MS/MS to identify the molecules that may be present in the extract (Fig. 4 & SI Table 1). LCMS/MS of HHA decoction revealed the presence of several metabolites including Palmatine (m/z 352): a protoberberine alkaloid with reported antiplasmodial activity to be the most abundant compound in the herbal combo (Fig. 4).

Given that *A. affinis* was the most potent component of the herbal mixture, it was also analysed by LC-MS/MS to identify the molecules present (Fig. 5 & SI Table 2). LCMS/MS metabolite fingerprint of *A. affinis* decoction revealed that it is rich in alkaloids (SI Table 2). And like HHA decoction, the protoberberine alkaloid Palmatine was observed to be the most dominant compound in *A. affinis* (Fig. 5 and SI Table 2). A comparison of intensity of peaks for Palmatine in HHA (Fig. 4) vs in *A. affinis* (Fig. 5) indicates the enormously higher amount of Palmatine in the latter vis a vis the former.

Although LCMS spectra of HHA and also *A.affinis* have shown that these two samples are rich in quaternary protoberberine alkaloids, this is not to mean that they are bereft of non - alkaloidal molecules or other alkaloids of a different subclass. Indeed, alkaloids of a subclass different from the protoberberine alkaloids detected were 7-alpha voacangine (identified in HHA), alpha-Codeimethine and Formosanine (both identified in *A.affinis*). Besides non-alkaloidal compounds detected included Olopatadine (2-[(11Z)-11-[3-(dimethylamino)propylidene]-6H-benzo [c][1]benzoxepin-2-yl]acetic acid), phenolic compounds like 4-(Trifluoromethyl) phenol and Caffeoyl quinic acid {(3R,5R)-1-[(E)-3-(3,4-dihydroxyphenyl) prop-2-enoyl]oxy-3,4,5-trihydroxycyclohexane-1-carboxylic acid} (all three identified in HHA) and 6-methoxy-7-hydroxycoumarin (a coumarin) and amidosulfuron (a phenol ether) (both identified in *A.affinis*). Besides, the number of unidentified low intensity peaks in the LCMS spectra of HHA and *A.affinis* are 35 and 17 respectively. Many of these peaks could be other phytochemicals which have been found to be present in these samples by phytochemical analysis (Table 5). In the sample of HHA, molecules coming from all seven plants would be present while in the sample of *A affinis*, the molecules coming from *A affinis* alone would be present. To lend credence to this, unlike *A. affinis* for which the protoberberine alkaloids were the only major peaks detected, in HHA, in addition to the protoberberine alkaloids, caffeoyl quinic acid, 7-alpa voacangine hydroxyindolenine and an unidentified compound with m/z of 637 were also major peaks detected (Figs. 4 and 5, supplementary Tables 1 and 2).

Given that only predicted compounds are gotten with LCMS, we subjected the crude methanol extract of *A.affinis* to acid-base-solvent extraction to isolate the alkaloidal fractions from which we were able to purify three protoberberine alkaloids using various chromatographic techniques (Erhunse et al. unpublished data). Of the three alkaloids isolated, both Berberine and Palmatine gave bright green fluorescence under long UV. Therefore, to be certain if both Quaternary Protoberberine Alkaloids (QPAs) are present in HHA, we ran a TLC of HHA along with pure Berberine and Palmatine standards using an intermediate polar/acidic solvent system (n-Butanol: Glacial acetic acid: Water) (14:3:4) which can separate both alkaloids. TLC metabolite fingerprint (Fig. 6) like LCMS (Fig. 4) confirmed Palmatine to be a protoberberine alkaloid present in HHA.

Solvent systems used: n-Butanol: Glacial acetic acid: Water (BGW) (14: 03: 04 v/v/v)

Amount loaded for HHA = 50 μg; Amount loaded for standards = 2.5 μg.

3.2. Biological assessment of HHA Soxhlet fractions: Antiplasmodial activity and cytotoxicity

The methods traditionally used for preparing herbal remedies may not be the best as regards efficacy. Therefore, other extraction methods were explored in a bid to improve on the antiplasmodial activity of HHA. By tinkering with HHA's preparation process using a polarity dependent sequential Soxhlet extraction method, fractions with better antiplasmodial activity than the herbal formula were gotten. The most potent of these fractions were Soxhlet methanol (SSM) (IC_{50} 3.79 ± 0.48 μg/ml), Soxhlet ethyl acetate (IC_{50} 4.14 ± 0.36 μg/ml) and Soxhlet acetone (IC_{50} 3.80 ± 0.08 μg/ml) with ~ thirteen-fold greater

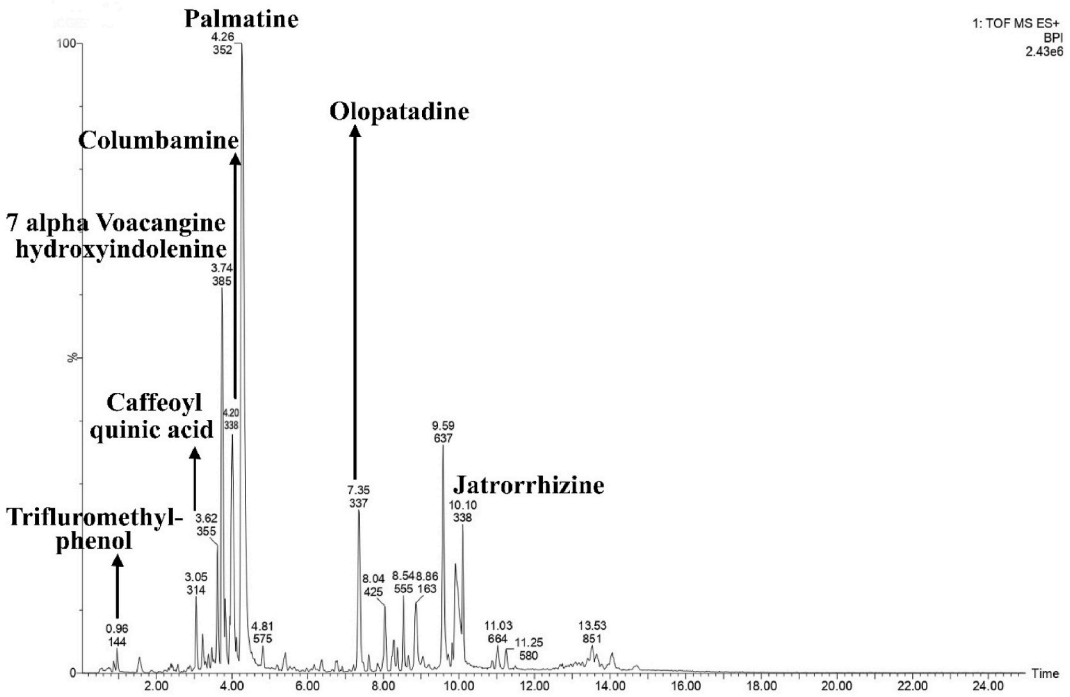

Fig. 4. LCMS spectra of HHA decoction indicating the major peaks.

