Isolation and structure elucidation of anti-malarial principles from *Terminalia mantaly* H. Perrier stem bark

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ABSTRACT

Emergence of malaria parasite resistance to drugs has raised global public health concerns for a compelling need to develop improved malaria therapy. This study is a bio-guided isolation of triterpenoid antimalarial compounds from *Terminalia mantaly*. Methanol extract of the plant was subjected to column chromatography, and eluted with a ternary solvent system gradient-wise. Two compounds, 1 and 2, were isolated and characterised by spectroscopic data (IR, 1H and 13C NMR, COSY, HMQC, HMBC) and by comparison with literature. Isolated compounds were investigated for antimalarial property by spectrophotometric determination of inhibition of β-Hematin formation, absorbance taken at 405 nm. Results were analysed using Graghpad Prism® (6.0) and presented as mean IC50 ±SEM. Statistical significance, determined using Student’s t-test and one-way ANOVA, set at p-value of 0.05. Quantitative β-Hematin formation inhibitory activities gave IC50±SEM values of (compound 1; 4.434 ±0.47), (compound 2; 5.140±4.2) with (chloroquine; 0.335±0.1 mg/ml). Compound 1 was identified as 2,3,19,23-tetrahydroxyolean-12-en-28-oic acid glucopyranoside (arjunglucoside I), and compound 2 as its aglycone, 2,3,19,23-tetrahydroxyolean-12-en-28-oic acid (arjungenin). This study provided credence for folkloric use of *Terminalia mantaly* to treat malaria, and this observed activity was probably due to these isolated triterpenoids.

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Keywords: β-Hematin, triterpenoids, nuclear magnetic resonance spectroscopy.

INTRODUCTION

Malaria is one of the most grievous parasitic infectious diseases caused by protozoa of the genus *Plasmodium*. It is one of the leading causes of death in many tropical regions including West African countries. About 74% of an estimated US$2.7 billion spent on malaria control and elimination globally by governments of malaria endemic countries and other international parties was said to be spent in WHO African Region (WHO, 2017). There is a close relationship between malaria and poverty. Majority of people affected by malaria live in world poorest regions. Malaria has been shown to be implicated in crippling poverty, less productivity, more work days missed and incurring of expenses due to treatment. In 2016, an estimated 216 million malaria cases were reported worldwide, with estimated death of 445,000. About 90% of the world malaria report in 2016 was said to be from WHO.
African Region, 7% from WHO South-East Asia Region, and 2% from WHO Eastern Mediterranean Region (WHO, 2017).

Nigeria is one of the most affected countries where morbidity and mortality rates due to malaria are high. The most vulnerable groups are children under five years (with an estimation of 75% malaria deaths in Nigeria between 1971-2015), and pregnant women. A recent assessment of malarial hazard and mortality in Nigeria showed that, despite decrease in malaria cases worldwide, malaria cases have increased exponentially in Nigeria resulting in deaths of many people especially children under age five (Kayode and Godwin, 2017).

*Terminalia mantaly* H. Perrier belongs to the family Combretaceae. It is widely grown as an excellent spreading shade and street tree. It is an ornamental plant locally known as Madagascar almond, French mantaly or umbrella tree. *Terminalia mantaly* has been used to treat fever, diarrhea, dysentery and bacterial infections (Ngouana et al., 2015). Biological screening efforts have revealed *Terminalia mantaly* to have antimalarial potential (Mbouna et al., 2018). Aqueous and ethanolic extracts from *Terminalia mantaly* stem bark have shown activity against clinical isolates of *Candida albicans* and *Aspergillus fumigatus* (Yapi et al., 2011). A study also showed fungicidal, synergistic inhibitory effects of *Terminalia catappa*, *Terminalia mantaly* and *Monodora tenuifolia* against clinical isolates of *Candida albicans* (Ngouana et al., 2015).

Medicinal plants have been a source of malaria therapy since the ancient times. It has been discovered that several plants have antimalarial properties (Ngbolua et al., 2011; Oluyemi and Samuel, 2017; Willcox et al., 2004). Extracts from bark of *Cinchona officinalis* and leaves of *Artemisia annua* are prototypical for the development of antimalarial drugs from natural products; while some plants have also demonstrated high level of antimalarial activities (Dawet et al., 2011; Adzu et al., 2008). However, resistance to currently available antimalarial agents has been reported (Wongsrichanalai and Meshnick, 2008).

