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In vitro evaluation of aliphatic fatty alcohol metabolites of *Perseaamericana* seed as potential antimalarial and antimicrobial agents

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Abstract

***Perseaamericana*Mill (Lauraceae) is a local medicinal plant used in Nigerian ethnomedicine as antimalarial. The aqueous decoction of the root part is a potent remedy against bacterial infections. Hence, the need to investigate the phytochemical and biological activities (antimicrobial and antiplasmodial) of the rootback of *Perseaamericana*. Chromatographic and spectroscopic techniques were used in the identification and purification of metabolites, which were assayed for antimalarial and antimicrobial activities using Plasmodium falciparum and a panel of microorganisms. From the seeds of *P. americana*, five known 1, 2, 4-dihydroxy derivatives aliphatic alcohols, called avocadenols were isolated and identified by spectroscopic methodsincluding 1D- and 2D NMR, and comparison withreported data in literature. Antifungal activity for 1, 2, 4-Trihydroxyheptadec-6-en-16-yne (5)(IC_{50}< 8 µg/mL) against all the fungal strains and *S. areus,* and antimalarial activity for compounds 1, 2, 4-Trihydroxyheptadec-16-ene (1) and 1, 2, 4-tetrahydroxyheptadecane-6, 16-diene(2)(IC_{50} = 1.6 and 1.4µg/mL for the D6 clone, respectively, and 2.1 and 1.4µg/mL for the W2 clone, respectively) was observed. The fatty alcohols 1, 2, 4-tetrahydroxyheptadecane-6, 16-diene(2); 1, 2, 4-Trihydroxyheptadec-16-yne(3) and 1, 2, 4-Trihydroxyheptadecane(4) also exhibited promising *in vitro* antibacterial activity against a panel of pathogenic bacteria *S.areus*, *methicillin resistant S. areus* and *E.coli* at IC_{50} values of 21.1, 8, 200µg/mL), (3.259, 86.32µg/mL) and (17.18, 8.26 and 200µg/mL), respectively.The results of this study provide evidence that the fatty alcohols are a promising class of antimalarial and antimicrobial agents.**

Keywords: metabolites, antimalarial, antimicrobial isolation, *Perseaamericana*, seeds, *Plasmodium falciparum*

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Introduction

Parasitic diseases such as malaria have a high mortality rate having a significant impact in developing countries and affecting several hundred millions of people worldwide.Malaria is one of the most important parasitic diseases in the world and is a major global health problem affecting over one hundred countries with disease prevalence escalating at an alarming rate, particularly in the last two decades. Rapid development of resistance by *Plasmodium falciparum* to the conventional drugs such as chloroquine necessitates the search for new antimalarials (Iwu*et al*., 1994; Wolf, 2002; Guerin *et al*.,

2002; Arguello, 1995; Fournet and Munoz, 2002). Malaria, a devastating infectious disease caused by highly adaptable protozoan parasites of the genus Plasmodium, has impacted on humans for more than 4000 years, causing illness andan estimated 1.5–2.5 million deaths each year. Malaria is endemic throughout the tropics, especially in sub-Saharan Africa and the developing world, threatening about 40 % of the world's population. Although four Plasmodium parasite species can infect humans, *Plasmodium falciparum* causes the majority of illnesses and deaths. Severe malaria, defined as acute malaria with major signs of organdysfunction or high levels of parasitemia, predominantly affectschildren and pregnant women (Pierce and Miller, 2009; Rosenthal, 2008; White, 2008).Chemotherapy is still at the forefront in the fight against malariadue to the unavailability of effective vaccines. Numerous drugshave been developed for the treatment of uncomplicated malaria,for example, mefloquine, primaquine, quinidine, proguanil (Genton, 2008; Vekemans and Ballou, 2008). There is still need to look inwards for newer and novel antimalarial agents from natural products via ethnopharmacological approach.

