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Research Article

Comparative antiplasmodial activity, cytotoxicity, and phytochemical contents of *Warburgia ugandensis* stem bark against *Aspilia africana* wild and *in vitro* regenerated tissues

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Abstract Malaria remains to be one of the most severe global public health concerns. Traditionally, *Aspilia africana* and *Warburgia ugandensis* have been used to treat malaria in several African countries for millennia. In the current study, *A. africana* calli (AaC), *A. africana in vitro* roots (AaIR), *A. africana* wild leaf (AaWL), and *W. ugandensis* stem bark (WuSB) were dried and pulverized. Fourier transform near-infrared spectroscopy was used to analyze the powdered samples, while 80% ethanolic extracts of each sample were assayed for antiplasmodial activity (against *Plasmodium falciparum* strains DD2 (chloroquine-resistant) and 3D7 (chloroquine-sensitive)) and cytotoxicity. WuSB showed the highest antiplasmodial activity (IC_{50} = 1.57 ± 0.210 μg/ml and 8.92 ± 0.365 μg/ml against *P. falciparum* 3D7 and DD2, respectively) and selectivity indices (43.90 ± 7.914 and 7.543 ± 0.051 for *P. falciparum* 3D7 and DD2, respectively). The highest total polyphenolic contents (total phenolic and flavonoid contents of 367.9 ± 3.55 mg GAE/g and 203.9 ± 1.43 mg RUE/g, respectively) were recorded for WuSB and the lowest were recorded for AaC. The antiplasmodial activities of the tested plant tissues correlated positively with total polyphenolic content. The high selectivity indices of WuSB justify its traditional applications in treating malaria and present it as a good candidate for discovering new antimalarial compounds. We recommend elicitation treatment for AaIR, which showed moderate antiplasmodial activity against *P. falciparum* DD2, to increase its secondary metabolite production for optimal antimalarial activity.

Keywords Antiplasmodial activity, *Aspilia africana*, Chloroquine sensitivity, Chloroquine resistance, *In vitro* regeneration, *Warburgia ugandensis*

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Introduction

Malaria continues to be among the most severe global public health concerns (Schultz et al. 2021; WHO 2022), although over recent years, its incidence and prevalence have been reported to be dramatically reduced in sub-Saharan Africa (Schultz et al. 2021; Snow et al. 2017). Globally, more than 600,000 people die of malaria every year, most of whom are children (WHO 2022). Approximately 241 million cases of malaria and 627,000 associated deaths in 2020 were reported (Monroe et al. 2022; WHO 2022). Between 2019 and 2021, there was an increase of approximately 13.4 million cases of malaria, which was attributed to the disruptions of the Covid-19 pandemic (WHO 2022).

In sub-Saharan Africa and the Americas, *Plasmodium falciparum* and *Plasmodium vivax* were not only the most pernicious Plasmodium parasites that caused malaria but were also the most prevalent parasites, accounting for up to 99.7% and 74.1%, respectively, in 2017 of all malaria cases (Okello and Kang 2019; WHO 2018) while in Southeast Asia, 70% of malaria cases were attributed to *Plasmodium knowlesi* (Barber et al. 2017). Common symptoms of malaria include fatigue, nausea, headache, muscle aches, and fever (Okello and Kang 2019).

Since time immemorial, medicinal plants have always been traditionally used to treat a number of diseases, including malaria (Rasool et al. 2020). World Health Organization estimates that about 80% of the global population depend on traditional medicine primarily for their healthcare demands (Gang and Kang 2022). In a number of first world countries, herbal medicine is becoming very popular as an alternative and complementary therapy because of its efficacy, fewer side effects, and affordability compared to conventional drugs (Okello and Kang 2019; Srivastava et al. 2019).

Ahuchaogu et al. (2018) explains that the potential use of herbal plants as a source of new therapeutic drugs is still greatly unexplored. In several African countries and for decades, *Aspilia africana* and *Warburgia ugandensis* have been used to treat malaria (Okello and Kang 2021; Waako et al. 2005). The whole plant, root, and leaf of *A. africana* and the root, leaf, and stem bark of *W. ugandensis* are the main parts used for malaria treatment across different communities (Were et al. 2020). According to Okello et al. (2019), decoctions are made from these plant parts and used to cure malaria.

Antimalarial activities of *in vitro* regenerated plant tissues have been previously reported, including *Spilanthes paniculata* callus (Rajendran et al. 2017) and calli from seven different species of *Piper* (Putri and Noli 2021). In addition, *in vitro* regenerated plant tissues such as the root, stem, leaf, and flower extracts of *Spilanthes acmella* possess significant larvicidal activity against malaria vectors (Pandey and Agrawal 2009).