Better understanding of the lifecycle of malaria parasite has offered a lot of advantages in rational approach to antimalarial drug discovery. During intraerythrocytic stage of *Plasmodium* life cycle, malarial parasite takes in hemoglobin from erythrocytes into the cytophosome. Many small tubular vesicles containing hemoglobin from the cytophosome fuse to food vacuole which is acidic (David et al., 2008). In the acidic pH of the food vacuole, hemoglobin is oxidized to methemoglobin and subsequently hydrolyzed by aspartic proteases into free heme (Fe$^{3+}$) and denatured globin. Further digestion takes place on globin via the actions of cysteine proteases, zinc containing metallopeptidase and exopeptidase in cytosol to produce amino acids for parasite protein synthesis and energy which are important for the development and the proliferation of the parasite (Rosa et al., 2005).

Accumulation of redox-active free heme (ferriprotoporphyrin IX [PPIXFe]) within the parasite food vacuole may reach as high level as 300–500 mM (Wright et al., 2001a). Free heme (Fe$^{3+}$) produce reactive oxygen species and may cause oxidative stress in the parasites. This toxic nature of free heme leads to parasite death (Thomas et al., 2014). Malaria parasite protects itself from heme-induced oxidative stress by effective heme detoxification mechanisms, primarily by converting free heme to hemozoin (β-hematin) in the food vacuole (Ziegler et al., 2001). Beta-hematin formation assay is an *in vitro* method which has been validated by existing antimalarial agents, such as chloroquine and quinine, and it has been used to investigate antiplasmodial activity of medicinal plants (Vargas et al., 2011).

This research work aimed at isolating the bioactive substances responsible for the anti-plasmodial property of *Terminalia mantaly* stem bark, and to elucidate their chemical structures using their nuclear magnetic resonance (NMR) spectroscopic data.
MATERIALS AND METHODS

Plant sample preparation

The stem bark of *Terminalia mantaly* H. Perrier was collected at the University of Ibadan botanical garden. The plant was authenticated and a voucher specimen deposited at the University of Ibadan Herbarium (UIH), Botany Department, with the number: UIH-22715.

Extraction

The plant sample was air-dried for 3 weeks and milled into powder. The sample was properly stored until used. About 1 kg was extracted with 7 litres of methanol using cold maceration method. The macerate was filtered and concentrated with rotary evaporator (Heidolph HB digital, Germany) at <40 °C. The concentrated extract was reduced to semi-solid using water bath at 40±10 °C to allow it dry completely. The percentage yield of the dried extract was computed.

Qualitative phytochemical screening

Phytochemical screening was performed on the crude extract for the presence of alkaloids, saponins, tannins, triterpenes, cardiac glycosides, anthraquinones, steroids and flavonoids using standard procedures (Ayoola et al., 2008).

Column chromatographic separation of crude extract

About 20 grams of the crude extract was loaded on a 85 cm x 6 cm column packed with 60-120 mesh size silica gel. It was then eluted with hexane, chloroform and methanol gradient-wise. The sample was initially eluted with hexane/chloroform (50:50), and polarity was gradually increased with the addition of chloroform and methanol as follows: Hexane/chloroform 20:80, 0:100; Chloroform/methanol 95:5, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80%. Two hundred and thirty, 30 ml fractions were collected. Eight fractions labelled F1 to F8 were obtained based on their TLC profiling. Active fraction F7 was further purified with ethyl acetate to give a white crystalline compound 1 (145 mg) and fraction F4 gave a white amorphous compound 2 (35 mg) by preparatory TLC.

Spectroscopic analysis of isolated compounds

1D and 2D NMR data were obtained by dissolving the samples in DMSO-d6. Melting point was determined on Barnstead Electrothermal IA9000 and Infrared spectra obtained on Perkin Elmer Spectrum BX FTIR.

