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Comparative Study on the Phytometabolites, in vitro Antiplasmodial Activity and Cytotoxicity of Stem Bark Extracts of *Annickia affinis* (Exell) Versteegh & Sosef and Annickia chlorantha (Oliv.) Setten & P.J. Mass

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Abstract
The similar-looking *Annickia affinis* and *Annickia chlorantha* are two closely related species of the genus *Annickia* that are difficult to tell apart. Literature to date has jointly referred to both as *Enantia chlorantha*. Amongst the many pharmacologic benefits ascribed to *E. chlorantha*, is its usefulness in the management of malaria. Given the closeness of both species, there is the likelihood of swapping one for another in the market of herbal drugs. This study thus set out to compare the phytometabolites, antiplasmodial activity and cytotoxicity of both species. *E. chlorantha* is known to be dominated by protoberberine alkaloids which are thought to confer it with its antiplasmodial property. Indeed, TLC metabolite fingerprinting of methanol and water stem bark extracts of both species revealed the presence of protoberberine alkaloids. However, these alkaloids were more abundant in *A. affinis* as compared to *A. chlorantha*. This was corroborated by the stark >2.82 fold difference in antiplasmodial IC_{50} of the respective methanol extracts being <0.78 µg/ml for *A. affinis* versus 2.2 µg/ml for *A.chlorantha*. Interestingly the selectivity indices for *A. affinis* (22.7) and *A. chlorantha* (24.3) determined using human hepatoma cell line were only marginally different. In conclusion, our study suggests that although both plants may be useful for managing malaria, consumers may get better and faster antimalarial relief with *A. affinis*.

Keywords: *Enantia chlorantha, Annickia chlorantha, Annickia affinis*, Protoberberine alkaloids, Antiplasmodial activity, Cytotoxicity.

Introduction
The genus *Annickia* Setten & Maas of plant family Annonaceae is comprised of eight (08) species viz. *A. affinis* (Exell) Versteegh & Sosef, *A. ambigua* (Robyns & Ghesq.) Setten & Maas, *A. chlorantha*, *A. kummerae* (Engl. &Diels) Setten & Maas, *A. lebrunii* (Robyns & Ghesq.) Setten & Maas, *A. le-testui* (Le Thomas) Setten & Maas, *A. pilosa* (Exell) Setten & Maas and *A. polycarpa* (DC) Setten & Maas. Of these, *A. affinis* and *A. chlorantha* are closely related species which are useful for the management of a number of diseases (Versteegh and Sosef, 2007). However, their great phenotypic similarity makes it difficult for herbal practitioners, their patrons as well as researchers to tell which is which (Olivier et al., 2015). Hence, there is likelihood of both plants being swapped for one another in the market of herbal drugs. Alternatively, both plants can be distinguished with confidence by DNA barcoding (Versteegh and Sosef, 2007). Besides, there are some salient features in their leaf and stembark which could be used to differentiate them (Versteegh and Sosef, 2007; Olivier et al., 2015). Thus, while *A. chlorantha* has a rough, grey bark, *A. affinis* possesses a smooth and greyish-brown bark (Figure 1) (Versteegh and Sosef, 2007). Further, while the simple hair under the leaf on the midrib of *A. affinis* point towards the apex, in *A. chlorantha,* hair on lower leaf surface is either simple, bifid, trifid or stellate and point in all directions (Versteegh and Sosef, 2007; Olivier et al., 2015). They can also be distinguished by the colour of their dry leaves since while the upper surface is brown to grey-green in *A. chlorantha*, it is grey-brown to almost black in *A. affinis* (Olivier et al., 2015). These were the criteria that were used by the botanist to authenticate *A. affinis* and *A. chlorantha*. Despite these differences between the two species, they are jointly referred to by a rather illegitimate name as *E. chlorantha*; a name substituted to *Annickia* in honour of Annick Le Thomas who previously revised the genus (Olivier et al., 2015). *E. chlorantha* is commonly known as African yellow wood. In Nigerian languages, it is known as *Awopa*, *Osu pupa* or *Dokitaigbo* (Yoruba), *Osomolu* (Ikale), *Kakerim* (Boki) and *Erenba-vbogo* (Edo). Given the differing reports of its antimalarial activity in publications (Agbaje and Onabanjo, 1991; Kimbi and Fagbenro-Beyioku, 1996; Adesokan and Akanji, 2010), this study set out to compare the phytometabolites, antiplasmodial activity and mammalian cell cytotoxicity of both species *in vitro*.