Similarly, an increasing number of multidrug-resistantmicrobial pathogens have become a serious problemparticularly during the last decade and provide theimpetus for the search and discovery of novel antibacterialand antifungal agents active against thesepathogens(Vekemans and Ballou, 2008).

Perseaamericana Mill commonly known as 'avocado pear' is a medium-sized, single-stemmed, terrestrial, erect, perennial, deciduous, evergreen tree of 15–20 m in height. The leaves and other morphological parts of *P. americana*possess medicinal properties, and are widelyused in traditional medicines of many African countries as antitussive,antimicrobial, antidiabetic, antiparasitic, anti-allergic, antihypertensive, analgesic and anti-inflammatory remedies (Adeyemi*et al*., 2002; Adeboye*et al*., 1999; Owolabi*et al*., 2005; Oberlies*et al*., 1998).

In this study, five aliphatic fatty alcohols metabolites isolated from the seeds of *P.americana* were evaluated for the first time for antimalarial activities. The antimicrobial activity was expanded to accommodate five bacteria and fungi.

Materials and Methods

Plant materials: The unripe fruit was collected from Edo State, Nigeria in January, 2014, identified and authenticated by Mr. Ugbogu O. A. and Shasanya O. S. of the Forest Research Institute of Nigeria (FRIN), Ibadan where voucher specimen is deposited in the herbarium.

Extraction: The seeds were removed, cut into small pieces, dried and pulverized in an oven at 30^{o}C for 4 days. The powdered (100 g) material of sample was extracted with 500 ml methanol for 48 hr(3 X) by cold maceration, filtered and the filtrate evaporated to dryness to obtained crude extract.

Isolation: Vacuum liquid chromatography VLC of total extract gave hexane 100% 3 g, Hexane :ethylacetate 50%, 4.3 g; ethylacetate 100%, 7.4 g; ethylacetate: methanol 50%, 6.5g and methanol 100%, 12.2 g. LH-20 sephadex of 50% hexane /EtOAc in Dichloromethane: methanol (1:1) gave 20 fractions. Fractions 5-10 pooled together based on similar R_f values, was subjected to HPLC reversed phase C-18 column, using MeCN-H_2O 95:5%, compounds **1**(8mg), **2** (5mg)and**3**(12mg) were resolved and eluted. Fractions 12 -15 was chromatographed on RP-C18, eluting with MeCN-H_2O (93:7) to obtain compounds **4** (6 mg) and **5** (4 mg).

Compound 1: 1, 2, 4-Trihydroxyheptadec-16-ene: white powder solid: 80 mg; mp 68-70^{o}C; $UV_{\lambda max}$ 202 nm: IR v_{max} (KBr) cm$^{-1,}$ 3320, 3297, 2920, 2850; HRCIMS *m/z* 287.2544 for $C_{17}H_{35}O_3$ 287.25387. ^{1}HNMR and ^{13}CNMR compared to Oberlies*et al*., 1998.

Compound 2: 1, 2, 4-tetrahydroxyheptadecane-6, 16-diene: colourlessprizms: 150 mg; mp 82-84^{o}C; $UV_{\lambda max}$ 208 nm: IR v_{max} (KBr) cm$^{-1,}$ 3435, 1649, 2918, 2850, 1470. HRCIMS *m/z* 325.2458 for $C_{17}H_{34}O_4$ (325. 2574). ^{1}HNMR and ^{13}CNMR compared to Ying-Chen *et al*., 2012.

Compound 3: 1, 2, 4-Trihydroxyheptadec-16-yne: white powdery solid: 35 mg; mp 74-76^{o}C; $UV_{\lambda max}$ 203 nm: IR v_{max} (KBr) cm$^{-1,}$ 3434, 3384, 3281, 2917; 2849, 1467, HRCIMS *m/z* 285.2464 for $C_{17}H_{33}O_3$ 287.25387. ^{1}HNMR and ^{13}CNMR compared to Oberlies*et al*., 1998.

