Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 December 2016 doi:10.20944/preprints201612.0044.v1

In Vitro and *in Vivo* Antiplasmodial Activity of Extracts from *Polyalthia suaveolens, Uvaria angolensis* and *Monodora tenuifolia* (Annonaceae)

Alvine N. Mfopa[1], Lauve R.Y. Tchokouaha[1,2,3], Cedric D.J. Mbouna[1], Marthe A.T. Tchuente[4], Rufin M.T. Kouipou[1], Patrick V.T. Fokou[1,2], Eugenie A.M. Kemgne[1], Raceline G. Kamkumo[1,5], Fabrice F. Boyom[1*]

[1] Antimicrobial & Biocontrol Agents Unit (AmBcAU), Laboratory for Phytobiochemistry and Medicinal Plants Studies, Department of Biochemistry, Faculty of Science, University of Yaoundé I, P.O. Box: 812, Yaoundé, Cameroon; Emails: ngmfalvine@yahoo.fr (ANM); yamthe_lauve@yahoo.fr (LRYT); cedrickjiatsa@yahoo.com (CDJM); toghueo.rufin@yahoo.fr (RMTK); tsouh80@yahoo.fr (PVTF); eugboyom@yahoo.fr (EAMK); fabrice.boyom@fulbrightmail.org (FFB).
[2] Noguchi Memorial Institute for Medical Research (NMIMR), P.O. Box LG 581. Legon, Accra, Ghana. Email: PTsouh@noguchi.ug.edu.gh (PVTF).
[3] Institute of Medical Research and Medicinal Plants Studies (IMPM), P.O. Box 6163, Yaounde, Cameroon
[4] Laboratory of Natural Products and Organic Synthesis, Department of Organic Chemistry, Faculty of Science, University of Yaoundé I, P.O. Box: 812, Yaoundé, Cameroon. Email: tch_aimee@yahoo.fr (MATT).
[5] Laboratory of Animal Physiology, Faculty of Science, University of Yaoundé I, P.O. Box: 812, Yaoundé, Cameroon. email: fotsingrace@yahoo.fr (RGK).

*Author to whom correspondence should be addressed:
E-mail address: fabrice.boyom@fulbrightmail.org; Tel.: +237 67727 6585.

Abstract: The present study aimed at investigating the *in vitro* and *in vivo* susceptibility of malaria parasites to crude extracts and fractions from *Polyalthia suaveolens*, *Uvaria angolensis*, and *Monodora tenuifolia*. The ethanolic extracts were prepared by maceration, and were further partitioned using water, dichloromethane, hexane, and methanol. The most promising fraction was subjected to column chromatography and the sub-fractions tested for activity *in vitro*. The antiplasmodial effect of extracts and fractions was tested against the Chloroquine resistant (*Pf*K1) strain in 96 wells microtiter plate format using SYBR green florescence assay. The promising fraction was further assessed for cytotoxicity and acute toxicity in Swiss albino mice and subsequently against the rodent malaria parasite, *P. berghei.* Qualitative phytochemical screening was also performed on the promising fraction. The methanol fractions exerted the overall better effect with that of the twigs of *P. suaveolens* (PStw(Ace)) showing the highest potency with a IC_{50} value of 3.24 µg/mL followed by the fractions of leaf of *M. tenuifolia* (MoTel(Ace), IC_{50}= 3.84 µg/ml) and stem bark of *P. suaveolens* (IC_{50}= 4.90 µg/ml). The phytochemical screening showed the presence of alkaloids, lactones, and phenols in the more active fraction of *P. suaveolens* (PStw(Ace)). The chromatographic fractionation of this fraction led to 12 sub-fractions with PS8 sub-fraction being the most active (IC_{50}= 4.42 µg/mL). *In vivo*, oral administration of 2000 mg/kg b.w of fraction PStw(Ace) in mice showed no signs of

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 December 2016 doi:10.20944/preprints201612.0044.v1

toxicity. Also, fraction PStw(Ace) at 400 mg/kg b.w exerted the highest suppressive effect against *P. berghei* strain B throughout the 4 days experiment (% parasitaemia below 5.2%). Overall, the results achieved supported the use of the three plants in the traditional treatment of malaria in Cameroon. More interestingly, the methanolic fraction of the twigs extract from *P. suaveolens* might be of interest in future development of an antimalarial phytodrug.

Keywords: *Polyalthia suaveolens*; *Uvaria angolensis*; *Monodora tenuifolia*; *Plasmodium falciparum K1*; toxicity; antimalarial

---

1. Introduction

Malaria mainly caused by *Plasmodium falciparum* and *Plasmodium vivax* represents a serious public health challenge since about 3.3 billion people are at risk of infection **[1].** Sub-Saharan Africa is the most affected region of the world with 90% of the 584 000 deaths recorded in 2013 **[1].** Cameroon with 71% of the total population living in high-transmission areas and 4500 deaths recorded annually is one of the most affected countries in Africa **[1].** These high mortality and morbidity caused by malaria have motivated the search for new drugs against plasmodium parasites given that the available treatments led by the Artemisinin-based Combination Therapies are impaired by emerging resistant *Plasmodium falciparum* strains **[2]**.

Fortunately, several studies have shown the evidence that plants traditionally used against malaria are valuable sources of potent antiplasmodial lead compounds **[3]**. Thus, *Monodora tenuifoia*, *Uvaria angolensis*, and *Polyaltha suaveolens* were selected and studied for *in vitro* and *in vivo* antiplasmodial activities.