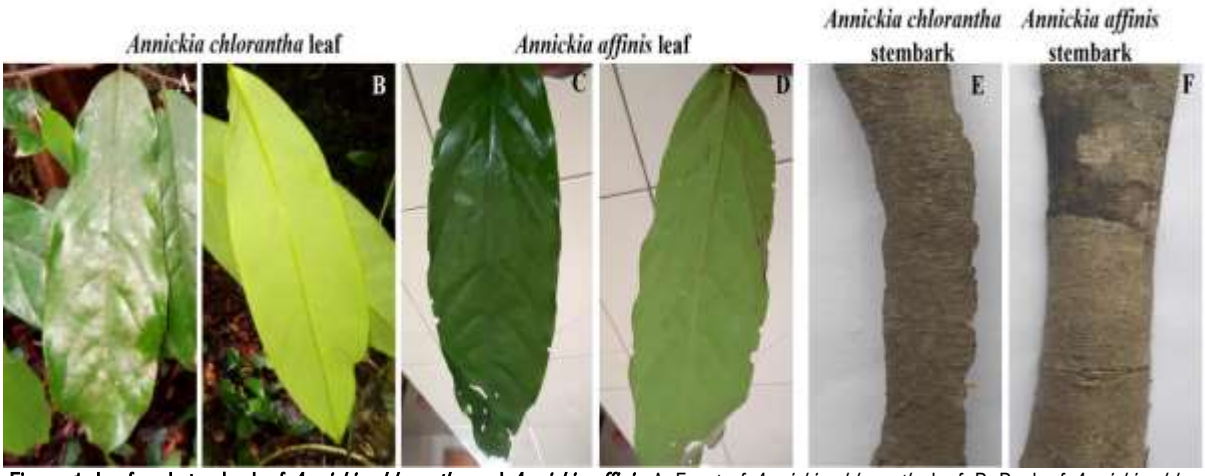

Figure 1. Leaf and stembark of *Annickia chlorantha* and *Annickia affinis*. A; Front of *Annickia chlorantha* leaf. B; Back of *Annickia chlorantha* leaf C; Front of *Annickia affinis* leaf. D; Back of *Annickia affinis* leaf. E; Rough grayish stembark of *Annickia chlorantha*. F; Smooth grayish-brown stem bark of *Annickia affinis* showing the cylindrical bole.

Materials and Methods

Sample Collection & Identification

Annickia chlorantha was collected from Akure Forest Reserve, Ondo East local government area of Ondo state, Nigeria (N07 277 36 ◦ E005 032 84 ◦ E = 306.5) by Mr. Omomoh Bernard Eromosele; a forest biologist with the Federal University of Technology, Akure, Ondo State, Nigeria. Garmin GPS was used to determine coordinates. *Annickia affiinis* was collected by Mrs. Bosede Benjamin; an expert herbal healer from its natural habitat in Benin city, Edo state, Nigeria. Both plants were collected in September, 2020 and submitted to Dr. Henry A. Akinnibosun of Plant Biology and Biotechnology Department, University of Benin, Nigeria for authentication. Thereafter, voucher specimens of both plant samples were deposited in the herbarium of same department. The voucher specimen numbers assigned were *A. affinis*, UBH-A511; *A. chlorantha*, UBH-A618.

Reagents and Chemicals

Malaria parasites used for the study included the chloroquine sensitive *Pf*3D7 (MRA102), chloroquine resistant *Pf*NDO (MRA819) and artemisinin resistant *Pf*Cam 3.II^{R539T} (MRA1240). These were obtained from Malaria Research and Reference Reagent Resource Centre. Human Hepatoma (HUH) cell line was obtained from American Type Culture Collection: Global bioresource centre. Roswell Park Memorial Institute (RPMI) 1640 medium, Albumax II, Gentamycin and Dulbecco's Modified Eagle's Medium (DMEM) were obtained from Gibco Life Technologies, USA. Hypoxanthine was obtained from Merck, India. Microculture tetrazolium (MTT) for tissue culture was obtained from Himedia, India. Pure berberine and palmatine used as TLC standards were obtained from Sigma Aldrich, India. All other reagents used were of analytical grade.