Many researchers have investigated the antiplasmodial activities of different parts of *A. africana* (Chono et al. 2009; Christian et al. 2012; Katuura et al. 2007; Waako et al. 2005) but there have been no reports on the antiplasmodial activities of *in vitro* regenerated tissues of *A. africana.* The current study aimed to investigate the antiplasmodial properties of *A. africana in vitro* regenerated tissues and compare them to those of the wild plant leaves of *A. africana* and the stem bark of *W. ugandensis*, which have been in use for centuries in different countries in Africa to treat malaria. Therefore, this study allows exploration of these traditionally used medicinal plants and *in vitro* regenerated tissues for the potential discovery of new antimalarial compounds. This study is timely, as resistance to a number of current antimalarial drugs has already been documented in regions such as Africa, the Americas, and Southeast Asia (WHO 2022).

Method and Materials

Explant preparation and calli induction

Aspilia africana plant shoots (95-125 mm) were cut from 3-month-old plants in growth chambers at the Herbal Medicine Resources Research Center, Korea Institute of Oriental Medicine (KIOM). The excised shoots of *A. africana* were cleaned in flowing tap water for 4 min then transferred to a clean bench. Using autoclaved double-distilled water, the shoots were again washed and sterilized with 100% (v/v) ethanol for 30 s and 70% (v/v) ethanol for 30 s, followed by 3 min in sodium hypochlorite (2% v/w). The plant shoots were rinsed four times with double-distilled autoclaved water. Apart from the terminal leaves of *A. africana*, all were cut off from shoots, segmented into 7 mm^2 pieces, and used for calli induction as explants. Nine leaf pieces were embedded in each Petri dish with MS culture medium gelled with 3 g/L gelrite containing vitamins and augmented with 1.0 mg/L 2,4-Dichloro-phenoxyacetic acid (2,4-D) and 0.5 mg/L benzylaminopurine (BAP). Sixty replications were performed, and the Petri dishes were kept at 25 ± 1°C in the dark at 75% relative humidity until calli formed. The calli were subcultured biweekly then transferred and kept in a 16-h photoperiod system (white cool fluorescent tubes provided light). The calli were maintained at 75% relative humidity and 25°C. The calli formed were cleaned with flowing water from a tap to remove media traces from their surface and then, using an oven, were dried for 48 h at 60°C. The calli (AaC) were then powdered by grinding and stored at 4°C until extraction. A summary of the sample preparation process is presented in Fig. 1.

Induction of In vitro root and sample preparation

The sterilized shoot apices of *A. africana* were cut (30-40 mm in length), and the end surfaces that had direct contact with sterilizing agents were removed. *A. africana* shoot apices were then planted in gelrite (3 g/L) MS plant tissue

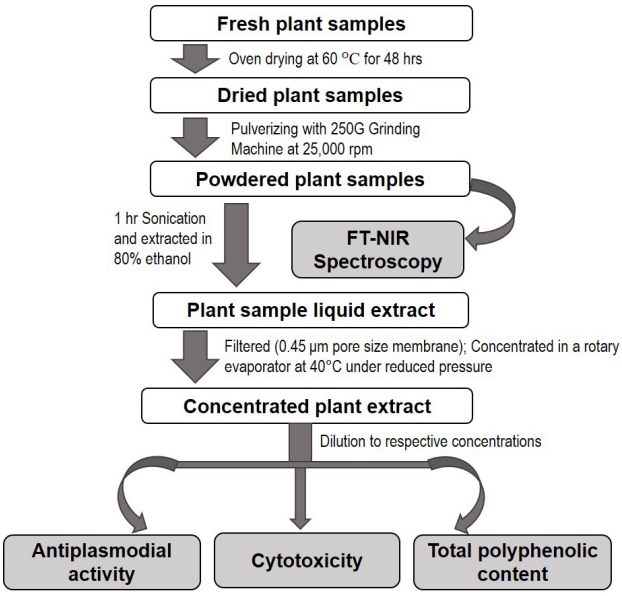

Fig. 1 Summary of preparation and processing of plant samples for biochemical assays

culture medium containing vitamins augmented with 0.1 mg/L 1-Naphthaleneacetic acid (NAA) to induce *in vitro* roots. Four shoot apices were inoculated in each polystyrene culture vessel (125 × 100 mm), and 100 replicates were performed. The inoculated shoot apices were then placed under light (provided by cool white tubes) under a 16-h photoperiod at 75% relative humidity and 25°C. The formed *A. africana in vitro* roots (AaIR) were harvested after six weeks, washed, dried, powdered, and stored at 4°C until extraction.

Collection and preparation of Wild *A. africana* leaf sample

Leaves from *A. africana* wild plants (AaWL) were collected from over 50 plants randomly from Pece, Gulu, Uganda. The leaves of *A. africana* were dried under the sun for six days and then ground to a fine powder. The leaf powder was posted to KIOM, South Korea, after packing in airtight bags and kept at 4°C until extraction and subsequent analysis, as shown in Fig. 1.