In vitro Beta-Hematin formation assay

Chemicals

Pyridine solution (Sigma), hydroxyethylpiperazine-N-[2-ethanesulphonic acid] (HEPES) solution 20 mM, 0.1M NaOH, 1M HCl, chloroquine phosphate, bovine heme [Fe(III)PPX], glacial acetic acid, sodium acetate (BDH Chemicals, England), methanol, saturated acetate solution (pH5.0).

Sample preparation

A concentration of 10 mg/ml of the eight column fractions (F1-F8) and the plant crude extract were made in methanol.

Beta-Hematin formation assay

The antimalarial potential of the plant sample was evaluated by the method described by Vargas et al. (2011). An aliquot, 10 µL, of each of the concentrations was placed inside four 2 ml sized Eppendorf’s tubes in triplicates. 1M HCl, 10 µL, was added to all tubes. Then 100 µL of freshly prepared bovine heme was added into the first two tubes. The plate was centrifuged at 900 rotations per minute for 5 minutes. Then 60 µL of pre-warmed sodium acetate solution (pH5.0) was added to all tubes. The tubes were incubated at 60 °C for 90 minutes. Then 750 µL of 15% pyridine in HEPES solution was added into first and third tubes and 750 µL of HEPES solution (pH7.5) was added into second and fourth tubes. The tubes were centrifuged at 900 rpm for 5 minutes and allowed to settle for 15 minutes. An aliquot (100 µL) of the supernatant was transferred into a non-sterilized 300 µL 96-well plate and its absorbance taken at 405 nm with Rayto scientific RT-6100 microplate reader.
Qualitative determination of the inhibition of β-hematin formation

Each concentration analysed (A_{Analysis}) [first tube] has a blank (A_{Analysis;Blank}) [second tube] which differed from the concentration by the addition of 750 µL HEPES 20 mM solution instead of 750 µL pyridine solution. They were all prepared in triplicates.

The absorbance, at 405 nm, of the complex due to the remaining hematin in wells was calculated by the formula:

\[ \Delta A_{Analysis} = A_{Analysis} - A_{Analysis;Blank} \]

For each tested concentration, a blank control (A_{CLT;Blank}) [third tube] as well as its control (A_{CLTBlank;Blank}) [fourth tube] were prepared in triplicates under the same experimental conditions as described above. The blank control (A_{CLTBlank;Blank}) had 750 µL HEPES 20 mM solution instead of 15% pyridine solution added to (A_{CLT;Blank}).

The residual absorbance, at 405 nm, of the sample independent from the inhibition of the beta-hematin complex was determined by the formula:

\[ \Delta A_{CLT;Blank} = A_{CLT;Blank} - A_{CLTBlank;Blank} \]

The resultant beta-hematin formation inhibitory effect induced by the sample being analyzed was computed by:

\[ I_{Analysis} = \Delta A_{Analysis} - \Delta A_{CLT;Blank} \]

Positive value for I_{Analysis} is considered a positive result or active concentration and negative value for I_{Analysis} is considered negative result or inactive concentration.

Quantitative determination of 50% inhibitory concentration (IC_{50} mg/ml)

The above procedure was repeated for the most active fractions in six concentrations (10 mg/mL, 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL, 0.625 mg/mL and 0.3125 mg/mL). The experiment was carried out in triplicates. Chloroquine was used as reference drug.

Statistical analysis

Experimental data (I_{Analysis}) expressed as mean ± standard error of the mean. Statistical significance was determined using unpaired t-test and one-way ANOVA followed by post hoc test. A P-value of <0.05 was considered significant. Activities were reported as IC_{50±SEM} mg/ml. GraphPad Prism® (6.0) was used.

Compound 1: 2α, 3β, 19α, 23-tetrahydroxyolean-12-en-28-oic acid glucopyranoside (Arjunglucoside I).
Compound 2: $2\alpha, 3\beta, 19\alpha, 23$-tetrahydroxyolean-12-en-28-oic acid (Arjungenin).

RESULTS
Extraction of Terminalia mantaly stem bark with methanol gave 137 grams of crude extract with percent yield of 13.7%.