An ethnopharmacological survey of Annonaceae plants in four areas of Cameroon revealed people's reliance on the three selected plant species to treat malaria and related symptoms, sexually transmitted diseases (*P. suaveolens*), dysentery, fever, muscle pain, headache (*M. tenuifolia*), typhoid, yellow fever and epilepsy (*U. angolensis*) **[4-6].** Overall for these species (*P. suaveolens, Uvaria sp.,* and *M. tenuifolia*) the recorded ethnopharmacological information indicated that 500 g of stem bark removed by scraping with a machete or 500 g of fruits are decocted/infused in 3 liters of water for 20 minutes, and 250 mL of the decoction/infusion taken orally 3 times daily for 7-15 days.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 December 2016 doi:10.20944/preprints201612.0044.v1

Moreover, these plants belong to the Annonaceae family that is well known for biosynthesis of bioactive compounds **[7-8]**, particularly the acetogenins that have a broad spectrum of biological activities including antiplasmodial effect **[9-13].**

2. Materials and Methods

2.1 Plant collection

Leaves, twigs and stem bark of each plant were collected at Mount Kalla (Yaoundé) in September 2014 and identified at the National Herbarium of Cameroon where voucher specimens were deposited under the identification numbers HNC 55313, HNC 500594 and HNC 1227 for *M. tenuifolia*, *U. angolensis* and *P. suaveolens* respectively.

2.2 Plants extraction and fractionation

Each plant part was cut into small pieces, dried at room temperature till constant weight and powdered. Five hundred g of each powder were macerated with 2000 mL of ethanol for 72 hours at room temperature. The ethanol extracts were evaporated to dryness under vacuum using a rotary evaporator (Rotavapor Buchi, Swizterland) and weighed. Each ethanol extract was fractionated by liquid-liquid partition **[12]**. Briefly, ethanolic residues were partitioned between dichloromethane and water (1:1) to yield water and dichloromethane fractions as well as interface precipitates. Then, dichloromethane fractions were further partitioned between hexane and methanol (1:1) to yield hexane and methanol fractions referred to as acetogenin-rich fractions. All extracts and fractions were subjected to antiplasmodial activity screening *in vitro* against *Plasmodium falciparum* chloroquine resistant strain K1 (*PfK1*)

From this screening, the methanol fraction from the twigs of *P. suaveolens* (PStw(Ace)) was found to be the most active. It was submitted to qualitative phytochemical screening as previously described **[14-18].** Furthermore, it was fractionated using silica gel column chromatography, eluting with solvent systems of increasing polarities, Hexane-Ethyl Acetate [100:0 – 0:100] and Ethyl Acetate - Methanol [95:5- 0:100]. Four hundred and thirty eight (438) sub-fractions of 100 mL each were collected and subsequently pooled on the basis of their thin layer chromatography (TLC) profiles into twelve (12) major sub-fractions PS1-PS12 [PS1-Hex 100%, PS2-Hex-EtOAc (98 :2), PS3-Hex-EtOAc (97 :3→ 95: 5), PS4-Hex-EtOAc (95 :5→93 :7), PS5-Hex-EtOAc (93 :7), PS6-Hex-EtOAc (93 :7 →85 :15), PS7-Hex-EtOAc (85 :15→78 :22), PS8-Hex-EtOAc (78 :22→60 :40), PS9-

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 December 2016 doi:10.20944/preprints201612.0044.v1

Hex-EtOAc (60 :40→ 50 :50), PS10-Hex-EtOAc (50 :50→20 :80), PS11-Hex-EtOAc (20 :80→10 :90) / EtOAc -MeOH (98 :2), PS12-EtOAc -MeOH (95 :5)/MeOH 100%]. All these sub-fractions were assessed *in vitro* for antiplasmodial activity and cytotoxicity as described below.

2.3 *In vitro* antiplasmodial activity of crude extracts, fractions and sub-fractions

Stock solutions of crude extracts, fractions and sub-fractions were prepared at 1 mg/mL in DMSO 100%, while solution of artemisinin was prepared in distilled water and tested at 1µM highest concentration. The stock solutions were serially diluted in 96-wells plates using RPMI 1640 to achieve the range of tested concentrations from 0.22 µg/mL to 125 µg/mL.

PfK1 (*P. falciparum* chloroquine-resistant *K1* strain) was continuously maintained in fresh O^{+ve} human erythrocytes suspended at 4% (v/v) heamatocrit in complete medium, consisting of 16.2 g/L RPMI 1640 (Sigma) containing 25 mM HEPES, 11.11 mM glucose, 0.2% sodium bicarbonate (Sigma), 0.5% Albumax I (Gibco), 45 µg/L hypoxanthine (Sigma) and 50 µg/L gentamicin (Gibco) and incubated at 37°C in an atmosphere of 5% O_2, 5% CO_2, 90% N_2. The used medium was replaced daily with fresh complete medium to propagate the culture. Giemsa-stained blood smears were examined microscopically under oil immersion to monitor cell cycle transition and parasitaemia **[19]**. Prior the antiplasmodial assay, the culture was synchronized into ring stage parasites by 5% sorbitol (w/v) treatment as previously described **[20]**.