Collection and preparation of *W. ugandensis* stembark sample

The stem bark was randomly collected from *W. ugandensis* trees at the Natural Chemotherapeutics Research Institute, Kampala, Uganda. The stem bark was dried in an oven at 38°C for three days, grinded to powder, then packed into airtight bags. The stembark powder was posted to KIOM, South Korea, and stored at 4°C until extraction and subsequent analysis (Fig. 1).

Preparation of samples for assay

One month after harvest, 2 g of each powdered sample (AaC, AaIR, AaWL, and WuSB) was extracted with 50 mL of ethanol (80%). The extracted samples were filtered with a syringe filter (0.45 µm membrane pore size). Then filtrate was concentrated at reduced pressure and 40°C using a rotary evaporator (EYELA N-1200B, Tokyo Rikakikai Co. Ltd., Japan).

In vitro antiplasmodial activity of samples

The plant sample extracts were assayed for their *in vitro* antiplasmodial activities using the Nitro Blue-Tetrazolium-based parasite lactate dehydrogenase (pLDH), as validated by Makler et al. (1993). The antiplasmodial activity of the extracts was studied against chloroquine-resistant (DD2) and chloroquine-sensitive (3D7) *Plasmodium falciparum* strains. Parasites were continuously cultured following the method of Trager and Jensen (1976), with some modifications. The culture medium consisted of Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco™ Paisley, Scotland, UK) containing 10% human serum, 6 g of *N*-(2-hydroxyethyl)-piperazine-*N*-2-ethanesulphonic acid (HEPES) (Sigma Chemical Co., St Louis, MO, USA), 44 mg of hypoxanthine (Sigma Chemical Co., St Louis, MO, USA), 50 mg of gentamycin (Sigma Chemical Co., St Louis, MO, USA), and 2.1 g of sodium bicarbonate (Sigma Chemical Co., St Louis, MO, USA). The cultures were maintained in 93% N_2, 4% CO_2, and 3% O_2 at 37°C. The plasmodia culture medium was changed every 24 h, and at the trophozoite stage, the parasites were fed fresh erythrocytes to keep parasitemia below 10%. The cultures were monitored frequently by Geimsa staining of thin blood smears.

For the antiplasmodial assay, flat bottomed 96-well microculture plates were used (Costar Glass Works, Cambridge, UK). Ten milligrams of each extract was added to 1 ml of dimethyl sulfoxide (DMSO) (Sigma Chemical Co., St Louis, MO, USA) and topped with distilled and autoclaved water to form a stock of 1000 µg/ml. The stock solution was diluted to obtain an initial concentration of 111 µg/ml for each plant extract. Each extract was subjected to a two-fold serial dilution (seven concentrations) against parasitized cultures of 2% parasitemia and 1.5% hematocrit. Well columns with no extract or drug served as negative controls. Chloroquine (CQ) and dihydroartemisinin (DHA) were positive controls.

The plates with the cultures were placed in an air-tight cabinet, gassed with 92% N_2, 5% CO_2, and 3% O_2, and then incubated for 48 h at 37°C. After incubation, 0.5 μCi [^{3}H]- hypoxanthine (specific gravity, 1 mCi/ml) (ICN Pharmaceuticals, Irvine, Calif, USA) was added to each well and the plates incubated for more 18 h. Cells were then harvested onto fiber mats using a cell harvester (Inotech Biosystems International, Inc., Rockville, Maryland), and the incorporated radioactivity was determined (in counts per min [cpm]) with a liquid scintillation and luminescence counter (Wallac 1450 Microbeta). The antiplasmodial assay was performed in duplicate. The growth inhibition percentage was calculated as follows: IC_{50} = antilog [log X_1 + (log Y_{50} - log Y_1) (log X_2 - log X_1) / (log Y_2- log Y_1)]. Where, Y_{50} is the cpm value midway between parasitized and non-parasitized control cultures and X_1, X_2, Y_1 and Y_2 are the concentrations and cpm values for the data points above and below the cpm midpoints (Sixsmith et al. 1984). The IC_{50} values above 100 μg/ml were considered inactive.

Total polyphenolic content determination

Phenolic contents

The total phenolic content (TPC) in the samples was determined following previous methods (Derakhshan et al. 2018, Okello et al. 2021) with minor modifications. A 0.3 mg/mL sample solution was prepared from each of the extracts. Using a pipette, 0.5 mL of each sample was transferred to a microcentrifuge tube (1.5 mL), mixed with 0.5 mL of Folin-Ciocalteu's reagent for 4 min. Then10% Na_2CO_3 (0.5 mL) was added to the microcentrifuge tube content and thoroughly mixed, then kept at 25°C in darkness for 60 min. Spectramax i3x (Molecular Devices, Wokingham, UK) was used for spectrophotometric measurements of the samples in triplicates, with absorbance readings at 725 nm. Gallic acid was used as the standard, and its calibration curve was used to determine the TPC (mg gallic acid equivalent [mg GAE/g]) of the samples.