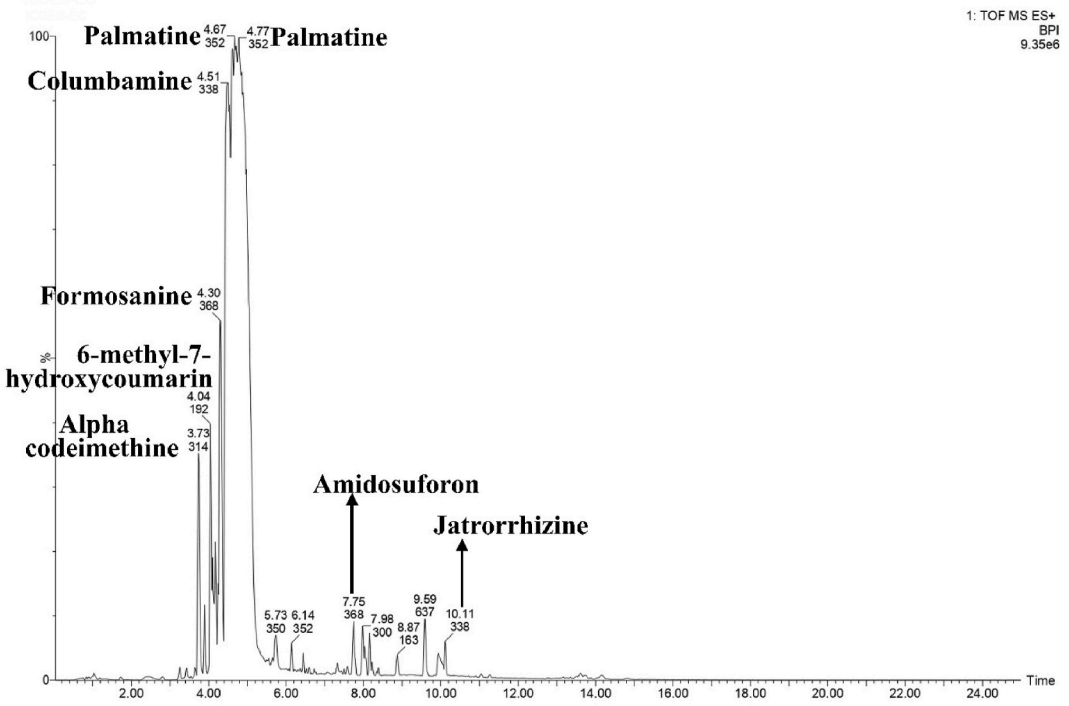

Fig. 5. LCMS spectra of *A. affinis* decoction indicating the major peaks. Note the dominance of protoberberine alkaloids.

Table 3
Antiplasmodial activity and cytotoxicity of polarity dependent sequential Soxhlet fractions of HHA.

HHA Soxhlet Fractions	Yield (g)	IC_{50} *Pf*3D7 (μg/ml)	CC_{50} HEK 293T (μg/ml)	S.I (CC_{50} HEK/ IC_{50} *Pf*3D7
Petroleum ether	1.84	nd	nd	–
Chloroform	1.79	8.04 ± 0.29	58.28 ± 6.25	7.25
Ethyl acetate	1.62	3.80 ± 0.36	>200	>52.63
Acetone	2.63	4.14 ± 0.08	>200	>48.31
Methanol	2.05	3.79 ± 0.42	>200	>52.77
Boiling water	2.99	>100	>200	>2
Cold water	2.23	>100	>200	>2

N. B– IC_{50}*Pf*3D7 of HHA decoction = 50 μg/ml.
Key: nd = Not determined (the petroleum ether fraction was insoluble in DMSO or water, consequently its antiplasmodial activity and cytotoxicity could not be determined). 145 g material was subjected to Soxhlet fractionation in a sequential manner such that the preceding marc was subjected to extraction with the succeeding solvent.

Table 4
Antiplasmodial activity and cytotoxicity of polarity dependent sequential Soxhlet fractions of *A. affinis*.

A. affinis Soxhlet Fractions	Yield (g)	IC_{50} *Pf*3D7 (μg/ml)	CC_{50} HEK 293T (μg/ml)	S.I (CC_{50} HEK/ IC_{50} *Pf*3D7
Petroleum ether	1.32	7.19 ± 0.66	90.00 ± 2.41	12.52
Chloroform	3.83	3.24 ± 0.21	80.00 ± 1.77	24.69
Ethyl acetate	1.03	3.76 ± 0.11	>200	>53.19
Acetone	0.87	1.38 ± 0.06	75.00 ± 1.92	54.35
Methanol	4.12	0.98 ± 0.04	105.00 ± 7.31	107.14
Boiling water	2.56	12.69 ± 0.82	>200	>15.76
Cold water	2.28	65 ± 2.92	>200	>3.07

145 g material was subjected to sequential Soxhlet fractionation in a manner that marc of the preceding solvent was subjected to extraction with the next solvent.

antimalarial parasite potency than is the case with HHA made using the traditional decoction method (IC_{50} 50 ± 4.22 μg/ml) (Table 2). IC_{50} values of other fractions ranged from (8.04 to >100 μg/ml) (Table 3). Soxhlet fractions of HHA were also assessed for selectivity (CC_{50} HEK 293T mammalian cell/IC_{50} *Pf*). Higher selectivity indices were seen for Ethyl acetate, Methanol and Acetone fractions than was the case with all the other fractions (Table 3)

Of all the HHA Soxhlet fractions obtained, the sequential Soxhlet methanol (SSM) fraction had the best antiplasmodial activity (IC_{50} 3.79 ± 0.48 μg/ml) (Fig. 7A–C). Intense parasite staining was also recorded upon treatment of trophozoite stage parasites with 100 μg/ml of extract @ 37 °C for 1 h (Fig. 7D). These results suggest that the proposed method of extraction is far better than the traditional method currently being used by the practitioners.