For drug activity screening, the SYBR green I based fluorescence assay was used as previously described **[21]**. This dye interacts with malaria parasite to produce a fluorescent complex. Ninety six microliters of synchronized ring stage parasites at 2% haematocrit and 1% parasitaemia were added into triplicate wells of 96-wells plates containing 4 µL of each inhibitor concentration. Negative control consisted of 0.4% DMSO and positive control of 1 µM Artemisinin (ART) highest concentration. The treated cultures were incubated for 48 hrs at 37°C in an atmosphere of 5% O_2, 5% CO_2, 90% N_2. Upon incubation, 100 µL of SYBR green I buffer [0.2 µL of 10,000 × SYBR Green I (Invitrogen) per mL of lysis buffer {Tris (20 mM; pH 7.5), EDTA (5 mM), saponin (0.008%; wt/vol), and Triton X-100 (0.08%; vol/vol)}] were added to each well and mixed gently and further incubated in the dark at 37°C for 1 h. Fluorescence was subsequently measured using a fluorescence multi-well plate reader (Perkin Elmer) with excitation and emission at 485 and 530 nm

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 December 2016 doi:10.20944/preprints201612.0044.v1

respectively. Fluorescence counts for ART were deducted from counts in each well and a dose–response curve was constructed by plotting fluorescence counts against the drug concentration and activity expressed as 50% inhibitory concentration (IC_{50}) using the IC Estimator-version 1.2 software (http://www.antimalarial-icestimator.net/MethodIntro.htm) where estimated parasite growth in the negative control (0.4% DMSO) is 100 %, and 0% in the positive control (125 nM ART) **[22-23]**.

2.4 Cytotoxicity of PStw(Ace) sub-fractions

The cytotoxic effects of the mother fraction (PStw(Ace)) and derived sub-fractions (PS1-12) on mammalian cells was determined using human foreskin fibroblasts (HFF) cells cultured in complete medium containing 13.5 g/L DMEM , 10% fetal bovine serum, 0.2% sodium bicarbonate (w/v) (Sigma) and 50 µg/mL gentamycin **[24]**. Cells ($5x10^3$cells/100 µL/well) were seeded into 96-well flat-bottom tissue culture plates in complete medium. After 24 h of seeding, 80 µL of each test solution were added and cells incubated for 48 h in a humidified atmosphere at 37°C and 5% CO_2. For each extract, the concentrations tested were ranged from 0.22-125 µg/mL. 0.4% DMSO v/v was tested as negative control. Twenty microliters of a kit solution of MTS/PMS (Promega) were added to each well, gently mixed and incubated for another 1.5 h at 37°C. After gentle supernatant removal and addition of 100 µL DMSO (quench agent) to the cell pellets, formazan formation was measured by recording optical density (OD) in each well using a microtiter plate reader ((Biotek EL800, USA) at 490 nm. Mean ODs were plotted versus drugs concentrations and the 50% cytotoxic concentration (CC_{50}) values were determined using the IC Estimator-version 1.2 software. Selectivity indices of extracts were subsequently calculated on the basis of their anti-plasmodial activities (IC_{50}) and HFF cell cytotoxicity (CC_{50}).

2.5 Safety and efficacy of twigs methanol fraction of *P. suaveolens* in mice

2.5.1 Experimental mice.

Six weeks old female Swiss albino mice (20–25 g) provided by the animal house of the Faculty of Medicine of the University of Yaoundé I were housed under standard conditions with 12 hours photoperiod and access to water and food *ad libitum* and used for the experiment. Animal welfare and ethical requirements were respected during the experiments.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 December 2016 doi:10.20944/preprints201612.0044.v1

2.5.2 Evaluation of the acute toxicity of the tested fraction

The acute toxicity of the methanol fraction from twigs of *P. suaveolens* was assessed in female Swiss albino mice prior to the *in vivo* antimalarial study. The study was performed according to the Organization for Economic Cooperation and Development guideline 423 **[25].** Mice were starved for about 4 h but allowed free access to water and weighed prior to test substances administration. Healthy mice were studied in 2 groups of 3 animals each. The test group received orally 1 mL of a single limit dose of 2000 mg/kg b.w of the fraction dissolved in 7% Tween 80/ 3% ethanol/ H_2O. The negative control group received 1 mL of 7% Tween 80/3% Ethanol/ H_2O solution. After the substances were administered, food was further withheld for 1 h, then animals were carefully observed for 4 h, and thereafter daily for 7 days during which several parameters such as mortality, moribund status, body weights, tremors, convulsions, salivation, diarrhea, lethargy, sleep, and other changes in the normal behavior were recorded daily.

2.5.3 *In vivo* antimalarial activity against *Plasmodium berghei*

The fraction PStw(Ace) that showed no signs of acute toxicity in mice was studied against *P. berghei* strain B (MRA-406, MR4, ATCC W Manassas Virginia) rodent malaria parasite model that was obtained from BEI Resources (www.beiresources.org) **[26]**. The parasites were maintained by several passages from infected to naïve mice monitored by Giemsa-stained smears observation. The antimalarial activity of PStw(Ace) fraction was studied through the suppressive test. Six groups of five animals each were used for this experiment. Briefly, animals were inoculated i.p. with 200 µL of 10^6 infected erythrocytes/mL normal saline and kept in observation for 4 h. Then, 4 groups individually received 1 mL of PStw(Ace) (dissolved in 7% Tween 80/ 3% Ethanol/ H_2O) at oral doses of 100, 200, 300, and 400 mg/kg b.w. respectively, daily for four consecutive days. Group 5 was treated as positive control and received 1 mL of daily dose of artemisinin at 14 mg/kg b.w. The last group considered as negative control received 1 mL of vehicle (7% Tween 80/ 3% Ethanol/ H_2O). The percentage of parasitemia was individually determined in infected mice using Giemsa-stained smears observation and infected erythrocytes counted under microscope at 100X magnification and mean values calculated each day for each dose. The higher the antiplasmodial potency of a dose, the stronger was considered its ability to suppress *P. berghei* parasitemia in mouse.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 December 2016 doi:10.20944/preprints201612.0044.v1