Flavonoid contents

The total flavonoid content in the samples was determined following a previous method by Okello et al. (2021) with minor modifications. A 1 mg/mL sample solution was prepared from each of the extracts. Using a pipette, 0.1 mL of each sample was transferred to a microcentrifuge tube (1.5mL) and mixed with 90% diethyl glycol (0.8 mL) and 1 N sodium hydroxide (10 μL) before being vortexed for 4 sec and kept in a water bath at 37°C for 60 min. Spectramax i3x (Molecular Devices, Wokingham, UK) was used for spectrophotometric measurements of the samples in triplicate, with absorbance readings at 420 nm. Rutin was used as the standard and its calibration curve was used to determine the total flavonoid content (mg rutin equivalent [mg RUE/g]) of the samples.

Fourier Transform Near-Infrared (FT-NIR) spectroscopy

An FT-NIR spectrometer (TANGO, Bruker Optics, Billerica, MA, USA) was used to analyze the pulverized AaC, AaIR, AaWL, and WuSB samples. A gold standard (1024957 type, ECL 01) and light trap (1002961 type, ECL 00) were used to calibrate the spectrometer, after which 2 g of each sample was put in 22 mm width glass vials and analyzed. At a wave number range of 12487-3948 cm^{-1}, the absorbance spectrum for each sample was obtained.

Cytotoxicity assay of the samples

The *in vitro* toxicity of the plant extracts was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in Vero cell lines. The cell lines were cultured in RPMI medium, containing 10% (v/v) fetal bovine serum (FBS), 0.5% fungizone, and 2% penicillin-streptomycin and incubated at 37°C and 5% CO_2 in a humidified environment. In a 96-well plate containing 100μl of the fresh culture medium, the Vero cell lines were seeded at a density of 1.0×10^6 cells/well and then incubated for 24h at 37°C in 5% CO_2. After the 24 h incubation, the medium was discarded, and cells were treated with the different plant sample extracts and then again incubated for 24 h in a humidifier at 37°C and 5% CO_2. After Next, 10 μL (5 mg/mL) MTT was added to each well and incubated at 37°C for more 4 h after which the supernatant was removed, and 100 μL DMSO was added to each plate and gently shaken for approximately 5 min to dissolve the formazan crystals. Each plant sample extract was treated in triplicates. The negative control was a medium containing 1% DMSO. Absorbance was measured using a spectrophotometer at 595 nm, and the level of cell viability was computed as follows: Cell viability = [A - B / C - B] × 100. Where A absorbance of the test compound/ plant extract, B = absorbance of the blank, and C = absorbance of the control. The 50% cytotoxic concentrations (CC_{50}) of the plant sample extracts were determined by regression analysis, and selectivity indices (SI) for the antimalarial activity were calculated as SI = CC_{50} *for extract cytotoxicity* / IC_{50}.

Results

Calli induction

Callus induction rate from the leaf explants was high (approximately 88 %) after weeks of culture. The induced calli were cream, pale yellow or brown, compact, and friable.

In vitro antiplasmodial activity of the samples

Generally, all the samples investigated exhibited antiplasmodial activity, although to varying degrees (Table 1). *Aspilia africana* samples (AaWL, AaIR, and AaC) had much lower activity against both chloroquine-resistant and-sensitive *P. falciparum* strains than *Warburgia ugandensis* stem bark samples (Table 1). WuSB had the highest antiplasmodial activity against both chloroquine-resistant and -sensitive *P. falciparum* strains, with IC_{50} values of 8.92 ± 0.365 μg/ml and 1.57 ± 0.210 μg/ml, respectively (Table 1). The antiplasmodial activity of WuSB was remarkably lower ($p < 0.05$) than that of the rest of the plant samples, but not from the positive standards (DHA and CQ). The least antiplasmodial activity against the chloroquine-sensitive strain (3D7) was exhibited by AaWL, with an IC_{50} value of 95.23 ± 4.045 μg/ml. Against the DD2 chloroquine-resistant plasmodium strain, the least antiplasmodial activity (IC_{50} = 77.2 ± 3.57 μg/ml) was exhibited by AaC (Table 1). Although AaIR had moderate activity (IC_{50} = 48.51 ± 3.880) against the *P. falciparum* DD2 strain, it had a weak antiplasmodial activity against 3D7, the chloroquine-sensitive strain (Table 1).