Sample preparation

Methanol extracts were prepared by macerating 100 g of the pulverized stem bark of *A. affinis* and *A.chlorantha* separately in 1 L of methanol each. The two samples were placed in a rotary shaker (120 rpm, 25^{o}C) for 72 hr. The extracts obtained were concentrated in a rotary evaporator and thereafter lyophilised. Water extracts of both plants were prepared by boiling 7 g/L of each plant in water for 1 hr at 100^{o}C as instructed by the herbal practitioner. Extracts were thereafter cooled and filtered through a 0.45-micron syringe filter after which they were frozen and lyophilized to obtain powdered extracts.

TLC Metabolite fingerprint of extracts

This was done using pre-coated aluminium Merck kieselgel 60 F_{254} silica plates. Briefly, 0.1 to 2 µl of samples (25 mg/ml in methanol for methanol extracts or water for water extracts) was loaded on the origin of the TLC plates and allowed to dry. The sample loaded plates were placed in a pre-saturated chromatographic tank containing developing solvent of choice. The chamber was tightly closed, and the solvent allowed to run till about 90% of the plate length. Plate was thereafter taken out, solvent front marked with a pencil, allowed to dry and visualized using different methods including visible light, short UV and long UV (Erhunse et al., 2023). For 2D TLC, after the first run with the first solvent system, the plates were air-dried, turned 90 degrees and placed in a chromatographic tank containing the second solvent system and solvent allowed to run in a direction perpendicular to the first dimension run.

In vitro antiplasmodial study

The SYBR Green I fluorescence-based assay was used for the antiplasmodial testing of samples (Smilkstein et al., 2004). Stocks (25 mg/ml) were prepared in either autoclaved water (water extracts) or DMSO (methanol extracts). The malaria parasite was cultured according to the method described by Trager and Jensen, (1976). The culture was synchronised at ring stage with 5% sorbitol and then exposed at 1% Parasitemia and 2% hematocrit to the extracts at various concentrations (0, 0.78, 1.56 and 3.13 µg/ml) in complete

medium comprising of 16.2 g/L RPMI 1640 powder, 0.2% sodium bicarbonate, 0.5% Albumax II, 50 mg/L hypoxanthine, and 10 mg/L gentamicin, in fresh O^+ erythrocytes. Incubation was done using a mixed gas system (5% O_2, 5% CO_2, and 95% N_2) for a total duration of 48 hr. at 37°C. Thereafter, 100 µl of SYBR Green I solution {0.2 µl of 10,000 × of SYBR Green I (Invitrogen) per ml of lysis buffer} was dispensed into each well to stain the parasite's DNA in the lysed red blood cells. The 96-well plate was then wrapped in foil and kept at 37° C in an incubator for 1 hr. Lysis buffer was prepared by adding 20 mM Tris (pH 7.5), 5 mM EDTA (pH 8.0), 0.008% (w/v) saponin and 0.08% (v/v) Triton X-100 into Milli-Q grade water. Fluorescence was measured at 485 nm excitation and at 530 nm emission and the 50% inhibitory concentrations (IC_{50}) were determined using the Antimalarial IC estimator version 1.2 software (Le Nagard et al., 2010).

In vitro cytotoxicity testing

HUH cells were cultured using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% foetal bovine serum until full confluence was achieved. Thereafter, they were trypsinized, seeded in a 96 well plate (10^4 cells/100 µl/well) and incubated at 37°C and 5% CO_2 for 12 hr. Next, spent media (90 µl) was replaced with fresh cDMEM (86 µl) followed by the addition of 4 µl extracts and plates were taken for a 24-hr incubation at 37°C in a 5% CO_2 incubator. DMSO (0.4% and 10%) were used as negative (full growth) and positive (zero growth) controls respectively. Post incubation, 20 µl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) prepared in PBS solution (5 mg/ml) was added to each well. After a 3 hr, 37°C incubation, media (120 µl corresponding to the amount of MTT (20 µl) plus 100 µl of extracts in DMSO/cDMEM) was aspirated out and 200 µl DMSO/well was added and solution mixed thoroughly to solubilize the formazan product. Absorbance was measured at 570 nm on a multi-well plate reader (Mosmann, 1983) and CC_{50} values were determined using the Antimalarial ICestimator version 1.2 software (Le Nagard et al., 2010).