3. Results and Discussion

3.1 Plant extraction, partition and antiplasmodial screening

A total of 36 extracts and fractions were prepared from organs collected from *P. suaveolens*, *M. tenuifolia* and *U. angolensis* with yields ranging from 0.02 to 4.94% (w/w) respectively for the interface precipitate of the twigs of *P. suaveolens* (PStw(I)) and the crude ethanolic stem bark extract of *U. angolensis* (UvAsb(EtOH)) (Table 1).

The results of the screening of extracts and fractions against *P. falciparum* (Table 1) indicated that from the 9 crude ethanolic extracts that were tested, 6 [PSl(EtOH), PStw(EtOH), MoTel(EtOH), MoTetw(EtOH), UvAl(EtOH), and UvAtw(EtOH)] exhibited antiplasmodial activity with IC_{50} values ranging from 4.53 to 10 µg/mL. The most active was the crude ethanolic extract from the leaves of *P. suaveolens* (PSl(EtOH), IC_{50}= 4.53 µg/mL). The subsequent fractions afforded through liquid-liquid partition of all extracts also exhibited varying effects on the parasites. Among the water fractions, only the leaves of *M. tenuifolia* (MoTel(H_2O)) and *U. angolensis* (UvAl(H_2O)) showed moderate antiplasmodial activity at IC_{50} values of 7.75 and 9.78 µg/mL respectively. Also, 2 interface precipitates from the stem bark of *P. suaveolens* (PSsb(I)) and from the leaves of *M. tenuifolia* (MoTel(I)) exerted good activities at the respective IC_{50} values of 3.84 and 4.53 µg/mL.

The acetogenin-rich (methanolic) fractions globally showed better potency compared to other types of fractions, and were the most active with 6 out 9 fractions [PStw(Ace), PSsb(Ace), MoTel(Ace), MoTetw(Ace), UvAl(Ace), and UvAtw(Ace)] showing IC_{50} values ranging from 3.24 to 7.78 µg/mL. Overall, the acetogenin-rich fraction of the twig extract of *P. suaveolens* (PStw(Ace)) showed the best antiplasmodial activity (IC_{50} = 3.24 µg/mL) and was therefore selected for further studies.

Table 1: Extraction yield and antiplasmodial activity of extracts and fractions from *P. suaveolens*, *M. tenuifolia* and *U. angolensis* (Annonaceae)

Plant & voucher specimen number	**Extracts/fractions**	**Plant parts**	**Extract's code**	***Extraction Yield (% w/w)**	****IC_{50} (µg/ml± SD) against *PfK1***
Polyalthia suaveolens HNC 1227/SRF/CAM	Crude Ethanolic	Leaves	PSl(EtOH)	3.28	4.53± 0.82
		Twigs	PStw(EtOH)	3.80	5.75 ±1.83
		Stembark	PSsb(EtOH)	4.30	>10
	Water	Leaves	PSl(H_2O)	0.42	>10
		Twigs	PStw(H_2O)	0.98	>10
		Stembark	PSsb(H_2O)	1.92	>10
	Interface precipitates	Leaves	PSl(I)	0.07	>10
		Twigs	PStw(I)	0.02	>10
		Stembark	PSsb(I)	0.06	3.84±0.86
	Methanolic	Leaves	PSl(Ace)	1.63	>10
	Acetogenin-rich fractions	Twigs	PStw(Ace)	1.65	3.24± 01
		Stembark	PSsb(Ace)	1.45	4.90 ± 0.73
Monodora tenuifolia HNC55313/SRF/CAM	Crude ethanolic	Leaves	MoTel(EtOH)	3.98	5.48±0.19
		Twigs	MoTetw(EtOH)	1.79	8.93±0.19
		Stembark	MoTesb(EtOH)	4.03	>10
	Water	Leaves	MoTel(H_2O)	2.11	7.75± 0.33
		Twigs	MoTetw(H_2O)	1.24	>10
		Stembark	MoTesb(H_2O)	1.94	>10

doi:10.20944/preprints201612.0044.v1

	Interface precipitates	Leaves	MoTel(I)	0.33	4.53±0.44
		Twigs	MoTetw(I)	0.82	>10
		Stembark	MoTesb(I)	0.12	>10
	Methanolic	Leaves	MoTel (Ace)	0.82	3.84± 0.37
	Acetogenin-rich fractions	Twigs	MoTetw (Ace)	1.23	5.02 ± 0.80
		Stembark	MoTesb (Ace)	1.21	>10
Uvaria angolensis	Crude ethanolic	Leaves	UvAl(EtOH)	3.27	9.98± 2.87
HNC50059/SRF/CAM		Twigs	UvAtw(EtOH)	3.83	10.00± 0.87
		Stembark	UvAsb(EtOH)	4.94	>10
	Water	Leaves	UvAl(H_2O)	0.76	9.78 ± 1.87
		Twigs	UvAtw(H_2O)	0.06	>10
		Stembark	UvAsb(H_2O)	0.05	>10
	Interface precipitates	Leaves	UvAl(I)	0.74	>10
		Twigs	UvAtw(I)	0.18	>10
		Stembark	UvAsb(I)	0.23	>10
	Methanolic	Leaves	UvAl(Ace)	1.39	5.78 ± 0.75
	Acetogenin-rich fractions	Twigs	UvAtw(Ace)	1.31	7.78 ± 0.13
		Stembark	UvAsb(Ace)	1.98	>10