Phytochemical screening of the crude extract indicated the presence of saponins, tannins, anthraquinones, terpenoids, steroids, flavonoids and alkaloids in varied degrees.

Column chromatographic separation gave compound 1 (145 mg; $R_f$ of 0.2 in CM 90%:10%) and compound 2 (35 mg; $R_f$ of 0.5 in CM 90%:10%). Compound 1 was a white crystalline powder, melting point rage of 219-223 °C.

Spectroscopic data

Compound 1 (0.725%): White crystalline powder, melting point 219-223 °C.

IR (KBr pellet): 1179.80 cm$^{-1}$, 1260.00 cm$^{-1}$, 1070.02 cm$^{-1}$, 1628.83 cm$^{-1}$, 1727.91 cm$^{-1}$, 2935.16 cm$^{-1}$, 3435.52 cm$^{-1}$.

$^1$H NMR (DMSO-d$_6$): $\delta$H 0.6031, 0.8423, 0.8660, 0.8889, 1.0790 and 1.2115 (each methyl group, s); 5.2377 (1H, dd, H-12). $^{13}$C NMR (DMSO-d$_6$): $\delta C$ 16.9331, 23.7920, 25.0781 and 28.5076; 143.6881 (C-13), 122.8184 (C-12), 80.5929 (C-3), 84.6654 (C-19), 43.3854 (C-18), 67.5180 (C-2), 64.2979 (C-23), 182.28 (C-28), 28.5076 (C-29); sugar moiety: $\delta C$ 94.7395, 78.2351, 76.9490, 72.8765, 69.8757, and 61.0577.

Compound 2 (0.175%): White amorphous powder. IR: 3419.00 cm$^{-1}$, 1018.29 cm$^{-1}$, 1643.76 cm$^{-1}$, 2979.00 cm$^{-1}$. $^1$H NMR (DMSO-d$_6$): $\delta$H 0.6605, 0.8163, 0.8741, 1.0772, 1.1988 and 1.2307 (each methyl group, s); 5.1319 (1H, brs, H-12). $^{13}$C NMR (DMSO-d$_6$); 124.59 (C-12), 144.69 (C-13), 81.4173 (C-3), 67.4601 (C-2), 48.1346 (C-9), 84.6382 (C-19), 182. 28 (C-28), 25.5883 (C-30), 64.3926 (C-23).

Antimalarial results
The results of the qualitative determination of β-hematin formation inhibitory property of the column fractions (F1 – F8) were shown in Table 1. Fraction F8 had the highest activity, followed by F4 and then F7 with $I_{\text{analys}}$ (Mean±SEM) of 0.201±0.0299, 0.181±0.0708 and 0.145±0.0122 respectively. F7 yielded compound 1 and F4 yielded compound 2. Compound 1 and compound 2 showed appreciably high activity at inhibiting beta-hematin formation. The mean IC$_{50}$ mg/ml ± standard error of the mean values and P-
values are given in Table 2. In determining the quantitative beta-hematin formation inhibitory activity at different concentrations, compound 1 with IC\textsubscript{50}±SEM of 4.434±0.47 mg/ml was more active than its aglycone (compound 2) with IC\textsubscript{50}±SEM of 5.140±4.2 mg/ml. Chloroquine was used as the standard (IC\textsubscript{50}±SEM = 0.335±0.1 mg/ml). When compared the activity of the active fractions with the activity of chloroquine using one-way ANOVA post analysis Tukey’s multiple comparison test, chloroquine was statistically more active at the same tested concentrations.

**Table 1:** Qualitative determination of β-hematin formation inhibition of column fractions and chloroquine.

<table>
<thead>
<tr>
<th>Fractions (10 mg/ml)</th>
<th>I\textsubscript{ANALYSIS} (Mean±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>0.115±0.0112</td>
</tr>
<tr>
<td>F2</td>
<td>0.103±0.0344</td>
</tr>
<tr>
<td>F3</td>
<td>0.079±0.0255</td>
</tr>
<tr>
<td>F4</td>
<td>0.181±0.0708</td>
</tr>
<tr>
<td>F5</td>
<td>0.102±0.0438</td>
</tr>
<tr>
<td>F6</td>
<td>0.135±0.0170</td>
</tr>
<tr>
<td>F7</td>
<td>0.145±0.0122</td>
</tr>
<tr>
<td>F8</td>
<td>0.201±0.0299</td>
</tr>
<tr>
<td>WE</td>
<td>0.167±0.0325</td>
</tr>
<tr>
<td>Chloroquine 4 mg/ml</td>
<td>0.337±0.0779</td>
</tr>
</tbody>
</table>

**Table 2:** IC\textsubscript{50} mg/ml determination of β-hematin formation inhibition of isolated compounds and chloroquine.