Results

TLC fingerprinting of test samples

The antiplasmodial activity of *Annickia chlorantha* has been linked to the presence of protoberberine alkaloids (Vennerstrom and Klayman, 1988; Imieje et al., 2017) which are berberine-like alkaloids that possess a number of pharmacological properties including antiplasmodial activity. Members of this subgroup include, berberine (the most common protoberberine alkaloid), palmatine, jatrorrhizine and columbamine. 1D and 2D TLC metabolite fingerprinting of extracts of both plants suggested that *A. affinis* has higher amounts of the protoberberine alkaloids (the green fluorescing spot under long UV) than *A. chlorantha* (Figures 2 and 3).

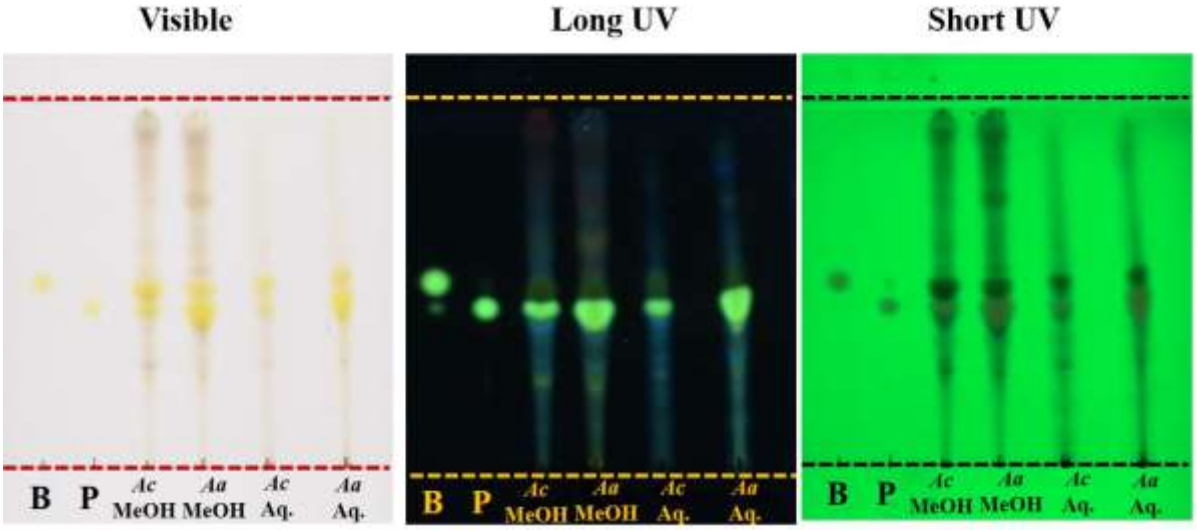

Figure 2. 1D TLC metabolite fingerprint of stem bark extracts of *Annickia affinis* and *Annickia chlorantha.* Protoberberine alkaloids appear as green fluorescent spots in the middle of the TLC plate under long UV (366 nm). Solvent system - Butanol: Glacial acetic acid: Water (14:3:4 v/v). Amount loaded = 50 µg for extracts and 2.5 µg pure compounds.

Key: B= Berberine, P = Palmatine, *Ac* meOH = methanol extract of *Annickia chlorantha*, *Aa* meOH= methanol extract of *Annickia affinis*, *Ac* aq. = aqueous extract of *Annickia chlorantha* and *Aa* aq. = aqueous extract of *Annickia affinis*