*Plant parts were extracted by maceration in ethanol and further partition using different solvents and the yield calculated in percentage relative to the weight of the starting materials. **50% inhibitory concentration; the susceptibility of *P. falciparum* K1 strain to plant extracts was assessed in culture using triplicate experiments; S.D. = standard deviation.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 December 2016 doi:10.20944/preprints201612.0044.v1

The potency of acetogenin-rich fractions has previously been reported **[9]** in a similar study targeting the Chloroquine-resistant *P. falciparum* W2 strain. In addition, studies by the same authors reported essential oils from the stem bark of *P. suaveolens* and *U. angolensis* as *in vitro* inhibitors of *P. falciparum* W2 **[27].** Globally, the observed inhibitory effect of methanolic fractions could be attributed to the presence of acetogenins. Indeed, these compounds have been shown to inhibit complex I (NADH: ubiquinone oxidoreductase) in mitochondrial electron transport systems **[28]**; in addition, they are potent inhibitors of NADH oxidase of plasma membranes **[29]**; these enzymes are all found in *P. falciparum* and the effects of inhibitors impair parasite oxidative and cytosolic ATP production, leading to apoptosis. Acetogenins have been also reported as potent cytotoxics with insecticidal, ascaricidal, fungicidal, antiparasitic, bactericidal and antiplasmodial activities **[10-13]**.

Little is known about the antiplasmodial activity of extracts from *M. tenuifolia.* However, the methanol (acetogenin-rich) fractions from a closely related species, *Monodora myristica* was previously showed to have moderate antiplasmodial activities with IC_{50} values ranging 5.52-9.03 µg/mL **[9].** Also, recent studies on other *Uvaria* spp., *viz. Uvaria lucida* and *Uvaria scheffleri* showed inhibition of *P. falciparum* W2 with IC_{50} values of 10.3 and 6.8 µg/mL respectively **[30].**

A qualitative phytochemical screening of the promising *P. suaveolens* (PStw(Ace)) fraction indicated the presence of three main classes of secondary metabolites, including alkaloids, phenols, and lactones. These groups of compounds have been previously reported to have significant inhibitory effects on *P. falciparum* **[31-33]**. This fraction was further fractionated to afford 12 major sub-fractions that were screened for antiplasmodial activity and cell cytotoxicity.

3.2 Antiplasmodial activity of sub-fractions from the twigs acetogenin-rich fraction of *P. suaveolens*

The results presented in table 2 indicated that from the 12 sub-fractions afforded from the twigs acetogenin-rich fraction of *P. suaveolens*, only PS7 and PS8 showed antiplasmodial activity. They were respectively eluted with Hex-EtOAc (85 :15→78 :22) and Hex-EtOAc (78 :22→60 :40). PS8 exerted the more potent inhibition of the growth of malaria parasites (IC_{50} = 4.42 µg/mL), compared to PS7 that rather exhibited a moderate antiplasmodial inhibition with IC_{50} of 8.55µg/mL. Apart from these two sub-fractions, all the others were not active against *P. falciparum* in culture. The phytochemical screening of the mother fraction (PStw(Ace)) indicated the presence of alkaloids, phenols, and lactones that might be

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 December 2016 doi:10.20944/preprints201612.0044.v1

individually and/or collectively responsible for the exhibited antiplasmodial activity. Functional terminal lactones or butenolide often characterize the acetogenins **[34]**. Previous studies have shown that lactones can be readily extracted using solvents systems in the same range as those used to afford sub-fractions PS7 and PS8 **[35]**. Also, it has been shown that Hex-EtOAc (80:20 v/v) is a suitable solvent combination to extract alkaloids **[36]**. It is therefore tentatively assumable that components of the active sub-fractions from PStw(Ace) might be alkaloid-like and or bear lactone functional groups. Moreover, the potent sub-fraction PS8 (IC_{50} = 4.42 µg/mL) also showed better selectivity (SI> 6.79) compared to PS7.

Table 2: Antiplasmodial and cytotoxic activities of sub-fractions of the *P. suaveolens* twigs acetogenin-rich fraction

Fraction/[a]sub-fractions	[b]IC_{50} (µg/ml± SD) against *PfK1*	[c]CC_{50} against HFF (µg/ml)	[d]SI (CC_{50}/IC_{50})
PStw(Ace)	3.24± 01	>30	>9.26
PS1	>10	-	-
PS2	>10	-	-
PS3	>10	-	-
PS4	>10	-	-
PS5	>10	-	-
PS6	>10	-	-
PS7	8.55 ± 2.05	>30	> 3,51
PS8	4.42 ± 0.87	>30	> 6.79
PS9	>10	-	-
PS10	>10	-	-
PS11	>10	-	-
PS12	>10	-	-
ART	0.005±0.0008	>30	>6000

[a]The 12 sub-fractions were afforded by column chromatography eluting with solvent systems of increasing polarity; [b]50% inhibitory concentration; the susceptibility of *P. falciparum* K1 strain to sub-fractions was assessed in culture using triplicate experiments; S.D. = standard deviation; [c]Cell cytotoxicity of sub-fractions was assessed on normal human foreskin fibroblast cells (HFF) using triplicate experiments; [d]Selectivity indices were calculated using the IC_{50} and CC_{50} of each sub-fraction; ART (Artemisinin) was tested as positive control.

