In vitro evaluation of aliphatic fatty alcohol metabolites of Persea americana seed as potential antimalarial and antimicrobial agents

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(Received 29:4:2014, Accepted 10:7:2014)

Abstract
Persea americana 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 Persea americana. 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 methods including 1D- and 2D NMR, and comparison with reported data in literature. Antifungal activity for 1, 2, 4-trihydroxyheptadec-6-en-16-yn-5 (IC50< 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) (IC50 = 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-yn(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 IC50 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, Persea americana, 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,
Falodun et al. / Nig J. Biotech. Vol. 27 (2014) 1 - 7

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 and an 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 organ dysfunction or high levels of parasitemia, predominantly affects children and pregnant women (Pierce and Miller, 2009; Rosenthal, 2008; White, 2008). Chemotherapy is still at the forefront in the fight against malaria due to the unavailability of effective vaccines. Numerous drugs have 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-resistant microbial pathogens have become a serious problem particularly during the last decade and provide their impetus for the search and discovery of novel antibacterial and antifungal agents active against these pathogens (Vekemans and Ballou, 2008).

Persea americana 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 widely used in traditional medicines of many African countries as antitussive, antimicrobial, antidiabetic, antiparasitic, anti-allergic, antihypertensive, analgesic and anti-inflammatory remedies (Adeyemiet al., 2002; Adeboyeet al., 1999; Owolabiet al., 2005; Oberlieset 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. Ugboogu 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°C for 4 days. The powdered (100 g) material of sample was extracted with 500 ml methanol for 48 hr at room temperature. The resulting mixture was filtered through a Whatman No. 1 filter paper 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%, 12.2 g; BH-20 sephadex of 50% hexane/EtOAc in Dichloromethane: methanol 100%, 7.4 g; ethylacetate: methanol 50%, 6.5 g and methanol 100%, 12.2 g. LH 20 sephadex of 50% hexane/EtOAc in Dichloromethane: methanol 100%, 7.4 g; ethylacetate: methanol 50%, 6.5 g and methanol 100%, 12.2 g.

Fractions 5–10 pooled together based on similar Rf values, was subjected to HPLC reversed phase C-18 column, using MeCN-H2O 95:5%, compounds 1 (8 mg), 2 (5 mg) and 3 (12 mg) were resolved and eluted. Fractions 12-15 was chromatographed on RP-C18, eluting with MeCN-H2O (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°C; UV \( \lambda_{\text{max}} \) 202 nm; IR \( \nu_{\text{max}} \) (KBr) cm\(^{-1}\): 3320, 3297, 2920, 2850; HRCIMS m/z 287.2544 for C\(_17\)H\(_{32}\)O\(_3\) 287.25387, \(^1\)HNMR and \(^{13}\)CNMR compared to Oberlieset al., 1998.

**Compound 2**: 1, 2, 4-Tetrahydroxyheptadecane-6, 16-diene: colourless prizms: 150 mg; mp 82–84°C; UV \( \lambda_{\text{max}} \) 208 nm; IR \( \nu_{\text{max}} \) (KBr) cm\(^{-1}\): 3435, 1649, 2918, 2850, 1470. HRCIMS m/z 325.2458 for C\(_{17}\)H\(_{32}\)O\(_4\) (325, 2574). \(^1\)HNMR and \(^{13}\)CNMR compared to Ying-Chenet al., 2012.

**Compound 3**: 1, 2, 4-Trihydroxyheptadec-16-yno: white powder: 35 mg; mp 74–76°C; UV \( \lambda_{\text{max}} \) 203 nm; IR \( \nu_{\text{max}} \) (KBr) cm\(^{-1}\): 3434, 3384, 3281, 2917, 2849, 1467, HRCIMS m/z 285.2464 for C\(_{17}\)H\(_{32}\)O\(_2\) 287.25387. \(^1\)HNMR and \(^{13}\)CNMR compared to Oberlieset al., 1998.

**Compound 4**: 1, 2, 4-Trihydroxyheptadecane: white powder solid: 50 mg; mp 79–81°C; UV \( \lambda_{\text{max}} \) 200 nm: IR \( \nu_{\text{max}} \) (KBr) cm\(^{-1}\): 3312, 3300, 2918, 2850, 1470. HRCIMS m/z 317.2084 for C\(_{18}\)H\(_{32}\)O\(_3\) (317.2074). \(^1\)HNMR and \(^{13}\)CNMR compared to Oberlieset al., 1998.

2
**Compound 5:** 1, 2, 4-Trihydroxyheptadec-6-en-16-ynne: colourless oil: 134 mg; UV$_{max}$ 208 nm; IR $\nu_{max}$ (KBr) cm$^{-1}$, 3368, 3314, 1662. HRCIMS m/z 305.2848 for C$_{21}$H$_{33}$O$_3$ (305.2870). $^1$H NMR and $^1$C NMR 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 Aspergillus fumigatus (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. intracellulararend A. fumigatuswas tested using an Alamar Blue method (Franzblau, 1998). All organisms were obtained from the American Type Culture Collection (Manassas, VA). Samples, dissolved in DMSO, were serially diluted in saline and transferred in duplicate to 96 well micro plates. Susceptibility testing was performed for all organcates to 96 well flat bottom micro plates. Microbial inocula were prepared by correcting the OD$_{630}$ of microbe suspensions in incubation broth to afford final target inocula. Controls [fungi: amphotericin B; bacteria: ciprofloxacin (ICN Biomedicals, OH)] were included in each assay. All plates were read at 530 or 544(ex)/590(em) nm (M. intracellulara and A. fumigatus) prior to and after incubation. Percent growth 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 one single concentration of 15.9 mg/mL only on the chloroquine-sensitive (D6) strain of P. falciparum. The compound showing >50% growth inhibition of the parasite was subjected to screening. For bioassay-guided fractionation, the column fractions were also tested only at single concentration. The pure compounds were subjected to additional testing for determination of IC$_{50}$ values. The standard antimalarial agents’ chloroquine and artemisinin were used as positive controls, with DMSO (0.25%) as the negative (vehicle) control. The selectivity indices (SI) were determined by measuring the cytotoxicity of samples on mammalian cells (VERO; monkey kidney fibroblast). All experiments were carried out in duplicate.