<table>
<thead>
<tr>
<th></th>
<th>IC\textsubscript{50} mg/mL mean±SEM</th>
<th>T-test P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arjunglucoside I</td>
<td>4.434 ± 0.47</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>Arjungenin</td>
<td>5.140 ± 4.2</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>0.335 ± 0.1</td>
<td>P&lt;0.05</td>
</tr>
</tbody>
</table>
DISCUSSION

Malaria is a life-threatening disease caused by Plasmodium parasites. The parasite digestion of hemoglobin in the food vacuole produces high quantity of free heme (Fe^{3+}); this redox active species is toxic to the parasite and can cause cell lysis (Sanjay et al., 2007). Consequently, the parasite converts the toxic free heme to non-toxic hemozoin (Egan, 2001). Hemozoin (β-hemin) formation is a validated pathway by with malaria parasite detoxifies free heme. Thus, it has been validated that classical antimalarial drugs such as chloroquine and quinine exhibit their antiplasmodial effect by enhancing heme toxicity through inhibition of hemozoin formation (Kumar et al., 2007). This in vitro synthesis of hemozoin in the presence of acetate solution at carefully optimized experimental conditions of temperature and pH (Egan et al., 2001) has been validated, absorbance taken at 405nm, for routine screening of plant extracts for anti-malarial activity (Vargas et al., 2011).

In this study, two triterpenoids of oleanane type were isolated from fractions 4 and 7 through chromatographic separation. They were identified as arjungenin and its glycoside, arjunglucoside I. Compound 1 IR (KBr pellet) spectrum gave signals at 1179.80 cm\(^{-1}\) and 1260.00 cm\(^{-1}\) which are indication of the presence of C-O. The signal observed at 1070.02 cm\(^{-1}\) was assigned to the presence of a glycosidic linkage in the molecule. Signal at 1628.83 cm\(^{-1}\) showed the presence of C=C group. The sharp absorption signal at 1727.91 cm\(^{-1}\) is indicative of an ester carbonyl group (C=O). There was a signal at 2935.16 cm\(^{-1}\) which is an indication of the presence of saturated C-H group while that at 3435.52 cm\(^{-1}\) showed presence of O-H bond.\(^1\)H and \(^13\)C NMR spectra data signals were characteristic of an oleanane-type triterpenoid with one sugar moiety. The \(^1\)H NMR (DMSO, \(d_6\)) gave signals for six methyl groups attached to quaternary carbons at \(\delta_H\) 0.6031, 0.8423, 0.8660, 0.8889, 1.0790 and 1.2115. HMQC revealed a cross-peak correlation of an unsaturated carbon with an olefinic hydrogen which resonates at \(\delta_H\) 5.2377 (d, 1H), assigned to C-12. An oxygenated hydrogen can be observed \(\delta_H\) 3.1292 assigned to C-3. \(^13\)C NMR (DMSO, \(d_6\)) indicated the presence of a methine carbon at \(\delta_C\) 122.8184 and a quaternary carbon at \(\delta_C\) 143.6891 assigned to C-12 and C-13 respectively (Abdullahi et al, 2012). This confirmed the olefinic proton observed at \(\delta_H\) 5.2377 which \(^1\)CNMR showed to be a methine. The chemical shifts of C-12 and C-13 suggests that compound 1 is a \(\Delta^{12}\)-unsaturated triterpenoid. C-3 gave a signal resonating at \(\delta_C\) 80.5929; and one anomic carbon signal resonating at \(\delta_C\) 94.7395, which is characteristic of the carbon forming the glycosidic linkage (Bharathi et al, 2013). The peaks at \(\delta_C\) 94.7395, 78.2351, 76.9490, 72.8765, 69.8757, and 61.0577 are indicative of the presence of hexose sugar unit carbon atoms with glycosidic ester linkage at C-28. NMR data suggested nine oxygen bearing carbons with four –OH– groups which are part of the triterpenoid skeleton – 67.5180 (C-2), 80.5929 (C-3), 84.6654 (C19), 64.2979 (C-23). A carbonyl carbon was observed at \(\delta_C\) 182.28. Extensive \(^1\)H and \(^13\)C NMR data comparison with those previously published showed that spectroscopic data of compound 1 were consistent with those reported for 2,3,19,23-tetrahydroxyolean-12-en-28-oic acid glucopyranoside (Arjunglucoside I) (Bharathi et al, 2013; Marthe et al., 2017).