Overall, from the bio-guided studies of the mother *P. suaveolens* twigs acetogenin-rich fraction (PStw(Ace)), it appeared that fractionation led to activity decline. Of note, PStw(Ace) (IC_{50}= 3.24 µg/mL) was more active and selective than the subsequent sub-fractions afforded from column chromatography. This decrease in potency through fractionation might result

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 December 2016 doi:10.20944/preprints201612.0044.v1

from rupture of synergistic interactions between its components, and also suggests its further development as potential antimalarial ameliorated plant extract. It is within this framework that the PStw(Ace) fraction was evaluated for safety in mice, and for antimalarial efficacy using the rodent model *Plasmodium berghei* parasites.

3.3 *In vivo* activities of the twigs acetogenin-rich fraction of *P. suaveolens* (PStw(Ace)).

3.3.1 Acute toxicity profile of PStw(Ace) fraction

The study of the promising twigs methanolic fraction of *P. suaveolens* showed safety at the dose of 2000 mg/Kg b.w. through oral administration in mice. Briefly, no death was recorded among experimental animals. Moreover, no major behavioral changes (moribund status, body weight loss, tremors, convulsions, salivation, diarrhea, persistent lethargy or sleep) were recorded during the 7 days observation beyond plant fraction administration. Overall, test animals globally behaved like controls (untreated mice). Therefore, the 50% lethal dose (LD_{50}) was > 2000 mg/Kg b.w, and the plant fraction categorized as nontoxic according to the OECD guidelines **[25]**.

3.3.2 Suppressive antiplasmodial activity of the *P. suaveolens* twigs acetogenin-rich sub-fraction in mice

From the four days activity assessment, the suppressive effect of the twigs acetogenin-rich fraction of *P. suaveolens* against *P. berghei* in mice was determined as presented in figure 1. On day one of the experiment, the percent parasitaemia was globally similar in all six groups of mice, indicating that infection was at the settlement stage in all inoculated animals.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 December 2016 doi:10.20944/preprints201612.0044.v1

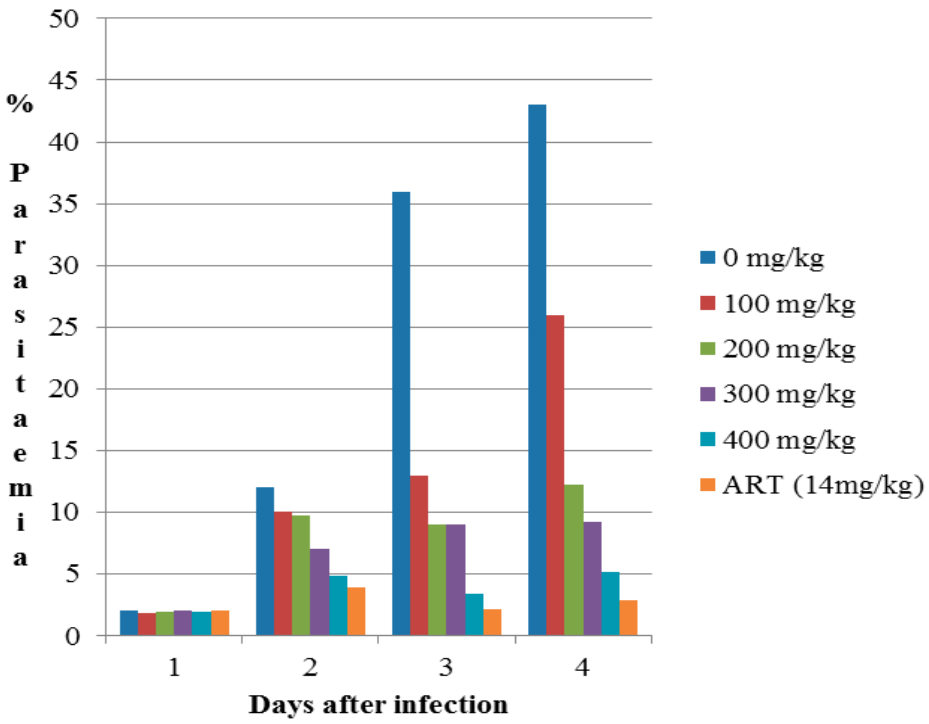

Figure 1: Suppressive effect of PStw(Ace) at different doses on *P. berghei* MRA-406 strain B in mice. Mice were treated with four doses of the fraction for four consecutive days. Mean % parasitaemias in each experimental group was determined daily by microscopic observation of tail blood thin smears stained with 5% Giemsa, and counting % erythrocytes infected with *P. berghei*.

Afterwards, the quality control assay (dose 0) indicated rising parasitaemia from day 1 to day 4 of the experiment (Figure 1). Besides, the PStw(Ace) exerted a dose-dependent suppression of the parasitaemia, and the more pronounced effect was observed at the highest dose of 400 mg/kg b.w. Although the reference Artemisinin showed to be more active, it is of note that its activity profile was quite comparable to that of PStw(Ace). In both cases, parasitaemia suppression was dose-dependent and more pronounced on day three after infection. These results further emphasized the promising feature of fraction PStw(Ace) that otherwise showed to be safe in mice at doses below 2000 mg/kg b.w.