Compound 4: 1, 2, 4-Trihydroxyheptadecane: white powder solid: 50 mg; mp 79-81^{o}C; $UV_{\lambda max}$ 200 nm: IR v_{max} (KBr) cm$^{-1,}$ 3312, 3300, 2918, 2850, 1470. HRCIMS *m/z* 317.2084 for $C_{19}H_{41}O_3$ (317. 2074). ^{1}HNMR and ^{13}CNMR compared to Oberlies*et al*., 1998.

Compound 5: 1, 2, 4-Trihydroxyheptadec-6-en-16-yne: colourless oil: 134 mg; $UV_{\lambda max}$ 208 nm: IR v_{max} (KBr) $cm^{-1,}$ 3368, 3314, 1662. HRCIMS *m/z* 305.2848 for $C_{17}H_{30}O_3$ (305. 2870). ^{1}HNMR and ^{13}CNMR compared to Ying-Chen *et al*., 2012.

Antimicrobial testing: In vitro antimicrobial activity against a panel of microorganisms, including fungi: *Candida albicans* (ATCC 90028), *Candida glabrata* (ATCC 90030), *Candida krusei*(ATCC 6258), *Cryptococcus . neoformans*(ATCC 90113) and*Aspergillusfumigatus* (ATCC 204305); and bacteria: *Staphylococcus aureus* (ATCC 29213), *methicillin-resistant S. aureus* (MRSa) (ATCC 33591), *Escherichia coli*(ATCC 35218), *Pseudomonas aeruginosa* (ATCC 27853) and *Mycobacterium intracellulare*(ATCC 23068), was determined using modified versions of the CLSI/NCCLS methods(NCCLS, 2000; NCCLS, 2002). *M. intracellulare*and *A. fumigatus*was tested using an Alamar Blue method(Franzblau, 1998).All organisms were obtained from the American Type Culture Collection (Manassas, VA). Samples, dissolved in DMSO, wereserially diluted in saline and transferred in duplicate to 96 well micro plates.Susceptibility testing was performed for all organcate to 96-well flat bottom micro plates. Microbial inocula were prepared by correcting the OD630 of microbe suspensions in incubationbroth to afford final target inocula. Controls [fungi: amphotericin B;bacteria: ciprofloxacin (ICN Biomedicals, OH)] were included in eachassay. All plates were read at 530 or 544(ex)/590(em) nm (*M. intracellulare* and*A. fumigatus*) prior to and after incubation. Percentgrowth was plotted versus test concentration to afford the IC_{50}using XLFit (Alameda, CA).

Antimalarial/Parasite LDH Assay: The *in vitro* antimalarial assay procedureutilized was an adaptation of the parasite lactate dehydrogenase (pLDH) assay developed by Makler*et al*., 1993. The assay was performed in a 96-well microplate and included two *P. falciparum* clones [Sierra Leone D6 (chloroquine-sensitive) and Indochina W2 (chloroquine- resistant)]. In primary screening the compounds were tested, in duplicate, at a single concentration of 15.9 *í*g/mL only on the chloroquine-sensitive (D6) strain of *P.falciparum*. The compound showing >50% growth inhibition ofthe parasite was subjected to screening. For bioassay-guidedfractionation, the column fractions were also tested only atsingle concentration. The pure compounds were subjected toadditional testing for determination of IC_{50} values. Thestandard antimalarial agents' chloroquine and artemisininwere used as positive controls, with DMSO (0.25%)as the negative(vehicle) control. The selectivity indices (SI) were determinedby measuring the cytotoxicity of samples on mammalian cells(VERO; monkey kidney fibroblast). All experiments were carried out in duplicate.

Results

The phytochemical investigation of *Persiaamericana* seeds led to the isolation and characterization of five compounds. Figure 1 displays the compounds isolated and characterized. The compounds were identified as 2*S* 4*S*-1, 2, 4-trihydroxyheptadec-16-ene(**1**); 1, 2, 4, 15-tetrahydroxyheptadec-6,16-diene(**2**); 1,2-4-trihydroxyheptadec-16yne(**3**); 1,2,4 trihydroxynonadecane(**4**) and 1, 2, 4-trihydroxyheptadecane 6-ene, 16-yne (**5**)).

