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Synergism of artemisinin with abyssinone –V from Erythrina abyssinica (Lam. ex) against Plasmodium falciparum parasites: A potential anti-malarial combination therapy

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A Plasmodium falciparum in vitro drug sensitive study was conducted in order to evaluate artemisinin in combination with potential anti-malarial drug from the stem bark of Erythrina abyssinica. Abyssinone-v was isolated and thus identified as a potential partner for a fixed combination therapy. Bioassay guided chromatographic separation of Ethyl acetate extract of Erythrina abyssinica led to the isolation of Abyssinone-v with IC50 value of 3.19 µg/ml against chloroquine-sensitive (D6) P. falciparum parasites. The structure of abyssinone-v was determined using nuclear magnetic resonance (NMR) and mass spectroscopic technique. The interaction of artemisinin and abyssinone-v was analyzed using combination ratios of 10:90 to 90:10 respectively against P. falciparum parasites. This led to the identification of anti-malarial combination therapy of artemisinin and abyssinone-v with sum of fraction inhibiting concentration (FIC) of 0.79 at a ratio of 2:3, respectively.

Key words: Erythrina Abyssinica, Plasmodium falciparum, abyssinone-v, artemisinin and combination therapy.

INTRODUCTION

Malaria is an infectious disease which causes about 275 to 350 million infections in human and approximately 1.3 million deaths annually (WHO, 2008). Sub-Saharan Africa accounts for 85% of these fatalities (Scott, 2005). Malaria mortality and morbidity are increasing in the Sub-Saharan region of Africa especially in the vulnerable groups such as children under five years and pregnant mothers (Snow et al., 2001), due to multi-drug resistance of Plasmodium falciparum parasites to available drugs. This has resulted in an economic constraint in malaria control (Bloland, 2001). Increase in the spread of multi-drug resistance of parasites to most of the available and affordable anti-malarial drugs is a major concern and requires innovative strategies in controlling it. There is an urgent need to discover and develop new anti-malarial drugs.

Traditional medicinal plants have been the main source of anti-malarial drugs with the two major drugs quinine and artemisinin drugs being used worldwide for treatment of malaria (Wright et al., 1990; Thomson, 1993). Quinine and artemisinin were obtained directly from Cinchona Sp and Artemesia annua plant respectively and were developed using plant-derived chemical structures as templates. Chemical modification of artemisinin has led to a series of potential anti-malarial drugs (artemether, artesunate and arteether) which are currently being used in combination with conventional drugs for treatment of malaria (Nicholas, 1994; Simon et al., 1990; Bai, 1993).

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The use of combination chemotherapy is the current innovative strategy in controlling malaria disease; it involves the use of a short half-life acting anti-malarial agent of artemisinin drug in combination with long half-life conventional drug, example being artemether-lumifantrine and artesunate-mefloquine drugs (Nosten, 2000). The artemisinin drug (short half-life) kills the parasites and excreted rapidly, resulting in re-emerging of the parasites after a period of time. Therefore use in combination with longer half-life anti-malarial drug result in achieving full eradication of the parasites preventing the recrudescence that occur with use of artemisinin mono-therapy (Walsh et al., 2007).

Currently, the drugs being used in combination with artemisinin drugs are the conventional drugs (lumifantrin, mefloquine and amodiaquine), which have developed resistance to P. falciparum parasites when used in monotherapy. The use of the conventional drugs in combination therapy might not solve the problem of multi-drug resistance of the parasites in the near future. There is an urgent need in discovering and developing new anti-malarial drugs from traditional medicinal plants that can be used in combination therapy with artemisinin drugs in order to reduce the development of resistance and the occurrence of recrudescence. Currently, the ongoing research is on developing artemisinin combination therapy from traditional medicinal drugs. Example, from recent research on artemisinin combination therapy with anti-malarial drugs from traditional medicinal plants was on artemisinin – flavanoids combination therapy. In this study, artemisinin –abyssinone-iv combination at ratio of 5:1 respectively showed synergism activity and artemisinin- abyssinone 4’-methyl ether combination showed additive activity against (D6 clone) P. falciparum parasites (Akala et al., 2010). In an effort to verify the efficacy of traditional anti-malarial drugs, a potential medicinal plant Erythrina abyssinica was screened for anti-malarial activity.

Phytochemical analysis of most active compound was done for identification of a natural product that has high potential of being used in combination therapy with artemisinin drug.