**Results**

The phytochemical investigation of Persia americana seeds led to the isolation and characterization of five compounds. Figure 1 displays the compounds isolated and characterized. The compounds were identified as 25 4S-1, 2, 4-trihydroxyheptadec-6-en-16-ynne (1); 1, 2, 4, 15-tetrahydroxyheptadec-6,16-diene (2); 1,2,4-trihydroxyheptadec-16-ynne (3); 1,2,4 trihydroxynonadecane (4) and 1, 2, 4-trihydroxyheptadecane 6-ene, 16-ynne (5).

The antimalarial activities of all isolated metabolites 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 Persia americana 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 unambiguously as 25 4S-1, 2, 4-trihydroxyheptadec-6-en-16-ynne (1); 1, 2, 4, 15-tetrahydroxyheptadec-6,16-diene (2); 1,2,4-trihydroxyheptadec-16-ynne (3); 1,2,4 trihydroxynonadecane (4) and 1, 2, 4-trihydroxyheptadecane 6-ene, 16-ynne (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 IC50 values of 16.0 and 8.5 ng/mL (for D6) and IC50 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 IC50 values >200 µg/mL. The five compounds demonstrated moderate activity against E. coli, P. aeruginosa, and Mycobacterium intracellulare at IC50 value of >200 µg/mL each. Compound 1 also exhibited strong activity against MRSA (IC50 13.81 µg/mL).

The antifungal activity of metabolites is presented in Table 2. Compounds 2, 3, 4 and 5 were fungicidal at IC50 values ranging from < 8 - 200 µg/mL. Compound 5 however showed the most promising antifungal 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 IC50/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 of metabolites of P. americana against Plasmodium falciparum

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>P. falciparum IC50</th>
<th>P. falciparum SI</th>
<th>P. falciparum IC50</th>
<th>P. falciparum SI</th>
<th>VERO IC50</th>
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<tbody>
<tr>
<td>1</td>
<td>29713.3</td>
<td>&gt;1.6</td>
<td>3448.3</td>
<td>&gt;1.4</td>
<td>&gt;47600</td>
</tr>
<tr>
<td>2</td>
<td>22683.7</td>
<td>&gt;2.1</td>
<td>34152.8</td>
<td>&gt;1.4</td>
<td>&gt;47600</td>
</tr>
<tr>
<td>3</td>
<td>25881.6</td>
<td>&gt;1.8</td>
<td>32544.6</td>
<td>&gt;1.5</td>
<td>&gt;47600</td>
</tr>
<tr>
<td>4</td>
<td>28194.4</td>
<td>&gt;1.7</td>
<td>40843.9</td>
<td>&gt;1.2</td>
<td>&gt;4760</td>
</tr>
<tr>
<td>5</td>
<td>&gt;47600</td>
<td>&gt;1</td>
<td>&gt;47600</td>
<td>1</td>
<td>&gt;47600</td>
</tr>
</tbody>
</table>
Figure 1: Chemical structures of metabolites isolated from *P. americana* seeds.
Table 2: Antimicrobial activities of metabolites of *P. americana* seed extract

<table>
<thead>
<tr>
<th>Test organism</th>
<th>IC$_{50}$ (µg/mL) of compounds</th>
<th></th>
<th></th>
<th></th>
<th>Amphotericin B</th>
<th>Ciprofloxacin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;8</td>
<td>0.27</td>
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<tr>
<td><em>C. glabrata</em></td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;8</td>
<td>0.39</td>
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<tr>
<td><em>C. krusei</em></td>
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<td>147.98</td>
<td>&gt;200</td>
<td>196.31</td>
<td>&gt;8</td>
<td>0.65</td>
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<tr>
<td><em>A. fumigatus</em></td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;8</td>
<td>1.18</td>
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<tr>
<td><em>C. neoformas</em></td>
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<td>&lt;8</td>
<td>8</td>
<td>32.94</td>
<td>&gt;8</td>
<td>0.24</td>
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<tr>
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<td>21.12</td>
<td>3259.1</td>
<td>17.18</td>
<td>&gt;8</td>
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</tr>
<tr>
<td><em>MRSA</em></td>
<td>31.6</td>
<td>&lt;8</td>
<td>86.36</td>
<td>8.26</td>
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<tr>
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<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>NT</td>
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<tr>
<td><em>P. aeruginosa</em></td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>NT</td>
</tr>
<tr>
<td><em>M. intracellulare</em></td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
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</tr>
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</table>

NT; not tested

**Acknowledgement**

This work was in part supported by a US- Senior Fulbright Award granted to Dr. A. Falodun to study at the University of Mississippi, USA, CIESCs for the Fulbright award, NIH, NIAID, Division of AIDS, Grant No. AI 27094 (antifungal) and the USDA Agricultural Research Service Specific Cooperative Agreement No. 58-6408-1-603 (antibacterial). Tertiary Education Trust Fund (TETFUND/DESS/RP/UNIV/BENIN/VOL.111 2013) and the University of Benin URPC VC. 23 is also highly acknowledged for the financial support.

**Conflict of Interest**

There are no conflicts of interest.

**References**