TLC metabolite fingerprint of HHA sequential Soxhlet fractions was done with three different solvent systems (non-polar through to polar) and visualized under long UV (Fig. 8). Better separation of the metabolites was achieved with the intermediate polar/acidic and polar/neutral solvents; Chloroform: Ethyl acetate: Formic acid (CEF) (10:8:2) and Ethyl acetate: Methanol: Water (EMW) (10:1.35:1) respectively (Fig. 8).

Key: Cl = chloroform fraction (Fr), Et = Ethyl acetate Fr, Ac = Acetone Fr, Me = Methanol Fr, Cw = Cold water Fr and Hw = Hot water Fr. TLC metabolite fingerprint of petroleum ether fraction could not be done as it was not soluble in methanol.

3.3. Biological assessment of A. affinis sequential Soxhlet fractions: antiplasmodial activity and cytotoxicity

In view of the fact that out of all seven HHA constituent plants, *A. affinis* decoction was the most potent (Table 2), pulverized *A. affinis* stem bark was subjected to a polarity dependent sequential extraction in a Soxhlet apparatus in the quest to obtain plant extracts with better antiplasmodial activity than HHA decoction. Just like in the case of HHA Soxhlet fractions, the Soxhlet methanol fraction of *A. affinis* was also found to be the most potent with an IC_{50} of 0.98 ± 0.04 μg/ml. IC_{50} values of other fractions (Petroleum ether, chloroform, acetone, ethyl

Table 5
Phytochemical composition of HHA, constituent plants and sequential Soxhlet methanol fractions of *A.affinis* and HHA.

	NC	PC	*A.indica*	*A.boonei*	*A.djalonensis*	*C.citratus*	*N.pobeguinii*	*S.bicolor*	*A.affinis*	*A.affinis* SSM fr	*HHA*	HHA SSM fr
Alkaloids	–	+++	–	+	–	–	+	+	+++	+++	+	+++
Flavonoids	–	nd	+	+	++	+	+	+	+++	+++	+	+++
Saponins	–	nd	–	–	–	–	–	–	–	–	–	–
Phenolics	–	nd	+	+	+	+	+	+++	+	+	+	+++
Terpenoids	–	nd	–	–	–	+	–	++	–	–	–	++
Steroids	–	nd	–	–	–	–	–	–	–	–	–	–
Tannins	–	nd	–	–	–	–	–	–	–	–	–	–
Quinones	–	nd	+	+	+	+	+++	–	+++	+++	++	+++
Cardiac glycosides	–	nd	–	–	–	–	–	–	–	–	–	–
Coumarins	–	nd	+	+	+	+	+	–	++	+++	+	–

Key: +++ = Very high, ++ = Moderate, + = Low, - = Absent. NC = Negative control, PC = Positive control, nd = not done, HHA SSM fr and *A.affinis* SSM fr represent Sequential Soxhlet methanol fractions of HHA and *A.affinis* respectively.

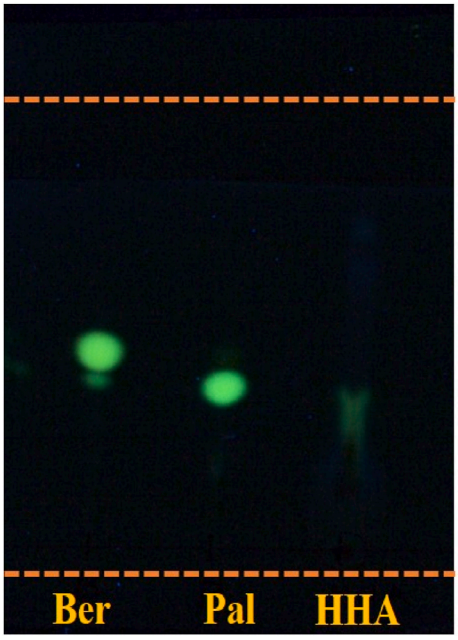

Fig. 6. TLC Metabolite fingerprint of HHA with Berberine and Palmatine standards as visualized under long UV. Rf of berberine = 0.55; Rf of Palmatine = 0.45; Rf of green fluorescing spot in HHA = 0.44. This confirms Palmatine to be the QPA in HHA. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

acetate, boiling water and cold water) ranged from (1.38–65 μg/ml) (Table 4). Soxhlet fractions of *A. affinis* were thereafter assessed for selectivity (CC_{50} HEK 293T mammalian cell/IC_{50}*Pf*). CC_{50} HEK293T ranged from (75 to >200 μg/ml) while selectivity index (S.I) ranged from 4 to 107 with the sequential Soxhlet methanol (SSM) fraction of *A. affinis* being the most selective (CC_{50} HEK293T = 105 μg/ml; S.I = 107) for the parasite (Table 4).

While the sequential Soxhlet methanol extract of *A. affinis* (IC_{50} 0.98 ± 0.04 μg/ml) was found to halt the parasite's growth at the trophozoite stage at 3.13 μg/ml (Fig. 9A–C), the sequential Soxhlet methanol fraction of HHA could achieve this feat only at 25 μg/ml (Fig. 7) strongly supporting that *A. affinis* must be the most potent inhibitory plant contributing to the efficacy of the HHA extract. Intense staining of the parasite was also observed upon treatment of trophozoite stage parasite with the *A. affinis* extract (Fig. 9D).

Like the Soxhlet fractions of HHA, better TLC separations were obtained also for *A. affinis* Soxhlet fractions with the intermediate polar/acidic and polar/neutral solvent systems Chloroform: Ethyl acetate: Formic acid (CEF) (10:8:2) and Ethyl acetate: Methanol: Water (EMW) (10:1.35:1) (Fig. 10).

Images were captured under long UV. Solvent system used Solvent systems used: Benzene: Ethanol: Ammonium hydroxide (BEA) (18:2:0.2 v/v/v); Chloroform: Ethyl acetate: Formic acid (CEF) (10:8:2 v/v/v); Ethyl acetate: Methanol: Water (EMW) (10:1.35:1 v/v/v). Amount loaded = 50 μg.

Key: Pe = Petroleum ether fraction (fr), Cl = chloroform fr, Et = Ethyl acetate fr, Ac = Acetone fr, Me = Methanol fr, Cw = Cold water fr and Hw = hot water fr.

Basic phytochemical screening of HHA, constituent plants and sequential soxhlet methanol fractions of HHA and *A.affinis* suggests that they are rich in phytometabolites of different classes (Table 5).

3.4. Acute toxicity study

No overt signs of toxicity (death inclusive) were noticed for mice in all treatment groups. Apart from itching around the mouth region experienced by mice treated with Soxhlet methanol extracts of HHA and *A. affinis* (which could be a result of the bitter taste of the protoberberine alkaloids), and changes in fur appearance (which could be a result of anxiety) by mice treated with HHA decoction, no other signs of toxicity were recorded. More so, these symptoms were observed only up to 24 h of drug administration (Table 6).