Cytotoxicity assay and Selectivity indices of the samples

To determine the toxicity of the plant samples, the same sample extracts that had previously been assayed for their antiplasmodial activities were further investigated using the MTT assay on Vero cells. The cytotoxicity results and the calculated selectivity indices are presented in Table 1. As per the criteria previously used in other studies (Afagnigni et al. 2020; Njeru et al. 2015; Njeru and Muema 2021), we adopted a threshold cytotoxic concentration (CC_{50}) of less than 20 μg/ml as toxic and above 20 μg/ml as nontoxic. All the samples investigated had CC_{50} values ranging from 51 to 96 μg/ml. AaIR not only had the highest CC_{50} value (96.22 ± 2.410 μg/ml) but was also significantly higher ($p < 0.05$) than the CC_{50} values of all other samples investigated (Table 1). The lowest CC_{50} value obtained was 51.21 ± 1.335 μg/ml for AaWL. Selectivity indices for *A. africana* samples were much lower than those for *W. ugandensis* stem bark. WuSB had the highest selectivity indices of 43.90 ± 7.914 and 8.92 ± 0.365, respectively, for antiplasmodial activities against 3D7 and DD2 strains. The SI value for WuSB was significantly higher ($p < 0.05$) than the SIs for all other samples investigated (Table 1). The lowest SIs (0.538 ± 0.009 and 0.884 ± 0.06 against 3D7 and DD2 Plasmodium strains, respectively) were recorded for AaWL (Table 1).

Total polyphenolic content

The highest total phenolic content (367.9 ± 3.55 mgGAE/g) was recorded in WuSB and was more than double the total phenolic content in AaIR (136.2 ± 5.46 mgGAE/g), which was highest among the *A. africana* tissues (Fig. 2a). Least total phenolic content was recorded in AaC (102.6 ± 1.12 mgGAE/g). There was no significant difference in total phenolic content between AaIR and AaWL (Fig. 2a). The total flavonoid content in all *A. africana* tissues was generally higher than the total phenolic content (Fig. 2).

Table 1 *In vitro* antiplasmodial activity, cytotoxicity screening, and selectivity indices of *A. africana* and *W. ugandensis* samples

Plant sample/ Reference drug	VERO CC50 (μg/ml)	IC50 (μg/ml), *P. falciparum* 3D7	Selectivity index	IC50 (μg/ml), *P. falciparum* DD2	Selectivity index
AaWL	51.21 ± 1.335^{c}	95.23 ± 4.045^{c}	0.538 ± 0.009^{b}	58.42 ± 5.905^{c}	0.884 ± 0.066^{c}
AaIR	96.22 ± 2.410^{a}	65.56 ± 3.305^{b}	1.472 ± 0.111^{b}	48.51 ± 3.880^{bc}	1.956 ± 0.165^{b}
AaC	78.98 ± 2.065^{b}	69.18 ± 2.955^{b}	1.200 ± 0.040^{b}	77.2 ± 3.570^{d}	0.990 ± 0.040^{c}
WuSB	67.27 ± 3.205^{b}	1.57 ± 0.210^{a}	43.90 ± 7.914^{a}	8.92 ± 0.365^{a}	7.543 ± 0.051^{a}
DHA		0.01528 ± 0.00041^{a}		< 0.0155^{a}	
CQ		0.29398 ± 0.0036^{a}		< 0.300^{a}	

Mean (± standard error) values in a column followed by the same letter are not significantly different based on Tukey's test and $p = 0.05$. CC_{50}, half-maximal cytotoxic concentration; IC_{50}, half-maximal inhibitory concentration; AaWL, *Aspilia africana* wild leaf; AaIR, *Aspilia africana in vitro* root; AaC, *Aspilia africana* callus; WuSB, *Warburgia ugandensis* stem bark; DHA, dihydroartemisinin; CQ, chloroquine.

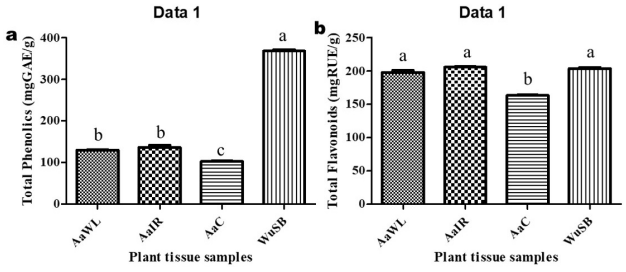

Fig. 2 Total phenolic and flavonoid contents in *A. africana* and *W. ugandensis* samples. (a) Total phenolic contents. (b) Total flavonoid contents. Values are presented as the mean ± standard deviation. Same letters indicate no significant differences based on Tukey's test and p = 0.05