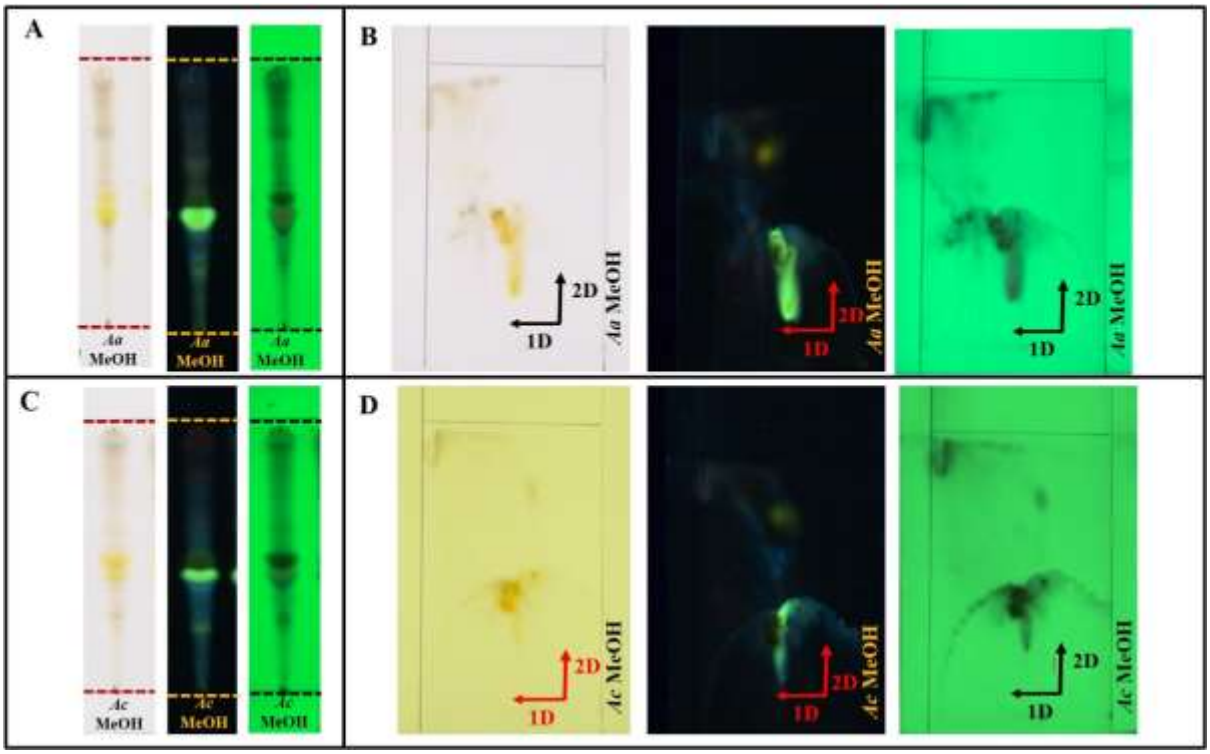

Figure 3. 1D & 2D TLC metabolite fingerprint of methanol stem bark extracts of *Annickia affinis* (A & B) and *Annickia chlorantha* (C & D).
Solvent system used for 1st run = Butanol: Glacial acetic acid: Water (14:3:4 v/v); Solvent system used for second run = Ethyl acetate: Methanol: Water (10:1.35:1v/v); Amount of sample loaded = 50 µg
Key: *Aa* meOH= methanol extract of *Annickia affinis*; *Ac* meOH = methanol extract of *Annickia chlorantha*.

In vitro antiplasmodial activity and cytotoxicity of extracts

The antiplasmodial activity of the extracts were tested against different strains of the parasite. Both aqueous and alcoholic extracts of *A.affinis* showed better antiplasmodial activity (IC_{50} <0.78 – 1.7 µg/ml) than the corresponding extracts from *A.chlorantha* (IC_{50} 2.08 – > 3.33 µg/ml) against all strains of the parasite studied (Table 1). Further, both extracts were quite selective (SI = >12) for the parasite as CC_{50} HUH ranged from 10 to >100 µg/ml.

Table 1. *In vitro* antiplasmodial activity and cytotoxicity of methanol and water stem bark extracts of *A. affinis* and *A. chlorantha*

Sample ID	IC_{50} (µg/ml) *Pf*3D7	IC_{50} (µg/ml) *Pf*NDO	IC_{50} (µg/ml) R539T	Resistance Index (IC_{50} *Pf*NDO/ IC_{50} *Pf*3D7)	CC_{50} (µg/ml) HUH	Selectivity Index (CC_{50} HUH/ IC_{50} *Pf*NDO)
Aa meOH	<0.78	0.44 ± 0.05	<0.78	<0.56	10 ± 0.65	22.73
Ac meOH	2.20 ± 0.04	2.39 ± 0.08	2.08 ± 0.48	1.09	58 ± 3.83	24.27
Aa Aq.	Nd	1.7 ± 0.01	Nd	-	21.05 ± 1.15	12.38
Ac Aq.	Nd	>3.13	Nd	-	>100	Nd

Aa meOH= methanol extract of *Annickia affinis*; *Ac* meOH = methanol extract of *Annickia chlorantha, Ac* aq. = aqueous extract of *Annickia chlorantha*; *Aa* aq. = aqueous extract of *Annickia affinis;* Nd = not done