Similarly, there was no significant difference in mean weights of mice before and after treatment with the extracts (Fig. 11). Since no mortality was recorded, the extracts were deemed to be safe up to 2 g/kg b.wt. as recommended by the OECD (OECD, 2001).

SSM = sequential Soxhlet methanol fraction, SSA = sequential Soxhlet acetone fraction.

3.5. Antimalarial study

3.5.1. Prophylactic study

CQ caused a 73.98% parasite suppression. Of all the extracts tested, HHA decoction showed the best ability to prevent parasite growth suppressing parasitemia by 56.76%. The next in line were HHA sequential Soxhlet acetone (47.13%) and methanol fractions (38.67%) followed by *A. affinis* sequential Soxhlet methanol extract (32.46%). This was reflected also in the ability of the test samples to increase the lifespan of the treated animals. CQ and HHA decoctions were the most successful in preventing death from the parasite as MSTs in both groups were about 23 days whereas an MST of about 12 days was recorded for the vehicle treated group (Table 7).

3.5.2. Suppressive study

The ability of extracts to suppress the growth of parasite was found (Table 7) by employing the Peter 4-day suppressive test. Microscopy of Giemsa stained smears revealed a 100% suppression of parasite growth by CQ. Despite HHA decoction being the least potent extract in *in vitro* studies (IC_{50} *Pf*3D7 50 ± 4.22 μg/ml), it was the best of the test extracts at suppressing the growth of the parasite by 56.76% *in vivo*. *A. affinis*

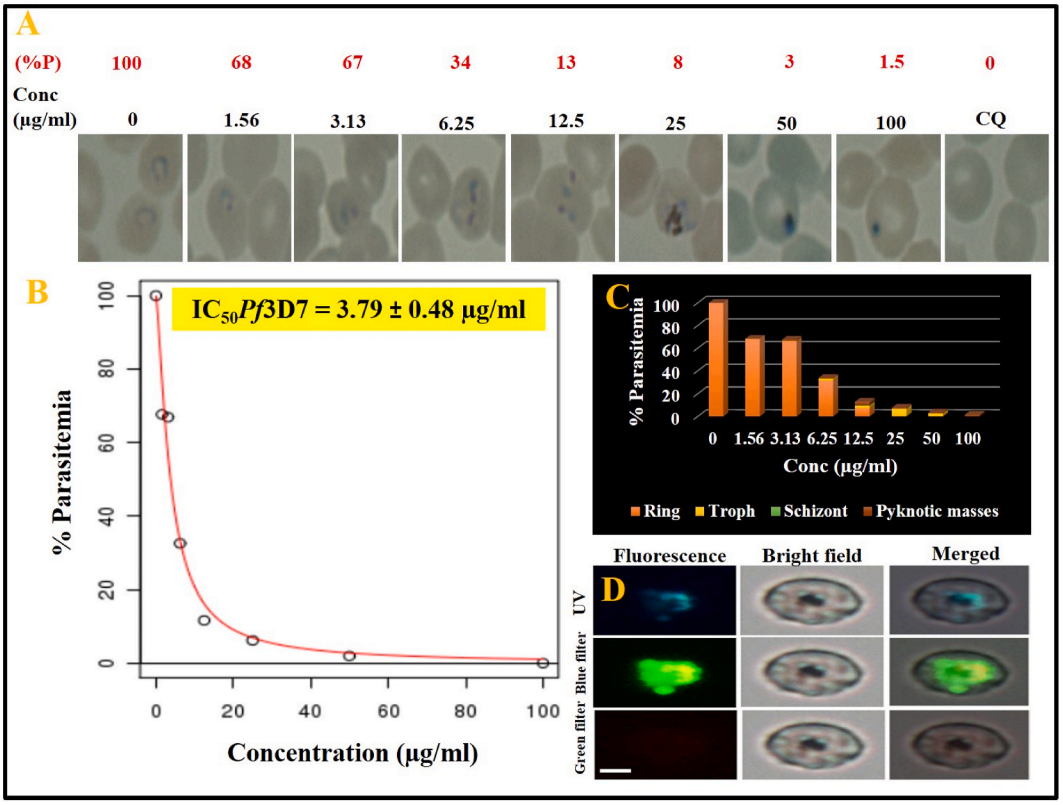

Fig. 7. Antiplasmodial activity, cytotoxicity, and fluorescence study of HHA sequential Soxhlet methanol extract. A; Giemsa microscopic evaluation of inhibition of parasite growth by different concentrations of extract. B; Quantitative data of the experiment described in A. C; Relative Parasite cell cycle stages under different concentrations of extract. D; Parasite staining by 100 µg/ml of extract incubated for 1 h @ 37 °C. Scale bar = 2 µm.