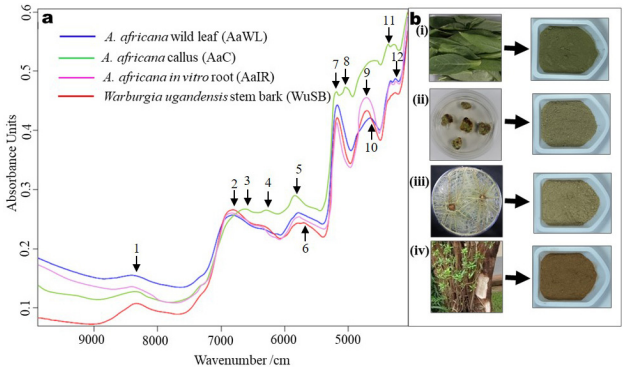

Fig. 3 (a) FT-NIR spectra for *A. africana* and *W. ugandensis* plant samples. (b) Samples used in the experiment: (i) *A. africana* wild leaf, (ii) *A. africana* callus, (iii) *A. africana in vitro* roots, and *W. ugandensis* stem bark. FT-NIR, Fourier transform near-infrared (FT-NIR) spectroscopy

The highest total flavonoid content was in AaIR (205.8 ± 0.96 mgRUE/g). However, this did not significantly differ from its contents in WuSB (203.9 ± 1.43 mgRUE/g) and AaWL (197.9 ± 3.28 mgRUE/g) but remarkably varied ($p < 0.05$) from total flavonoid contents in AaC (Fig. 2b).

Fourier Transform Near-Infrared (FT-NIR) spectroscopy

The FT-NIR spectra demonstrated some extent of chemical similarity between the samples (Fig. 3). Between 9000 and 5000 cm^{-1}, the spectra of AaWL and AaIR had five peaks 1, 2, 4, 5, and 7 at respectively 8295, 6867, 6337.5, 5775, and 5172 cm^{-1} and very closely resembled but slightly different from the spectra of AaC with additional peaks 3 and 8 between 7000 and 5000 cm^{-1}, and WuSB with three peaks (5, 6 and 7) between 6000 and 5000 cm^{-1} (Fig. 3). From 5000 to 4000 cm^{-1} wavelengths, only AaIR and WuSB each had a peak (9) at 4751 cm^{-1} whereas AaWL and AaC each had a peak (10) at 4584 cm^{-1} (Fig. 3). AaC was the only sample with a peak (8) at about 4950 cm^{-1}. In addition, while the spectra of AaIR, AaWL, and AaC had peaks 11 and 12 at respectively 4323 and 4253 cm^{-1}, WuSB only had a peak (12) at 4253 cm^{-1} (Fig. 3).

Discussion

Ethanol is a common solvent used to extract medicinal plant materials for bioactivity and efficacy assays (Inbaneson et al. 2012; Mallik and Akhter 2012; Taha and Alsayed 2000). Indeed, 80% ethanol has been effectively used to extract medicinal plant materials for antiplasmodial activity bioassays (Andrade-Neto et al. 2004; Ichino et al. 2006; Oliveira et al. 2004). Furthermore, in our previous study Okello et al. (2021), 80% ethanol extract showed very good antioxidant activity in *A. africana* tissues, thus justifying its use in the current study.

Warburgia ugandensis is a very useful traditional medicinal plant because of its pharmaceutical qualities; in fact, *W. ugandensis* is ranked second among high-priority medicinal plants (Mwitari et al. 2013; Okello and Kang 2019). In Africa, several communities use the roots, stem bark, or leaves of *W. ugandensis* to treat malaria (Okello and Kang 2019). To treat the disease, the plant parts are boiled in water, and decoctions are taken orally (Okello and Kang 2019).

Several studies have previously investigated the antimalarial activities of different plant parts of *A. africana* (Christian et al. 2012; Katuura et al. 2007; Waako et al. 2005) and *W. ugandensis* (Irungu et al. 2007; Okello et al. 2018; Were et al. 2020). In one study, different leaf extracts (methanol, ethyl acetate, hexane, and water) of *A. africana* were investigated against chloroquine-sensitive (D 10) plasmodium and chloroquine-resistant (K 1) strains (Waako et al. 2007). In their study, ethyl acetate extract had the most potent antimalarial activity against both strains with IC_{50} values of respectively 9.3 ± 7.5 µg/ml and 11.5 ± 2.8 µg/ml on chloroquine-sensitive (D 10) and chloroquine resistant (K 1) plasmodium strains (Waako et al. 2007). Waako et al. (2007) obtained IC_{50} values below 30 µg/ml for all extracts, except for the hexane extract, which was inactive. In another *in vitro* antiplasmodial study, *A. africana* leaf ethyl acetate/petroleum ether extract had an IC_{50} value of 30.3 µg/ml, exhibiting good antimalarial activity, whereas the methanol extract was inactive (Chono et al. 2009). The observed differences in these results from this study could be due to the differences in the solvents used for extraction, in plant parts used, and in the sensitivities of the different strains used in the studies.