**MATERIALS AND METHODS**

**Experimental**

The melting point of compound 1 (Figure 1) was determined using an electro-thermal melting point apparatus with a thermometer range of 0 – 360°C and was uncorrected. An infrared spectrum was generated using IR-408 shimadzu spectrometer and the compound was isolated with KBr as discs. 

$^1$H and $^{13}$C NMR analysis were done using mercury Vx. (200 MHZ) NMR spectrometer in deuteriated chloroform with TMS as the internal standard. An electron impact mass measurement was recorded on a VG – 12 – 250 UPGREDE mass spectrometer machine. Silica gel (5 to 40 µm mesh) was used for dry column chromatography while 220 to 440 mesh silica was used for flash chromatography.

P. falciparum parasites (D6, Chlroquine-sensitive strain) from Sierra Leone were used for anti-malarial test. The strains were obtained from the Division of Experimental Therapeutics, Walter Reed Army Institute of Research (WRAIR), Nairobi, Kenya. The root bark of *E. abyssinica* was collected from Uasin-Gishu District, 320 Km West of Nairobi, Kenya. The staff of Botany Department Herbarium, Moi University, where a voucher specimen was deposited, identified the plant.

**Collection, solvent extraction and chromatography**

Following a survey on the ethno uses of medicinal plants in Uasin-Gishu District in the Rift-Valley province of Kenya through interviews with traditional health practitioners, *E. abyssinica* was identified to be the most commonly used medicinal plant for treatment of malaria. *E. abyssinica* was selected for the study. Botanical identification was done by a taxonomist and a voucher specimen deposited at the Botany Department Herbarium, Moi University, Eldoret - Kenya. The stem bark of *E. abyssinica* were chopped into small pieces, air dried at room temperature for two weeks, and ground into powder form.

Two Kilogram of the powder was soaked in cold methanol for three days to extract the compounds. The resulting dark yellow filtrate was concentrated under reduced pressure using Buchii R110 rotary evaporator to a dark brown semi-solid and was subjected to anti-malarial test as methanolic crude extract. Thirty grams of methanolic crude extract was subjected to solvent partitioning using n-hexane, ethyl acetate and the residue dissolved in methanol to obtain three solvent fractions, concentrated and subjected to anti-malarial test.

The Ethyl acetate fraction (5 g) was the most active and was chromatographed over silica gel, eluting under pressure with increasing amount of ethyl acetate in n-hexane. The eluted fractions were monitored by thin layer chromatography using n-hexane and ethyl acetate (6:4) mixture as a developing solvent. Four fractions were obtained and were subjected to anti-malarial test.

The most active fraction 3 (10 ml), was concentrated and further purified by flash chromatography using ethyl acetate in n-hexane as eluting solvent.

This led to the isolation of compound 1, which was re-crystallized using 10% of n- hexane in acetone.
Anti-malarial test

Stock solution

Stock solution (100 µg/ml) for in vitro assay of methanolic extract, solvent fractions and compound 1 were prepared in deionized autoclaved water and re-sterilized by passing through 0.22 μm micro-filters in a laminar flow hood. The water-insoluble extracts were dissolved in 100% DMSO (dimethylsulfoxide) from Sigma Chemical Co; St Louis, MO USA, followed by a subsequent dilution to lower the concentration of DMSO to less than 1% to avoid solvent carrying over solvent effect (Dominique et al., 2001). Stock solutions (1 µg/ml) of chloroquine diphosphate and artemisinin (MW = 282.332) were similarly prepared for use as reference drugs. Each drug was filter-sterilized with syringe adaptable 0.22 µml filters into sterile Bijoux bottles and was stored at -20°C until required for bioassay.

Parasite culture

Test samples were screened against P. falciparum D6 (CO-sensitive) strain from Sierra Leone. The strains were obtained from the Division of Experimental Therapeutics, Walter Reed Army Institute of Research (WRAIR), Nairobi. The parasites were cultivated by a previously described in vitro technique (Trager et al., 1976). Complete culture medium (CMS) was prepared and consisted 10.4 g/l of RPMI 1640 (Rosewell Park Memorial Institute), 25 mM (5.94 g/l) HEPES buffer and 31.5 mM sodium bicarbonate incubation period, the radio-labeled cultures were harvested onto In vitro anti-plasmodium assay of 0.4% by applying 200 µl, 1.5% hematocrit, parasitemia (expressed as the percentage of erythrocytes infected) solvent effects. The susceptibility tests were carried out with initial range. Negative controls treated with solvent (DMSO) alone were added to each set of experiment (Azas et al., 2001) to check for any extracts and compound 1) to the first row wells, a Titertek motorized followed by addition in duplicate of 25 µl of the test solution (plant solutions (1 µg/ml) of chloroquine diphosphate and artemisinin (MW = 282.332) were similarly prepared for use as reference drugs. Each drug was filter-sterilized with syringe adaptable 0.22 µml filters into sterile Bijoux bottles and was stored at -20°C until required for bioassay.