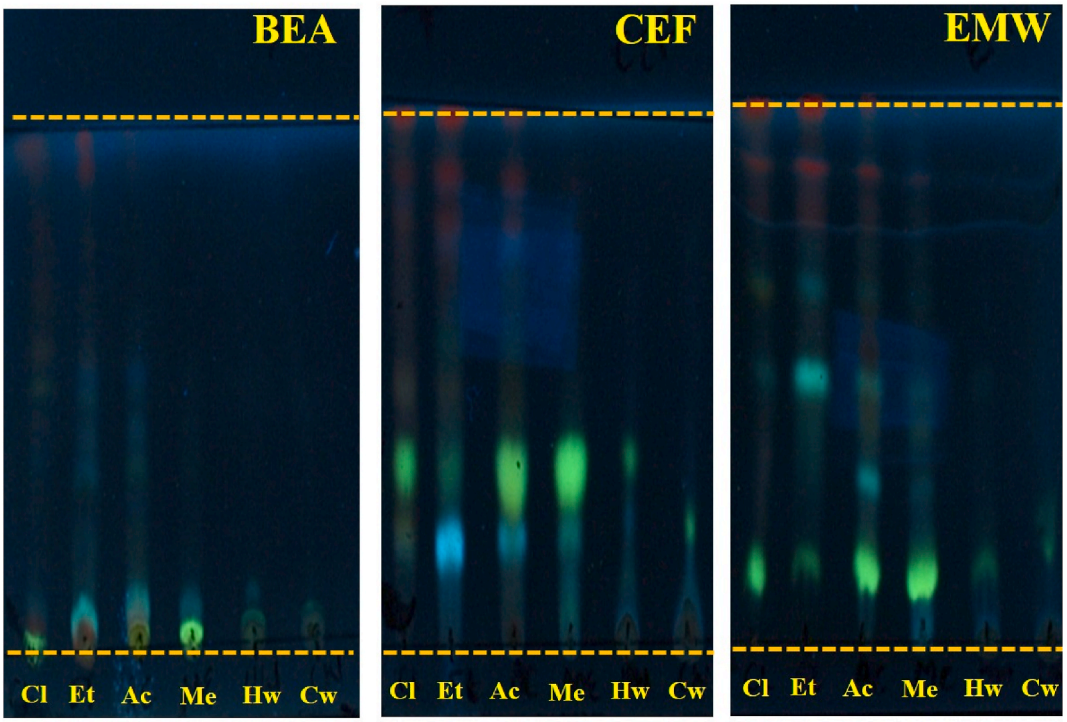

Fig. 8. TLC metabolite fingerprint of HHA sequential Soxhlet fractions. Images were captured under long UV. Solvent systems used: Benzene: Ethanol: Ammonium hydroxide (BEA) (18:2:0.2 v/v/v); Chloroform: Ethyl acetate: Formic acid (CEF) (10:8:2 v/v/v); Ethyl acetate: Methanol: Water (EMW) (10:1.35:1 v/v/v). Amount loaded = 50 µg.

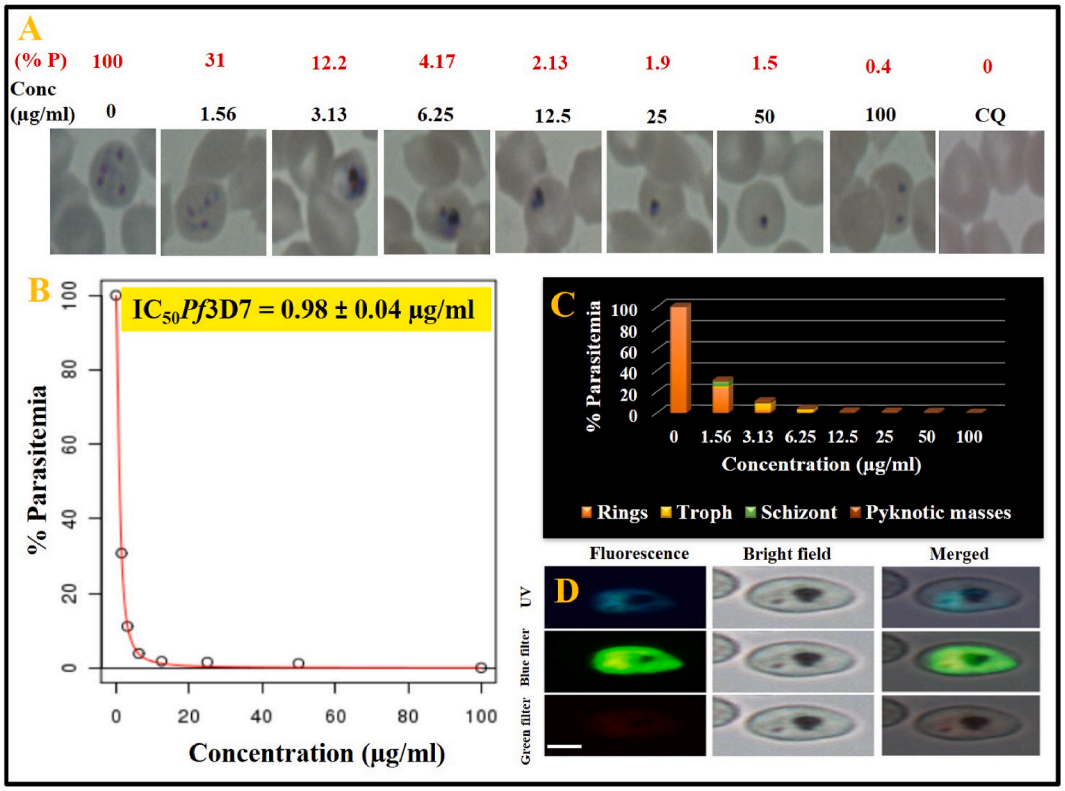

Fig. 9. Antiplasmodial activity, cytotoxicity, and fluorescence study of *A. affinis* sequential Soxhlet methanol extract. A; Inhibition of parasite growth by different concentrations of extract. Images shown are from smears made at 48 h of culture. B; Parasite growth curve after treatment with different concentrations of extract. C; % Parasitemia and frequency of Parasite cell cycle stages at different concentrations of extract. D; Parasite staining by 100 µg/ml of extract incubated for 1hr @ 37 °C. Scale bar = 2 µm.

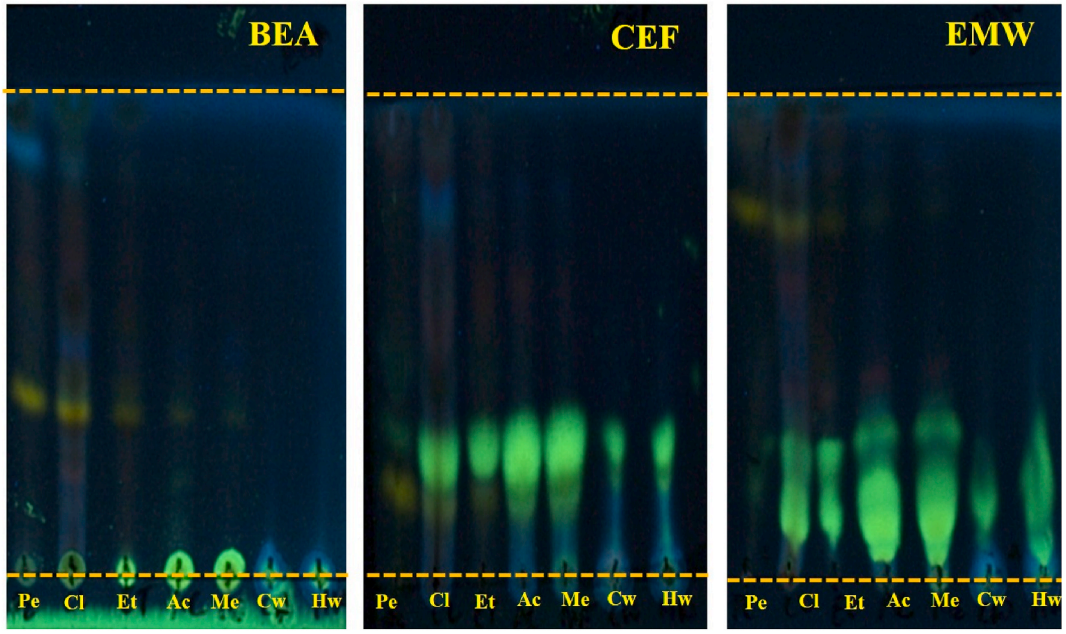

Fig. 10. TLC metabolite fingerprint of *A. affinis* Soxhlet fractions.