Compound 2 is a white amorphous solid. IR data showed a very strong and broad absorption at 3419.00 cm\(^{-1}\) which is indicative of the presence of O-H group. The prominent absorption at 1018.29 cm\(^{-1}\) for C-O bond confirms alcoholic OH. Signal at 1643.76 cm\(^{-1}\) is indicative of C=O group. The spectrum also showed the presence of saturated C-H group at 2979.00 cm\(^{-1}\). The NMR data for compound 2 are similar to those recorded for compound 1 with only signals for sugar moiety missing. The \(^1\)H NMR and \(^13\)C NMR (DMSO, \(d_6\)) data gave signals that were characteristic of \(\Delta^{12}\)-unsaturated oleanane triterpenoid (Singh et al, 2004). The \(^1\)H NMR has six methyl singlets at \(\delta_H\) 0.6605, 0.8163, 0.8741, 1.0772, 1.1988 and 1.2307. A characteristic down-field proton at \(\delta_H\) 5.1319 (s, br, 1H) gave a correlation with an olefinic carbon at \(\delta_C\) 124.59 in the HMQ
iterpenes are the main plasmodial activity. The compounds were recently identified and isolated from Combretum racemosum (Gossan et al., 2013) and four enzymes of \textit{Terminalia bellerica} (Min et al., 2014), \textit{Terminalia chebula} (Bharathi et al., 2013), \textit{Combretum racemosum} (Gossan et al., 2016). The compounds were recently identified and screened against three pathogenic yeasts (\textit{Candida albicans}, \textit{Candida parapsilosis} and \textit{Candida krusei}) and four enzymes of metabolic significance (glucose-6-phosphate dehydrogenase, human erythrocyte carbonic anhydrase I and II, and glutathione S-transferase) with activity comparable to the drug standard used (Marthe et al., 2017). Arjungenin, isolated from \textit{Terminalia chebula}, was reported to have potent inhibitory effect on Epstein-Barr Virus Early Antigen (EBV-EA) induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) in Raji cells; its activity higher than that of arjunglucoside I. Arjungenin also showed inhibitory effect in an in vivo carcinogenesis test conducted to promote skin tumour on mouse skin. It also showed moderate cytotoxic effect against HL60 cells (Manosroi et al., 2013).

The results of this study showed that there is scientific basis for the ethnomedical usage of the \textit{Terminalia mantaly} to treat malaria.

### Conclusion

This study reported isolation and structural characterization of two oleane-type triterpenoids identified as arjungenin and its glycoside, arjunglucoside I with antimalarial properties against in vitro β-Hematin synthesis. Therefore, this study provided support for the traditional use of \textit{Terminalia mantaly} stem bark for the treatment of malarial fever. Further isolation of other antimalarial compounds from \textit{Terminalia mantaly} stem bark; and insilico studies towards the development of a lead compound for the treatment of malaria are ongoing. Chronic and acute toxicity profiles of the extracts and isolated compounds must be clearly established before they can be considered for application in humans.

### COMPETING INTERESTS

The authors declare that there is no competing interest.

### AUTHORS’ CONTRIBUTIONS

BS designed and supervised the study. YAA carried out the chemical and biological
aspects of the research and drafted the manuscript. The manuscript was revised and its final version agreed on by both authors.

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