Conclusion

The results achieved from this investigation have indicated that some plant extracts from the studied Annonaceae have antiplasmodial activity. Of particular note, the methanol fraction PStw(Ace) (acetogenin-rich) obtained from liquid-liquid partition of the *P.*

 doi:10.20944/preprints201612.0044.v1

suaveolens twigs ethanol crude extract has shown promising antiplasmodial activity. Further study of this fraction has also indicated its safety in mice, and suppressive potential of *P. berghei* parasitaemia over a four days study. This acetogenin-rich fraction should be studied in details to determine its qualitative and quantitative compositions in an attempt to formulate a standardized phytodrug against malaria.

Author contributions

FFB designed and supervised the study; ANM, LRYT, CDJM, MATT, RMTK, PVTF, EAMK, and RGK performed the chemical and biological (*in vitro* and *in vivo* studies) parts of the study and drafted the manuscript; FFB, EAMK, and RGK critically revised the manuscript. All authors agreed on the final version of the manuscript for submission to *Medicines,* ***Special Issue*** *"Plant Medicines for Clinical Trial".*

Compliance with ethical standards

The authors declare no competing interest.

Acknowledgement

The authors acknowledge the support by the Seeding Labs' Instrumental Access Grant (SL2012-2) to FFB.

References

1. World Malaria Report 2014, World Health Organization, Geneva, www.who.int/malaria/publications/world_malaria_report_2014/en/.

2. Dondorp, A.M.; Nostern, F.; Yi, P.; Das, D.; Phyo, A.P.; Tarning, J.; Lwin, K.M.; Ariey, F.; Hanpithakpong, W., Lee, S.J.; Ringwald, P.; Silamut, K.; Imwong, M.; Chotivanich, K.; Lim, P.; Herdman, T.; An Sen, S.; Yeung, S.; Singhasivann, P.; Day, N.P.J.; Lindergardh, N.; Socheat, D.; White, N.J. Artemisinin-Resistant *Plasmodium falciparum* Malaria. *N. Engl. J. Med.* **2009**, 361, 455-467.

3. Ginsburg, H.; Deharo, E. A call for using natural compounds in the development of new antimalarial treatments–an introduction. *Malaria J.* 2011, 10, 1-7.

4. Tsabang, N. ; Tsouh, F.P.V.; Tchokouaha, L.R.Y.; Noguem, B.; Bakarnga-Via, I.; Dongmo, N.M.S.; Nkongmeneckc, B.A.; Boyom, F.F. Ethnopharmacological survey of Annonaceae medicinal plants used to treat malaria in four areas of Cameroon. *J. Ethnopharmacol.* **2012**, 139, 171–180.

5. Bele, M.Y.; Focho, D.A.; Egbe, E.A.; Chuyong, B.G. Ethnobotanical survey of the uses of Annonaceae around mount Cameroon. *Afr. J. Plant Sci*. **2011**, 5(4), 237-247.

6. Burkill, H.M. *The useful plants of west tropical Africa*, 2nd edition. Royal Botanic Gardens, Kew, UK, **1985**.

7. Adeoye, A.O.; Oguntim, B.O. 3-Dimethylallylindole: An Antibacterial and Antifungal metabolite from *Monodora tenuifolia*. *J. Nat. Prod*. **1986,** 49(3), 534-537.

8. Leboeuf, M.; Cavé, A.; Bhaumik, P.K.; Mukherjee, R. The phytochemistry of the Annonaceae. *Phytochemistry* **1982**, 21, 2783-2813.

9. Boyom, F.F.; Tsouh, F.P.V.; Tchokouaha, Y.L.R.; Ngoutane, M.A.; Madiesse, K.E.; Mbacham, F.W.; Tsamo, E.; Amvam, Z.P.H.; Gut, J.; Rosenthal, P.J. Potent antiplasmodial extracts from Cameroonian Annonaceae. *J. Ethnopharmacol*. **2011**, 134, 717–724.

10. Rakotomanga, M.; Razakantoanina, V.; Raynaud, S.; Loiseau, P.M.; Hocquemiller, R.; Jaureguiberry, G. Antiplasmodial activity of acetogenins and inhibitory effect on *Plasmodium falciparum* adenylate translocase. *J. Chemother*. **2004**, 16, 350–356.

11. Guadaño, A.; Gutiérrez, C.; DelaPeña, E.; Cortes, D.; González, C.A. Insecticidal and mutagenic evaluation of two annonaceous acetogenins. *J. Nat. Prod.,* **2000**, 63, 773-776.

12. Alali, F.Q.; Liu, X.X.; Mc Laughlin, J.L. Annonaceous acetogenins: recent progress. *J. Nat. Prod.,* **1999**, 62, 504–540.

13. Rupprecht, J.K.; Chang, J.M.; McLaughin, J.L. Annonaceous acetogenins: a review. *J. Nat. Prod.,* **1990**, 53, 237-278.

14. Odebeyi, O.O.; Sofowora, E.A. Phytochemical screening: Nigeria medical plants. *Lloydia* **1978**, 41, 234-325.

15. Harbone, J.B. *Phytochemical methods. A guide of modern techniques of plants analysis*. Chapman and Hall, 1st Edition,London, St Edmuds Burg Press. **1976**, 1-295.