sequential Soxhlet methanol extract suppressed the parasite growth by 32.46% despite being ~50 fold more potent than HHA decoction *in vitro* (IC_{50} *Pf*3D7 0.98 ± 0.04 µg/ml). Similarly, HHA Soxhlet methanol extract which was ~14 fold more potent than HHA decoction *in vitro* (IC_{50} *Pf*3D7 3.78 ± 0.48 µg/ml) gave the least parasite suppression (23.05%) of all the test extracts. This also played out in survival time of the animals as the MSTs for mice fed CQ, HHA and *A. affinis* sequential Soxhlet methanol fraction were 24.2 ± 6.02, 14.8 ± 9.34 and 9 ± 1.41 days respectively.

Table 6
Acute Toxicity Study of HHA decoction and potent Soxhlet fractions of HHA and *A. affinis* @ 2 g/kg b.wt.

	Mortality	Toxic symptoms				
Group ID	Dead/ Treated mice	30 mins-3 h	4 h	12hr	24 h	Day 2–14
Control	0/3	None	None	None	None	None
HHA decoction	0/3	Rapid heart rate followed by sleep with one mouse displaying stereotypies	Withdrawal from food and changes in fur appearance	Withdrawal from food and changes in fur appearance	Change in fur appearance	None
HHA SSM fraction	0/3	Itching around mouth region followed by sleep	None	None	None	None
HHA SSA fraction	0/3	Lethargy	None	None	None	None
A. affinis SSM fraction	0/3	Stereotypies and itching around mouth region	None	None	None	None

Key: SSM = sequential Soxhlet methanol; SSA = sequential Soxhlet acetone.

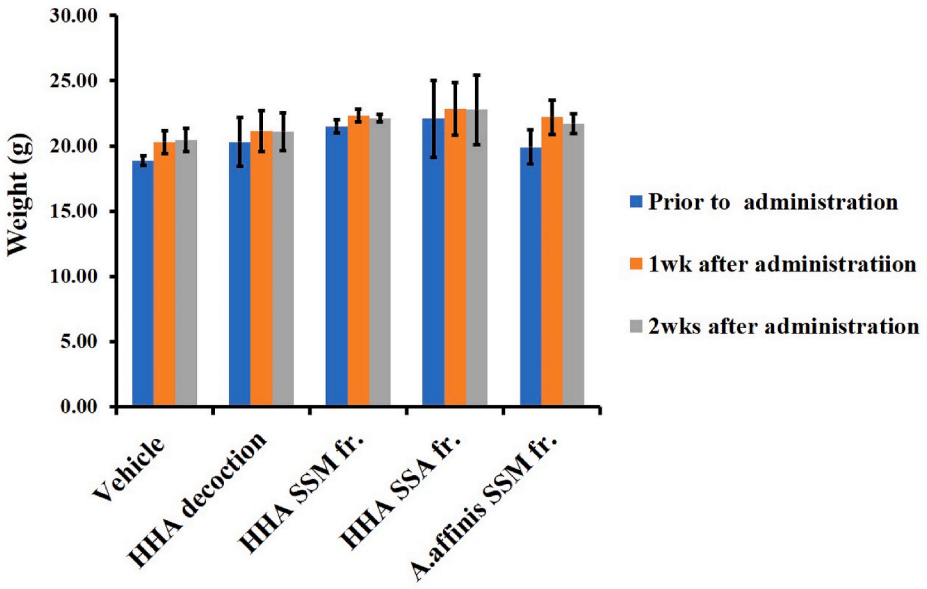

Fig. 11. Mean weights of 3 animals determined before the administration of test substances and weekly thereafter. Results are presented as mean ± SD.

Table 7
In vivo antimalarial activity of HHA, potent Soxhlet fractions of HHA and *A. affinis*.

Sample ID	% P Suppression (Prophylactic study, day 3 p.i)	MST (days)	% P Suppression (Suppressive study, day 4 p.i.)	MST (days)	% P Suppression (Curative study day 8 p.i)	MST (days)
Untreated (-ve control)	00.00 ± 00.00	12.40 ± 1.52	00.00 ± 00.00	9.20 ± 0.84	00.00 ± 00.00	11.20 ± 0.84
CQ (+ve control)	73.98 ± 2.09***	21.80 ± 7.73	100 ± 00.00***	24.2 ± 6.02	100 ± 0.00***	26.60 ± 5.08
HHA SSM fr	38.67 ± 9.88*	23.60 ± 2.41	23.05 ± 9.68*	8.20 ± 2.77	24.41 ± 3.43*	11.60 ± 5.81
HHA SSA fr	47.13 ± 5.57*	21.60 ± 2.30	46.92 ± 8.25**	8.20 ± 0.45	44.63 ± 3.27**	17.80 ± 8.98
A.affinis SSM fr	32.46 ± 0.86*	22.20 ± 2.86	42.50 ± 4.00**	9.00 ± 1.41	00.00 ± 00.00	10.40 ± 4.28
HHA Decoction	56.76 ± 10.86**	23.60 ± 4.56	58.20 ± 6.20**	14.8 ± 9.34	44.45 ± 15.65**	16.20 ± 4.60

Results are presented as mean ± SD. Samples were tested at 100 mg/kg b.wt., CQ was tested at 25 mg/kg b.wt.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ in comparison with untreated vehicle control.
Key: CQ = chloroquine, HHA = Hepta-herbal *Agbo-iba*, SSM = sequential Soxhlet methanol fraction, SSA = sequential Soxhlet acetone fraction.

3.5.3. Curative study

Despite being very potent *in vitro* (IC_{50} *Pf*3D7 0.98 μg/ml), the sequential Soxhlet methanol extract of *A. affinis* was inactive in mice model of the disease causing no suppression of % parasitemia. This was followed by HHA sequential Soxhlet methanol extract which suppressed parasite growth by 24.41%. HHA decoction on the other hand was the most potent extract tested causing a 44.63% suppression of parasite growth. The MSTs were 11.2 ± 0.84 days (Vehicle treated), 26.6 ± 5.08 days (CQ), 10.4 ± 4.28 days (*A.affinis*) and 16.2 ± 4.60 days (HHA decoction).