In vitro anti-plasmodium assay

The in vitro anti-malarial test was based on the inhibition of [G-³H] – hypoxanthine (Amersham International, Buckinghamshire, UK) uptake by P. falciparum cultured in human blood (Desjardins, 1979). Twenty five microliters of aliquots of the complete culture medium (CMS) were added to all the wells of 96-well flat bottomed micro-culture plates (Falcon, Becton Dickinson, Franklin Lakes, NJ), followed by addition in duplicate of 25 µl of the test solution (plant extracts and compound 1) to the first row wells, a Titertek motorized hand diluter (Flow laboratories, Uxbridge, UK) was used to make serial twofold dilutions of each sample over a 64-fold concentration range. Negative controls treated with solvent (DMSO) alone were added to each set of experiment (Azas et al., 2001) to check for any solvent effects. The susceptibility tests were carried out with initial parasitemia (expressed as the percentage of erythrocytes infected) 0.4% by applying 200 µl, 1.5% hematocrit, P. falciparum culture to each well.

Two hundred microliters of culture media without parasites was added into four wells on the last row of each plate to serve as a background (controls). Parasitized and non-parasitized erythrocytes were incubated at 37°C in a gas mixture containing 3% CO₂, 5% O₂ and 92% N₂ for 48 h after which 25 µl of culture medium containing 0.5 µCi, of [G-³H] – hypoxanthine was added to each well. The culture plates were further incubated for 18 h. At the end of incubation period, the radio-labeled cultures were harvested onto glass-fiber filters using a 96-well cell harvester. [G-³H] hypoxanthine uptake was determined using a micro-beta trilux liquid scintillation and luminescence counter (Wallac MicroBeta Trilux). The drug concentration capable of inhibiting 50% of the P. falciparum (IC₅₀) was determined by logarithmic transformation of drug concentration and radioactive counts per minute (cpm) using the formula:

\[ \text{IC}_{50} = \text{antilog} \left( \log X_1 + (\log Y_{50} - \log Y_1) \times (\log X_2 - \log X_1) / (\log Y_2 - \log Y_1) \right) \]

Where Y₅₀ is the cpm value midway between parasitisized and non-parasitisized control cultures and X₁, Y₁, X₂, and Y₂ are the concentrations and cpm values for the data points above and below the cpm midpoints (Sixsmith et al., 1984). The analysis of IC₅₀ values were done using the computer software called GraFit-4 database.

Drug combination bioassay

Template plates were used in preparation of drugs combinations. Test drugs were dispensed into an extra plate in various ratios of blends. The combined test samples were well mixed and transferred to multiple daughter plates or test plates, such that 2 daughter plates were made per two drugs pair. One daughter plate was used for the assay and the other kept at -20°C for running a repeat test on a different day at the end of which the average of the two experiments was given as the final result.

The method described by Canfield et al. (1995) was adopted. The solutions of initial concentrations 20 to 50 times the estimated IC₅₀ values were combined in various ratios of various drugs. Thus fixed ratios of predetermined concentrations needed to inhibit parasite growth by 50% (IC₅₀) was used to determine the interaction of two drugs. Single and combined drug solutions were dispensed into the 96 well microtitre plates to give nine combinations ratios of 90:10 to 10:90 (extract A:extract B) (Fivelman et al., 1999). Incubation and subsequent procedures were followed as previously described in section of in vitro anti-plasmodium assay. Corresponding IC₅₀ values were determined for each drug alone and in combination using the method of Sixsmith et al. (1984). The degree of synergy was evaluated according to the method of Berenbaum (1978). Fraction inhibition concentration (sum FIC) was calculated using the formula:

\[ \frac{A_c/A_d + B_c/B_d} = K \text{ (sum FIC)} \]

where Aₐ and Bₜ are the equally effective concentrations (IC₅₀) when used in combination, and Aₐ and Bₜ are the equally effective concentrations when used alone. When FICs < 1 it indicates synergism, sum FICs ≥ 1 and < 2 it indicates additive interaction and sum FIC ≥ 1 it indicates antagonism (Gupta et al., 2002).