The antimalarial activities of all isolatedmetabolites are reported as IC_{50}values against chloroquine-sensitive (D6) and chloroquine-resistant (W2) strains of *P. falciparum* in Table 1.The results of the antimicrobial activity of the compounds are shown in Table 2.

Discussion

The Powdered sample of *Perseaamericana* was subjected to activity guided isolation technique. Column chromatography, LH-20 sephadex and HPLC were used in isolation of compounds **1-5** which were characterized by 1D NMR and comparison of physical properties and spectroscopic data with those reported in literature. The pure isolates were assayed for antimalarial and antimicrobial activities against a panel of microorganisms. These aliphatic alcohol metabolites isolated and characterized, possessed various degrees of unsaturation. The presence of a 1, 2, 4 trihydroxy alcohol was diagnostic in the metabolites. The compounds were established unambiquously as 2*S* 4*S*-1, 2, 4-trihydroxyheptadec-16-ene(**1**); 1, 2, 4, 15-tetrahydroxyheptadec-6, 16-diene (**2**)1,2-4-trihydroxyheptadec-16-yne (3)1,2,4,trihydroxynonadecane (**4**) and 1, 2, 4-trihydroxyheptadecane 6-ene, 16-yne (**5**) which were in agreement with previously isolated compounds (Oberlies*et al*., 1998; Ying-Chen *et al*., 2012).

All purified compounds **1-5** were evaluated for *in vitro* antimalarial activity (against chloroquine sensitive (D6) and resistant (W2) clones of *Plasmodium falciparum*), cytotoxicity and for antimicrobial activity.Determination of *in vitro* antimalarial activity was based on the assay of plasmodial LDH activity. Among the series, compound **1** was the most active against both strains of plasmodium. Chloroquine and artemisinin were used as positive controls which showed IC_{50} values of 16.0 and 8.5 ng/mL (for D6) and IC_{50} of 150.0 and 9.0 ng/mL (for W2), respectively. None of the tested compounds or fractions had cytotoxic effects towards mammalian kidney fibroblasts (Vero cells) up to a concentration of 23.8 mg/mL.*In vitro* cytotoxicity of all the metabolites was determined against mammalian kidney cell line(Vero) up to a highest concentration of 10 µg/mL by neutral red assay. None of the compounds were found cytotoxic indicating a selectivity of antimalarial action. This is the first report of the antimalarial activity of this class of metabolites.

Compounds **1** and **2** also possessed moderate activity against *S.aureus* with IC_{50} values >200 µg/mL. The five compounds demonstrated moderate activity against *E. coli, P.aeruginosa,* and *Mycobacterium intracellulare* at IC_{50} value of >200 µg/mL each. Compound **1** also exhibited strong activity against MRSA (IC_{50} 13.81 µg/mL).

The antifungal activity of metabolites is presented in Table 2. Compounds **2, 3, 4** and **5** were fungicidal at IC_{50} values ranging from < 8 - 200 µg/mL. Compound **5** however showed the most promising antifungal activity against all the fungi used in this study at IC_{50} of < 8 µg/mL.Marked activity against *C. neoformans* was displayed at the tested concentration. These metabolites probably will be very useful in HIV/AIDS patients where the dominant pathogenic organism is *C. neoformans*. The positive control amphotericin B gave IC_{50}/MIC values of 0.2/0.6 mg/mL, respectively. Compound **1** exhibited moderate activity against all the fungi at the tested concentration. Further *in vivo* studies will be useful in the future to establish the possible mechanism of action.

Conclusion

The antimalarial activities of *P. americana* have never been conducted. This is the first report of the antimalarial activity of the fatty alcohols metabolites of *P. americana*. The metabolites were found to be possess potent activity at the concentration tested.