4. Discussion

The present study assessed the potential of a Nigerian HHA for managing malaria. We report that of the seven HHA constituent plants, only *A.affinis* (IC_{50} 1.49 ± 0.19 μg/ml) and *S.bicolor* (IC_{50} 50 ± 7.43 μg/ml) have antiplasmodial activity and with ~34 fold better activity for *A. affinis* over *S.bicolor*, our investigation suggests that *A.affinis* is the major contributor of the antiplasmodial activity of the herbal combo of HHA. The *in vitro* antiplasmodial activity of solvent extracts of four (*A. indica*, *A. djalonensis*, *C. citratus*, and *A. boonei*) of the other HHA component plants has previously been reported: (ethanol extracts of *A.boonei* IC_{50} FCB1 12.3 μg/ml and *A.djalonensis* >50 μg/ml (Guédé et al., 2010), methanolic extract of *A.indica* IC_{50} *Pf*3D7 & Dd2 < 5 μg/ml (Tahir et al., 1999), ethanol and water extracts of *C.citraus* IC_{50} *Pf*3D7 (μg/ml) 28.75 and 723.30 respectively, IC_{50} *Pf*Dd2 (μg/ml), 54.84 and 141.00 respecively) (Tarkang et al., 2014a). The report by Tarkang et al. (2014a) is consistent with our observation for the water extract of *C.citratus* (i.e. IC_{50} > 100 μg/ml). Further, in our study, decoctions of the other three plants (*A. indica*, *A. djalonensis* and *A. boonei*) and *N. pobeguinii* were inactive (IC_{50} >100 μg/ml) against *P. falciparum* in culture. The inactivity of these other extracts may be due to the method of preparation which could have negatively affected their activity by reducing the amounts of bioactive principle(s) present (Neag et al., 2018; Van Der Kooy and Verpoorte, 2011). Indeed, Gedunin (IC_{50} 1 μM) has earlier been isolated from the methanol extracts of fruits and bark of *A indica* (Khalid et al., 1989). However in the HHA studied by us, it is decoction of the leaves of *A.indica* for which the IC_{50} found by us is > 100 μg/ml. This difference of source material as well as solvent and method of extraction could be the reason why we found no antimalarial promise in *A indica*. Also, literature records Betulinic acid from stem bark of *Alstonia boonei* as potent *in vitro* (Steele et al., 1999). However, there are discrepancies in the *in vivo* reports by Steele et al. (1999) and Olanlokun et al. (2021). While the later reports it to be very potent even at a low dose of 25 mg/kg b.wt., the former reports it to be ineffective at suppressing malaria parasite growth at 250 mg/kg b.wt. Betulinic acid was isolated from the chloroform fraction of a methanol extract as against the water decoction studied by us. Further, the fact that 2.3 g Betulinic acid was isolated from 2 Kg of *Alstonia boonei* stem bark indicates that it is present in the miniscule amount of 0.001% (Olanlokun et al., 2021). No wonder that at such low levels, activity may not be picked up in a crude extract especially when the intrinsic antiplasmodial activity of the molecule in question is moderate {IC_{50} against chloroquine resistant (K1) and sensitive (T9-96) *Plasmodium falciparum* were found to be 19.6 μg/mL and 25.9 μg/mL, respectively} (Steele et al., 1999). According to the classification of plants based on potency, plant extracts with IC_{50} < 5 μg/ml are highly active, those with IC_{50} 5–15 μg/ml are promising, the ones with IC_{50} 15–50 μg/ml are moderately active and those with IC_{50} > 50 μg/ml are inactive (Kraft et al., 2003; Pink et al., 2005). Therefore, while HHA and *S. bicolor* with IC_{50} of 50 μg/ml are moderately active, *A. affinis* with IC_{50} 1.49 ± 0.19 μg/ml is highly active. *A. affinis* thus has a ~34-fold higher antiplasmodial activity than the combo HHA. Our study thus reports for the first time the antiplasmodial activity of decoctions of *A.affinis* and *S.bicolor*. *A. affinis* is closely related to *Annickia chlorantha*. Both these plants have been jointly referred to as *Enantia chlorantha* in the literature (Olivier et al., 2015). Although *E.chlorantha* is known to possess antiplasmodial activity (Boyom et al., 2009; Imieje et al., 2017; Vennerstrom and Klayman, 1988), the information available in the literature cannot be said to belong to either *A. affinis* nor *A. chlorantha* (Olivier et al., 2015). Thus, in our study, for the first time, we have taken care to do a DNA barcoding of this sample (Erhunse et al. unpublished data) and observed it to share an 88% sequence identity with *Annickia chlorantha*. This sequence was deposited in the repository https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA648657&0=acc_s%3Aa with the accession number **PRJNA648657** after which the sample was authenticated as *Annickia affinis* by a botanist. We tinkered with the method of preparation by subjecting dried samples of each of the seven plants parts in HHA taken together and *A. affinis* plant part taken singly to exhaustive extraction in a Soxhlet apparatus. This impacted the type/amounts of phytochemicals as shown in the results of phytochemical screening (Table 5). Successive Soxhlet methanol extraction of HHA resulted in better activity (IC_{50} 3.79 ± 0.48 μg/ml) than HHA water decoction (IC_{50} 50 ± 4.22 μg/ml) making the extraction method-based difference in activity to be ~13 fold. With regard to *A. affinis,* where the IC_{50} values were 1.49 ± 0.19 μg/ml (water decoction) and 0.98 ± 0.04 μg/ml (Methanol Soxhlet extraction), this difference was only 1.5-fold.

Intriguingly, the intensity of parasite staining done at identical concentration (100 μg/ml) and conditions (2% H, 4% P in 1 ml cRPMI) {Fig. 1D (HHA decoction, IC_{50} 50 ± 4.22 μg/ml), 2D (*A.affinis* decoction IC_{50} 1.49 ± 0.19 μg/ml), 7D (Soxhlet HHA IC_{50} 3.79 ± 0.48 μg/ml) and 9D (Soxhlet *A.affinis* IC_{50} 0.98 ± 0.04 μg/ml) was directly proportional to the antiplasmodial activity of the extracts. This suggested that the compound(s) responsible for the antiplasmodial activity of the extracts may be responsible for parasite staining.

Interestingly, the fact that observed antiplasmodial potency was not due to non-specific toxicity is evident from both *in vitro* high selectivity indices (Tables 2 and 4) and from the results of the acute toxicity study (Table 6 and Fig. 11) which suggest that HHA and indeed all the tested extracts are non-toxic to Balb/C mice when doses as high as 2 g/kg b.wt. are administered orally.