Several studies have demonstrated that various solvent plant extracts differ in their antiplasmodial/antimalarial activities (Bantie et al. 2014; Martinez-Correa et al. 2017;

Waako et al. 2007). The ethanolic leaf extract of *A. africana* was found to have a significant chemo-suppressive effect of 92.23% at a concentration of 400 mg/kg on *P. berghei* during a four-day early infection stage (Christian et al. 2012). Christian et al. (2012) further emphasized that the leaf extract of *A. africana* suppressed the growth of Plasmodium parasites to undetectable levels in red blood cells. The researchers noted that the antimalarial property exhibited was consistent with the traditional use of the plant as a remedy for malaria and could be a potential source of antimalarial agents. Christian et al. (2012) attributed the antimalarial activity of the plant to its flavonoid, terpene, and alkaloid content.

Similar to our findings, previous studies on *W. ugandensis* have reported good activity against *Plasmodium* parasites (Okello and Kang 2021; Were et al. 2010; 2020). Were et al. (2010) assayed various solvent extracts of stem bark of *W. ugandensis* against *P. knowlesi* and recorded the lowest IC_{50} (3.14 ± 0.12 μg/ml) for chloroform extract. The slightly lower value of IC_{50} observed for WuSB in our study could be because of the differences in the extracting solvent, plant age, plant growth environment, and Plasmodium strains used. Furthermore, in agreement with our study, an *in vivo* study demonstrated the potent antimalarial activity of *W. ugandensis* stem bark (Were et al. 2020).

In our study, WuSB demonstrated the best antiplasmodial activity against 3D7 (IC_{50} =1.57 ± 0.210) and DD2 (IC_{50} =8.92 ± 0.365) plasmodium strains, possibly because of its very high antimalarial phenolic and flavonoid contents. WuSB IC_{50} values for both strains did not significantly differ ($p < 0.05$) from those of the standard drugs used, and the plant sample also had the highest polyphenolic content. Phenolic and flavonoid compounds have been associated with good antiplasmodial activity in medicinal plants (Cudjoe et al. 2020; Ntie-Kang et al. 2014). The antimalarial activities of other medicinal species, such as *Ocimum santum* (Inbaneson et al. 2012), *Alchornea cordifolia* (Cudjoe et al. 2020) and *Securidaca longepedunculata* (Karama et al. 2020) have also been linked to the phenolic and flavonoid contents of the plants.

The antiplasmodial activities of phenolics and flavonoids have been attributed to the presence of hydroxyl groups in these compounds (Karama et al. 2020). Because of its very high total phenolic and flavonoid contents, WuSB had the most potent antiplasmodial activity. The antiplasmodial activities of the samples matched their total flavonoid and phenolic contents; therefore, AaC, with the lowest total phenolics and flavonoids, had the least antiplasmodial activity. Unlike previous studies, the antiplasmodial activities of AaWL were quite low, possibly because the sample materials were stored for a longer time before the assay. Thus, some bioactive principles were degraded (Grace et al. 2014). Antimalarial compounds, such as phenolic compounds, including chlorogenic acid, are present in *A. africana* plant tissues (Okello et al. 2021), and flavonoids were observed to decrease in concentration with longer storage duration of the plant materials (Mediani et al. 2014). Based on the IC_{50} values and according to the criteria used in previous studies (Bero et al. 2009; Dolabela et al. 2008), we adopted the following to classify the antiplasmodial activities of the investigated plant materials: $IC_{50} < 10$ μg/ml as good, IC_{50} of 10-50 μg/ml as moderate, and $IC_{50} > 100$ μg/ml as inactive.

From previous studies, calli from different plant tissues contained various secondary metabolites and demonstrated potent biological activities, including antimalarial potential. Some of these plants are, *Phyllanthus niruri* (Cimanga et al. 2004), *Harpagophytum procumbens* (Grąbkowska et al. 2016), *Vigna unguiculata* (Vats et al. 2012), *Randia echinocarpa* (Valenzuela-Atondo et al. 2020), and *Oroxylum indicum* (Faraz et al. 2020). We produced calli from leaf explants of *A. africana* then them as part of our material for study. *A. africana* calli had low antiplasmodial activity, with IC_{50} values of 69.2 ± 2.955 μg/ml and 77.2 ± 3.57 μg/ml against 3D7 and DD2 strains, respectively. The antimalarial potency of calli from previous studies have been reported, including *P. niruri* (IC_{50} = 16.3 ± 2.5 μg/ml) (Cimanga et al. 2004) and *Sonchus arvensis* (IC_{50} = 5.9 μg/ml) (Wahyuni et al. 2021). The differences in the antiplasmodial potentials of calli could be due to differences in media and hormone combinations, plant species, explant parts, and callus culture duration (Bucchini et al. 2013; Grąbkowska et al. 2016).

FT-NIR analysis is a technique that captures chemical data related to O-H, S-H, C-H, and N-H bonds in a sample (Okello et al. 2021; Páscoa et al. 2019). FT-NIR spectrometry gives vital data for phytochemical analysis and phytochemical content quantification in medicinal plants (Okello et al. 2021; Páscoa et al. 2019). The phytochemical content of medicinal plants responsible for their biological activities contributes to the NIR spectra (Okello et al. 2021).