Table 1: Activity ofmetabolites of *P.americana*against *Plasmodium falciparum*

	IC_{50}(µg/mL) of metabolites				
Metabolites	*P. falciparum* D6 IC_{50}	*P. falciparum* D6 SI	*P. falciparum* W2 IC_{50}	*P. falciparum* W2 SI	VERO IC_{50}
1	29713.3	>1.6	3448.3	>1.4	>47600
2	22683.7	>2.1	34152.8	>1.4	>47600
3	25881.6	>1.8	32544.6	>1.5	>47600
4	28194.4	>1.7	40843.9	>1.2	>4760
5	>47600	>1	>47600	1	>47600

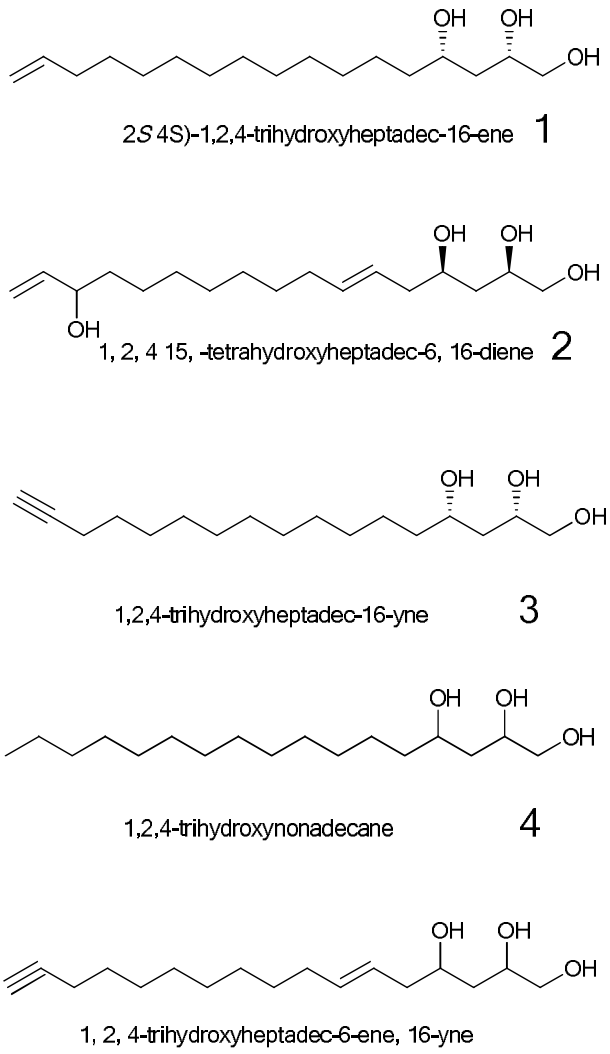

Figure 1: Chemical structures of metabolites isolated from *P.americana*seeds

Table 2: Antimicrobial activities of metabolites of *P. americana* seed extract

Test organism	IC_{50} (µg/mL) of compounds						
	1	2	3	4	5	Amphotericin B	Ciprofloxacin
C.albicans	>200	>200	>200	>200	>8	0.27	NT
C. glabrata	>200	>200	>200	>200	>8	0.39	NT
C. krusei	>200	147.98	>200	196.31	>8	0.65	NT
A. fumigatus	>200	>200	>200	>200	>8	1.18	NT
C.neoformas	>200	<8	8	32.94	>8	0.24	NT
S. aureus	>200	21.12	3259.1	17.18	>8	NT	0.12
MRSA	31.6	<8	86.36	8.26	>200	NT	0.10
E.coli	>200	>200	>200	>200	>200	NT	0.006
P. aeruginosa	>200	>200	>200	>200	>200	NT	0.09
M.intracellulare	>200	>200	>200	>200	>200	NT	0.40

NT; not tested

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Conflict of Interest

There are no conflicts of interest.

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