In the suppressive study, HHA showed a ~1.7-fold better ability to suppress parasite growth (56.76% parasite suppression) than the sequential Soxhlet methanol extract of *A. affinis* (32.46% parasite suppression). Against the fact that a significant synergy is said to occur with a ≥ 2-fold increase in activity, lower levels of synergy are considered as significant for herbal medicines (Rasoanaivo et al., 2011). Even greater synergy was observed in a curative study, where at 100 mg/kg b.wt. while the extract of HHA decoction suppressed parasitemia by 44.45 %, the sequential Soxhlet methanol extract of *A. affinis* was inactive (0% parasite suppression) indicating that there was no correlation between *in vitro* antiplasmodial activity and *in vivo* antimalarial activity.

LCMS revealed that *A. affinis* decoction is dominated by alkaloids with the protoberberine alkaloid Palmatine being the most abundant compound. The abundance of palmatine was also recorded in HHA. Since LCMS based identities of compounds are at best good predictions that need further confirmation, following systematic procedures (Grycová et al., 2007), we isolated alkaloids from *A. affinis* and by using proton NMR confirmed one of the alkaloids to be Palmatine which happened to be the most dominant compound (Erhunse et al. unpublished data). This strongly suggests that Palmatine is a major contributor to both the *in vitro* antiplasmodial and the *in vivo* antimalarial activity of HHA. Previously, berberine; the precursor for palmatine has been reported to stain human mast cells (Timoshanko et al., 2006), as well as cow sperm and oocyte (Reyes et al., 2004). Being a structural analog of berberine and sharing its fluorescence characteristics, Palmatine is very likely responsible for parasite staining by the extracts studied by us.

In traditional medicine, whole plants or mixtures of plants are used rather than isolated compounds. Notwithstanding much better *in vitro* antiplasmodial activity of *A. affinis* Soxhlet methanol extract than was the case with HHA decoction, the better ability of HHA decoction to suppress parasite growth as compared to the sequential Soxhlet methanol extract of *A. affinis* (Table 7) *in vivo* suggests an improvement in the pharmacokinetics of QPAs in HHA by other phytoconstituents in the herbal combo. We surmised that inhibitors of drug transporters may exist in HHA. It is noteworthy that QPAs are substrates of Permeability glycoprotein (P-gp) (Zhang et al., 2011). The Pgp transporter is highly expressed on the luminal surface of enterocytes where it limits the concentration of substrate drugs when ingested via oral routes. This could have impacted the poor activity of *A. affinis in vivo* despite being very potent *in vitro*. One of HHA's component plants; *N.pobeguinii* contains resveratrol and its derivative; resveratrol β-D-glucopyranoside (Kuete et al., 2015; Seukep et al., 2016). Thus, resveratrol, a

non-flavonoid polyphenol which is a confirmed Pgp inhibitor (Bedada et al., 2015; Li et al., 2016), may have improved the bioavailability of QPAs in HHA by suppressing Pgp's expression at the transcription level (Ganesan et al., 2021). Further, plant secondary metabolites are known to possess varied pharmacologic activities (Velu et al., 2018). Previous studies reported the presence of phenolics, flavonoids, and alkaloids in *A.boonei*, *A.djalonensis*, *A.affinis* and *N.pobeguinii* (Dawodu et al., 2014; Erhunse et al., 2016; Njoya et al., 2017). These were confirmed by our experimental findings (Table 5) and may have contributed to the antimalarial activity of HHA *in vivo*. Similar observations of synergy have been reported with single herbs as well as Multi-herbals. For example, dried *Quassia amara* leaf tea is much more active *in vivo* than *in vitro* (Bertani et al., 2007). Likewise, despite Nefang (a polyherbal formulation used in Cameroon) being inactive *in vitro* (Tarkang et al., 2014a), it was better at suppressing parasitemia *in vivo* than its most potent plant constituent *Psidium guajava* (Tarkang et al., 2014b). The mechanism for better *in vivo* than *in vitro* activity was however not studied by these researchers. Our observation is also reminiscent of the studies done by Elfawal et al. (2015) which showed the greater promise of whole *Artemisinin annua* plant than Artemisinin. In the polyherbal system studied by us, it seems that the magic bullets (QPAs) are largely provided by one plant (*A.affinis*) while the facilitators of orally given QPA's bioavailability are provided by the other herbs. Thus, the paradox of greater *in vitro* activity but poorer *in vivo* antimalarial activity of *A.affinis* versus poorer *in vitro* activity but greater *in vivo* antimalarial activity of HHA may be the result of synergy among specialized secondary metabolites coming from HHA constituent plants. Elements of synergy may stem from the presence of pgp inhibitors like resveratrol from *N. pobeguinii* which are expected to improve the bioavailability of QPAs (Bedada et al., 2015; Li et al., 2016). Also, the antimalarial activity of HHA could have been enhanced following intestinal biotransformation of β-carboline alkaloids from *N. pobeguinii* (Peeters et al., 2022) and/or via immunomodulation (Benson et al., 2013) and anti-anaemic properties (Oladiji et al., 2007) of HHA constituent plants such as *Sorghum bicolor*. Being a water extract, HHA may contain high molecular weight polysaccharide(s) that could contribute to its antimalarial activity by priming the immune system of the host (Bip et al., 2005; Dénou et al., 2019; Yu et al., 2018) to kill the parasite (Awasthi et al., 2003). The presence of prodrug(s), Pgp inhibitor(s) and the possibilities of immunostimulation by HHA constituent plants need further exploration.

5. Conclusion

This study for the first time provides scientific evidence for the use of HHA towards the management of malaria in Benin city, Nigeria. While a moderate potency was recorded *in vitro*, excellent activity was seen *in vivo*. Of all the HHA constituent plants, *A.affinis* was the only plant studied by us in detail because it gave the best antiplasmodial activity against the malaria parasite in culture. However, *in vivo* studies with HHA suggest the valuable contribution of ancillary molecules coming from other HHA constituent plants that may assist the drug action of QPAs in diverse ways. Identification of the constituent plants contributing these molecules is thus advocated as it could guide the development of HHA into an improved traditional medicine (ITM).

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CRediT authorship contribution statement

Nekpen Erhunse: Conceptualization, Methodology, Resources, Investigation, Data curation, Formal analysis, Writing - original draft, Writing - review & editing. **Ehimwenma Sheena Omoregie:** Conceptualization, Resources. **Dinkar Sahal:** Conceptualization, Methodology, Funding acquisition, Resources, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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