Discussion and Conclusion

Annickia affinis and *Annickia chlorantha* are closely related look alike species making it difficult to identify one from the other. This is the first study which compares the metabolite fingerprints and antiplasmodial activity of these two plants. Plant samples are said to be highly active against the parasite if IC_{50} < 5 µg/ml, promising if IC_{50} is between 5 - 15 µg/ml, moderately active if IC_{50} is between 15 - 50 µg/ml and inactive if IC_{50} > 50 µg/ml (Kraft et al., 2003). Further, a selectivity index (SI) < 10 indicates nonspecificity of action (Peeters et al., 2022). Thus, with IC_{50} <0.78 to >3.13 µg/ml and SI >12, extracts of both plants were highly active against various strains of the malaria parasite as well as selective. This is consistent with reports of good antiplasmodial activity of *Enantia chlorantha* by other researchers (Vennerstrom and Klayman, 1988; Boyom et al., 2009; Imieje et al., 2017). However, in the present study we have found that *A. affinis* has much better antiplasmodial activity than *A. chlorantha*. Further the results of our study suggest that the antiplasmodial activity reported for *E. chlorantha* in the literature (IC_{50} < 1 µg/ml) may likely be that of *A. affinis*. Indeed, *A. affinis* is the more frequently occurring species (Olivier et al., 2015). However, since both plants are frequently confused, literature reports of *E. chlorantha* may refer to *A.chlorantha* or even other species of the *Annickia* genus (Olivier et al., 2015).

Although *E. chlorantha* is reported to contain protoberberine alkaloids that confer promising antiplasmodial activity *in vitro* (Vennerstrom and Klayman, 1988; Phillipson and Wright, 1991; Imieje et al., 2017), these alkaloids have not been proven to demonstrate antimalarial activity *in vivo* (Phillipson and Wright, 1991). Their limited oral bioavailability as substrates of P-glycoprotein (P-gp) is however established (Maeng et al., 2002; Tarabasz and Kukula-Koch, 2020). P-gp is an efflux pump which is expressed in a wide variety of tissues where this pump plays a role in the absorption, distribution and excretion of xenobiotics. Given the role of P-gp in influencing cellular concentrations of drugs, several methods including the use of inhibitors have been exploited to overcome P-gp mediated efflux (Dewanjee et al., 2017). P-gp inhibitors are classified into four generations with plant-derived P-gp inhibitors regarded as 4th generation P-gp inhibitors (Dewanjee et al., 2017).

Literature has differing records for the *in vivo* antimalarial activity of *E.chlorantha*. For instance, Agbaje and Onabanjo, (1991) reported complete parasite clearance on day 15 by a 95% ethanol extract given to mice subcutaneously (IC_{50} 0.34 mg/g). However, a zero-parasite clearance by the aqueous extract of the plant against *Plasmodium yoelii nigeriensis* infected mice was recorded via the oral route (Agbaje and Onabanjo, 1991). In contrast, Agomo et al., (1992) observed an early suppression of % parasitemia which did not lead to increased survival for the leaf and bark decoctions of the plant administered orally to *Plasmodium yoelii nigeriensis* infected mice. Against the same parasite strain, decoctions of the stembark administered intraperitoneally cleared the parasite in a dose dependent manner (Kimbi and Fagbenro-Beyioku, 1996). Against *P. berghei* (NK 65 strain), 400 mg/kg aqueous stembark extract administered via oral route showed an activity similar to chloroquine and caused 100% parasite clearance with 60% mortality after 17 days (Adesokan and Akanji, 2010). Apart from solvent of extraction, route of administration may have played a role in these differing reports since the bioactive agents (protoberberine alkaloids) are substrates of P-gp. For instance, it was recently reported that a hepta-herbal antimalarial *Agbo-iba* formula comprising of *A. affinis* and six other plants displayed a better antimalarial activity than *A. affinis* alone even though the latter had an over 50-fold better *in vitro* antiplasmodial activity than the herbal combo (Erhunse et al., 2023). It is also possible that one of either *A. chlorantha* or *A. affinis* contains P-gp inhibitor(s) or that one has a higher amount of P-gp inhibitor(s) than the other because some berberine containing plants have been reported to possess P-gp inhibitory properties thereby improving bioavailability (Stermitz et al., 2000; Ma et al., 2016). Hence, a study to compare the *in vivo* antimalarial activity of both plant extracts administered via various routes is advocated as this may guide their proper use.

Conflict of Interest

None declared

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