RESULTS AND DISCUSSION

Structural elucidation

Abyssinone-v (compound 1, Figure 1), a white powder re-crystallized from fraction 3 of ethyl acetate extract from E. abyssinica was the most active fraction against P. falciparum parasites, with a melting point of 168-9°C. The spectroscopic data (IR, ¹H-NMR, ¹³C-NMR and mass spectra) were as follows: IR spectral data (Table 1) gave a broad absorptions at 3600 cm⁻¹ corresponding to chelated hydroxyl groups, strong peak at 1700 cm⁻¹ corresponding to conjugated carbonyl group, a medium peak at 1650 and 1610 cm⁻¹ corresponding to C=C of alkenes and benzene ring respectively, and a C-O stretch peak at 950 cm⁻¹.

¹H-NMR spectral data (Table 2) indicate that the compound is a flavones with two prenyl groups. The
Table 1. IR parameters of abyssinone-v (Figure 1).

<table>
<thead>
<tr>
<th>Wavenumbers (cm(^{-1}))</th>
<th>950</th>
<th>1650/1610</th>
<th>1700</th>
<th>2800-3000</th>
<th>3800</th>
</tr>
</thead>
<tbody>
<tr>
<td>Functional groups</td>
<td>C-O</td>
<td>C=C</td>
<td>C=O</td>
<td>CH(_3) or CH(_2) or CH</td>
<td>O-H</td>
</tr>
</tbody>
</table>

Table 2. \(^1\)H-NMR and \(^{13}\)C-NMR parameters of abyssinone – v (compound 1).

<table>
<thead>
<tr>
<th>Position of C</th>
<th>(^{13})C-NMR (\delta) (ppm)</th>
<th>(^1)H-NMR (\delta) (ppm), (m, J) (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-2</td>
<td>79.6</td>
<td>5.4, (d, J=8.0)</td>
</tr>
<tr>
<td>C-3</td>
<td>43.3</td>
<td>3.1 (dd, (J = 13, 4.2 \text{ (axial)}))</td>
</tr>
<tr>
<td>C-4</td>
<td>196.6</td>
<td></td>
</tr>
<tr>
<td>C-5</td>
<td>164.4</td>
<td></td>
</tr>
<tr>
<td>C-6</td>
<td>96.8</td>
<td>5.96 s</td>
</tr>
<tr>
<td>C-7</td>
<td>165.1</td>
<td>OH = 7.25 s</td>
</tr>
<tr>
<td>C-8</td>
<td>95.7</td>
<td>6.6 s</td>
</tr>
<tr>
<td>C-9</td>
<td>163.6</td>
<td></td>
</tr>
<tr>
<td>C-10</td>
<td>103.3</td>
<td></td>
</tr>
<tr>
<td>C-1'</td>
<td>127.8</td>
<td></td>
</tr>
<tr>
<td>C-2'</td>
<td>121.8</td>
<td>7.04 s</td>
</tr>
<tr>
<td>C-3'</td>
<td>126.3</td>
<td></td>
</tr>
<tr>
<td>C-4'</td>
<td>153.6</td>
<td>OH = 8.2 br, s</td>
</tr>
<tr>
<td>C-5'</td>
<td>126.3</td>
<td></td>
</tr>
<tr>
<td>C-6'</td>
<td>121.8</td>
<td>7.04 s</td>
</tr>
<tr>
<td>C-1''</td>
<td>29.9</td>
<td>3.3 (d, J=7.0)</td>
</tr>
<tr>
<td>C-2''</td>
<td>129.9</td>
<td>5.3 m</td>
</tr>
<tr>
<td>C-3''</td>
<td>135.1</td>
<td></td>
</tr>
<tr>
<td>C-4''</td>
<td>26.0</td>
<td>1.75 s</td>
</tr>
<tr>
<td>C-5''</td>
<td>18.1</td>
<td>1.71 s</td>
</tr>
</tbody>
</table>