In the FT-NIR spectra, peaks from 4200 to 4900 cm^{-1} are due to stretching and deformation modes attributed to O-H and C-H groups belonging to phenolic rings (Carbas et al. 2020; Okello et al. 2021). WuSB followed by AaIR exhibited the most potent antimalarial activity against both 3D7 and DD2 strains, and were the only plant samples

with peaks at 4751 cm^{-1} indicating the presence of important antimalarial phenolic compounds. The spectral peaks at 5050-5200 cm^{-1} were attributed to the combination modes of the O-H group in phenols and the corresponding aromatic ring-related vibrations (Carbas et al. 2020).

The FT-NIR spectral peaks from 5400 to 6000 cm^{-1} were attributed to overtones of the C-H stretching modes from the corresponding aromatic rings (Oliveira et al. 2004). The spectral peaks from 6050 to 7200 cm^{-1} are due to overtones resulting from C=O stretching in flavonols and O-H combinations in phenols (Oliveira et al. 2004; Wiedemair et al. 2019). As reflected in the spectra, all samples had two peaks between 6050 and 7200 cm^{-1}. indicating the presence of polyphenolic compounds partly responsible for the antimalarial properties of the plant samples investigated.

Vero cell lines have been used as *in vitro* models for toxicity studies (Afagnigni et al. 2020; Njeru and Muema 2021). The choice of Vero cell lines to detect basal cytotoxicity is because the cell lines are readily available, are easy to culture, and have high sensitivity (Njeru and Muema 2021). As previously reported (Afagnigni et al. 2020; Njeru and Muema 2021), we adopted a threshold cytotoxic concentration (CC_{50}) value of less than 20 μg/mL as toxic and above 20 μg/mL as nontoxic. The CC_{50} values of all samples investigated ranged from 51.21 ± 1.335 to 96.22 ± 2.410, within acceptable limits of toxicity.

With SI, drug candidates can be characterized and optimized for efficacy and safety (Muller and Milton 2012; Touret et al. 2020; Zhang and Hamada 2019). Selectivity indices have been routinely employed in drug discovery as important parameters to assess the balance of safety and efficacy profiles of drug candidates for intended purposes (Schultz et al. 2021; Touret et al. 2020). *W. ugandensis* stem bark (WuSB) had significantly higher ($p < 0.05$) SI values for both 3D7 (43.90 ± 7.914) and DD2 (7.543 ± 0.051) *P. falciparum* strains compared to all other plant samples and indeed would be a good candidate for antimalarial drugs. Although AaWL had CC_{50} values above 20 μg/mL, the SI values for both 3D7 and DD2 were very low (below 1), implying that although the cytotoxicity levels were low, the antiplasmodial activities of the sample against both strains were also low and thus may not be a good candidate for antimalarial drugs, even though the plant has been used for a long time to treat malaria in many communities.

Conclusion

WuSB exhibited the most promising antiplasmodial activity (IC_{50} = 1.57 ± 0.210 μg/ml and 8.92 ± 0.365 μg/ml against 3D7 and DD2 *P. falciparum* strains, respectively). The high selectivity indices of WuSB for 3D7 and DD2 *P. falciparum* strains at 43.90 ± 7.914 and 7.543 ± 0.051, respectively, not only justify its use for centuries traditionally to treat malaria in many African communities but also present it as a good candidate that could be exploited for the discovery of new antimalarial compounds. Furthermore, *in vitro* regenerated tissue (AaIR) has been demonstrated to possess better antimalarial activities than AaWL, which has been used for centuries to treat malaria. The highest total polyphenolic contents (367.9 ± 3.55 mgGAE/g and 203.9 ± 1.43 mgRUE/g for total phenolic and flavonoid contents, respectively) were recorded for WuSB and least (102.6 ± 1.12 mgGAE/g and 163.3 ± 0.65 mgRUE/g for total phenolic and flavonoid contents respectively) for AaC. The antiplasmodial activities of the investigated medicinal plant tissues were positively correlated with total polyphenolic content. Since the secondary metabolite contents of *in vitro* tissues can be increased, we recommend elicitation treatment for AaIR with moderate antiplasmodial activity against the DD2 *P. falciparum* strain to boost its secondary metabolite contents for optimal antimalarial activities.

Authors' Contributions

DO conceived the research idea, designed the experimental plan, participated in every stage and all parts of the research work, did the statistical analyses, and wrote the manuscript. JG and AW participated in the antimalarial and cytotoxicity assays. RK prepared samples and wrote the manuscript writeup. YC performed total polyphenolic content analysis. RG prepared samples, participated in FTNIR analysis and wrote the manuscript. FO read and improved the manuscript. YK provided technical guidance, supervised the whole research work, read and improved the manuscript. All authors read and approved the final manuscript.

Availability of Data and Materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing Interests

Authors declare that they have no competing interests.

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