16. Trease, G.E.; Evans, W.C.A. *Textbook of Pharmacognosy*. 14th Ed., Bailliere Tindall Ltd, London. **1996**, 60-75.

17. Oloyede, O.I. Chemical profile of unripe pulp of *Carica papaya*. *Pakist. J. Nutrition* **2005**, 4, 379-381.

18. Aromdee, C.; Wichitchote, P.; Jantakun, N. Spectrophotometric determination of total lactones in *Andrographis paniculata* Nees. *Songklanakarin J. Sci. Technol*. **2005**, 27(6), 1227-1231.

19. Trager, W.; Jensen, J.B. Human malaria parasites in continuous culture. *Science* 1976, 193, 673–675.

 doi:10.20944/preprints201612.0044.v1

20. Lambros, C.; Vanderberg, J.P. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *J. Parasitol*. **1979**, 65, 418-420.

21. Smilkstein, M.; Sriwilaijaroen, N.; Kelly, J.X.; Wilairat, P.; Riscoe, M. Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. *Antimicrob. Agents & Chemother*. **2004**, 48, 1803–1806.

22. Le Nagard, H.; Vincent, C.; Mentré, F.; Le Bras, J. Online analysis of in vitro resistance to antimalarial drugs through nonlinear regression. *Comput Methods Programs Biomed*, 2011, 104(1),10-8.

23. Kaddouri, H.; Nakache, S.; Houzé, S.; Mentré, F.; Le Bras, J. Drug Susceptibility of *Plasmodium falciparum* Clinical Isolates from Africa using *Plasmodium* Lactate Dehydrogenase Immunodetection Assay and inhibitory Emax model for precise IC_{50} measurement. *Antimicrob. Agents & Chemother*. **2006**, 50, 3343-3349.

24. Boyom, F.F.; Fokou, P.V.T.; Tchokouaha, L.R.Y.; Spangenberg, T.; Mfopa, A.N.; Kouipou, R.M.T.; Mbouna, C.D.J.; Donfack, V.F.D.; Zollo. P.H.A. Repurposing the Open Access Malaria Box to discover potent inhibitors of *Toxoplasma gondii* and *Entamoeba histolytica*. *Antimicrob. Agents & Chemother*. **2014**, 58(10), 5848–5854.

25. Organization for Economic Co-operation and Development (OECD). *Guideline for Testing of Chemical, Acute Oral Toxicity –Up-and-Down Procedure*. **2001**, 423:1-14.

26. Fidock, D.A.; Rosenthal, J.P.; Croft, L.S.; Brun, R.; Nwaka, S. Antimalarial drug discovery:efficacy models for compound screening. *Nat. Rev. Drugs Discov*. **2004**, 10, 509-520.

27. Boyom, F.F. *Huiles essentielles d'Annonaceae Camerounaises: Analyse chimique, Activités antioxydante et Antiplasmodiale*. Doctorate thesis, Biochemistry, University of Yaoundé I. 2004, pp. 67-172.

28. Lewis, M.A.; Arnason, J.T.; Philogene, B.J.R.; Rupprecht, J.K.; McLaughlin, J.L. Inhibition of respiration at site I by asimicin, an insecticidal acetogenin of the Pawpaw *Asimina triloba* (Annonaceae). *Pest. Biochem. Physiol*. **1993**, 45,15-23.

29. Morré, D.J.; DeCabo, R.; Farley, C.; Oberlies, N.H.; McLaughlin, J.L. Mode of action of bullatacin, a potent antitumor acetogenin: inhibition of NADH oxidase activity HeLa and HL-60 but not liver, plasma membranes. *Life Sci*. **1995**, 56, 343-348.

30. Muthaura, C.N.; Keriko, J.M.; Mutai, C.; Yenesew, A.; Gathirwa, J.W.; Irungu, B.N.; Nyangacha, R.; Mungai, G.M.; Derese, S. Antiplasmodial potential of traditional antimalarial phytotherapy remedies used by the Kwale community of the Kenyan Coast. *J. Ethnopharmacol*. **2015**, 170, 148–157.

31. Tasdemir, D.; Lack, G.; Brun, R.; Ruedi, P.; Scapozza, L.; Perozzo, R. Inhibition of *Plasmodium falciparum* fatty acid biosynthesis: evaluation of FabG, FabZ, and FabI as drug targets for flavonoids. *J. Med. Chem*. **2006**, 49 (11), 3345–3353.

32. Kaur, K.; Jain, M.; Kaur, T.; Jain, R. Antimalarials from nature. *Bioorg. & Med. Chem*. **2009**, 10, 1-28.

33. Jimenez-Romero, C.; Ortega-Barría, E.; Arnold, A.E.; Cubilla-Rios, L. Activity against *Plasmodium falciparum* of lactones isolated from the endophytic fungus *Xylaria sp. Pharm. Biol*. **2008**, 46(10-11), 1–4.

34. Li, N.; Shi, Z.; Tang, Y.; Chen, J.; Li, X. Recent progress on the total synthesis of acetogenins from Annonaceae. Beilstein J. Org. Chem. **2008**, 4 (48), 1–62.

35. Khetarpal, V.K.; Storbeck, L.S.; Wells, D.; Meacham, R.H. Jr. Method for separation and determination of lactone and hydroxy acid forms of a new HMG CoA reductase inhibitor (RG 12561) in plasma. *J. Chromatography* **1991**, 567(2), 491-497.

36. Bett, S. 2015. at https://www.quora.com/Why-is-a-mixture-of-hexane-and-ethyl-acetate-used-in-this-alkaloid-extraction-procedure, Feb. 26, 2015.