\(^1\)H-NMR spectrum of compound 1 showed resonance for two protons, H-3 (axial) at \(\delta = 3.1 \, dd\), \(J = 13, 4.2 \text{ Hz}\) and H-3 (equatorial) at \(\delta = 2.8 \, dd, J = 14.2, 3.0 \text{ Hz}\), H-2 proton at \(\delta = 5.4 \, d, J = 8.0 \text{ Hz}\), a chelated 5-hydroxyl proton at \(\delta = 12.06 \, s\). It also showed an aromatic singlet at \(\delta = 7.04 \text{ Hz}\) integrating for two protons assigned to H-2' and H-6' allowing the two prenyl groups at C-3' and C-5' in ring B. The prenyl group were confirmed by a resonance H-1'' proton exhibiting an AXY spin system at \(\delta = 3.3 \, d, J = 7.0 \text{ Hz}\) and a multiplet at \(\delta = 5.3 \text{ corresponding to one alkene's proton H-2''}$. The \(^{13}\)C-NMR spectral data (Table 2) showed distinctive peaks at \(\delta = 196.6 \text{ corresponding to a carbonyl carbon atom, the alkenes carbon atoms at }\delta = 129.9 \text{ and 135.1 corresponding to C-2'' and C-3'')}$. Compound 1 was identified as 5, 7, 4'-'trihydroxy-3', 5'-diprenyflavon (trivial name is abyssinone - v). The \(^1\)H-NMR and \(^{13}\)C-NMR values in Table 2 were comparable to reported literature values of Abyssinone-v (Kamat et al., 1981).

Compound 1 was verified to be Abyssinone - v with molecular formula \(C_{25}H_{28}O_5\) by electron impact mass spectral data which showed a molecular ion peak at \(m/z\) 408.

### Anti-plasmodium results

The anti-plasmodium activity criteria in the \textit{in vitro} assay were defined as high when IC\(_{50}\) value was below 10 \(\mu\)g/ml, moderate when between 10 – 50 \(\mu\)g/ml and low when between 50 – 100 \(\mu\)g/ml. Drug samples with IC\(_{50}\) > 100 \(\mu\)g/ml was considered to be inactive. The anti-plasmodium activity of the methanolic crude extract of \textit{E. abyssinica}, solvents extracts (n-hexane, ethyl acetate and residual methanolic extract) and compound 1 against CQ sensitive \textit{P. falciparum} (D6 strain) are shown in Tables 3 and 4.

The Ethyl acetate fraction (Table 3) of the stem bark of \textit{E. abyssinica} showed the highest anti-malarial activity of IC\(_{50}\) value = 1.00 \(\mu\)g/ml. This shows that most of the anti-malarial potency of \textit{E. abyssinica} is concentrated in the ethyl acetate fraction. The chromatographic separation of ethyl acetate fraction led to isolation of the most active
compound 1 in fraction 3 with anti-malarial activity of IC\textsubscript{50} value = 3.20 µg/ml. This shows that compound 1 has a lower activity than the ethyl acetate fraction due to loss in synergism with compounds separated out.

**Anti-plasmodial activity of combination of drugs**

Drug combination is one of the effective means to counter parasite resistance in anti-malarial chemotherapy (White and Olliaro, 1996). Combinations also help reduce risk of drug resistance development (Olliaro and Taylor, 2003; Anne et al., 2001). The abyssinone-v (compound 1) of *E. abyssinica* was further subjected to synergistic analysis with pure compound of artemisinin (ART) and the following results in Table 5 were obtained.

The interaction of abyssinone-v with the purified Artemisinin showed synergistic effect at all the combination ratios with highest synergism at IC\textsubscript{50} = 2.46 and 0.33 µg/ml respectively. Therefore abyssinone-v drug has high potential to be used in combination with artemisinins drugs to help in the reduction of recrudescence that take place after use of artemisinins drugs only. Combination chemotherapy for malaria should take advantage of synergistic interactions, as these would enhance therapeutic efficacy and lower the risk of resistance emergence since the combined drugs protect each partner against emergence of resistance (Woodrow et al., 2005).

**Conclusion**

The most active compound abyssinone-v (Figure 1) isolated from *E. abyssinica* had anti-malarial activity of IC\textsubscript{50} = 3.19 µg/ml. Although the compound abyssinone-v (1) showed lower activity than pure artemisinin (ART) drug with IC\textsubscript{50} = 2.76 ng/ml, a combination of abyssinone-v and artemisinin showed a remarkable synergistic activity of sum FIC = 0.79 at a ratio of 2: 3 of the combination with each having IC\textsubscript{50} = 2.46 µg/ml and 0.33 ng/ml respectively. Abyssinone-v/artemisinin combination has high potential of being developed into a combination therapy for treatment of malaria. This combination...
justifies the use of natural products in developing combination drugs.

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