Potentiation of antimalarial activity of arteether in combination with Vetiver root extract

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In malaria, development of resistance towards artemisinin derivatives has urged the need for new drugs or new drug combinations to tackle the drug resistant malaria. We studied the fresh root extract of Vetiver zizanioides (Linn.) Nash (VET) with a CDRI-CIMAP antimalarial α/β arteether (ART) together for their antimalarial potential. Our results showed additive to synergistic antimalarial activity of VET and ART with sum fractional inhibitory concentrations Σ FICs 1.02±0.24 and 1.12±0.32 for chloroquine sensitive (CQS) and chloroquine resistant (CQR) strain of Plasmodium falciparum (William H. Welch), respectively. Further, these combinations were explored against multidrug resistant rodent malaria parasite i.e. P. yoelii nigeriensis. Analysis of in vivo interaction of ART and VET showed that 10 mg/kg×5 days of ART with 1000 mg/kg of VET ×5 days cured 100% mice infected with MDR parasite, while the same dose of ART could produce only up to 30% cure and VET fraction was not curative at all. Synergism/additiveness, found between VET and ART is reported for the first time. The curative dose of ART in the combination was reduced to its one fourth, and thus limits the side effects, if any. Although antimalarial potential of ART was enhanced by VET, action mechanism of later needs to be elucidated in detail.

Keywords: ACT, Khus grass, Malaria, MDR, Plasmodium falciparum, Plasmodium yoelii nigeriensis, Vetiver

Malaria remains one of the most threatening diseases in the Least Developed and the Developing Countries. According to the World Health Organization (WHO), about 3.2 billion people are at risk of malaria. In 2015 alone, 214 million new cases and 438000 deaths have been reported; 88% from the African region followed by the South-East Asia (10%) and the Eastern Mediterranean region (2%)1,2. About 57 countries have been reported to have reduced their malaria cases by 75% by 2015, in line with the target set by the World Health Assembly in 20053. WHO Member States have endorsed that by adopting the Global Technical Strategy for Malaria 2016-2030, a bold vision of a world free of malaria, have set a new target of reducing the global malaria burden by 90% by 20304.

Though there are studies on controlling the malarial mosquito vector, Anopheles stephensi, including its genetic diversity4,5, till date no effective malaria vaccine is available6 and chemotherapy remains the most feasible alternative towards treatment of the disease7. Furthermore, no new class of antimalarial has been introduced since 19968,9. Drug resistant strains of P. falciparum have been found in many endemic areas of the world10. Artemisinin and its derivatives are the most important class of antimalarial drugs, effective for both uncomplicated and severe malaria11. These act both as blood schizontocidal and gametocytocidal agents12. The most extensively used artemisinin derivatives are arteether, artemunate, artemether and dihydroartemisinin. Although artemisinin derivatives are effective against all Plasmodium species, combination therapies consisting of artemisinin and other standard antimalarial drugs (chloroquine, amodiaquine, mefloquine and sulfadoxin-pyrimethamine) have been demonstrated to have better parasite clearance and efficacies13,14. The use of artemisinin derivatives as monotherapy as well as combination therapies, have recorded decrease in reported admission and deaths around the world between 2000-201015. In contrast, treatment failure of artemunate-mefloquine have also been reported16,17.
There were subsequent reports of artesunate resistant infections from Western Cambodia and western border of Thailand.

History reveals that plants have always been considered as an important source of medicines against malaria. Artemisinin derivatives are now recommended by WHO worldwide in ACT (artemisinin combination therapy) as the first line treatment of malaria. Vetiveria zizanioides (L.) Nash (Poaceae), popularly known as Khus grass or miracle grass, is an important aromatic plant commonly found in India and has been used traditionally in the treatment of mouth ulcers, fever, urinary tract infections including fungal and bacterial infections. It is the major source of the well known vetiver oil which is used in medicine to treat depression, insomnia, rheumatism, arthritis and in perfumery. A hexane bioactive fraction obtained from the roots of this plant inhibited the growth of multi drug resistant bacterial pathogenesis. Luqman et al. also reported antibacterial activity in spent roots of this plant. Simonsen et al. revealed the in vitro antimalarial activity as high as IC₅₀ as 56 µg/mL in whole plant against Plasmodium falciparum. Considering the above findings, in the present study, root extracts were used for antimalarial assessment.

Materials and Methods

In vitro cultivation of P. falciparum

Two laboratory strains of Plasmodium falciparum, P3D7 (Chloroquine sensitive) and PfK1 (Chloroquine resistant) were used for the study. Parasite strains were cultivated in RPMI-1640 (HEPES modified) medium supplemented with 0.5% AlbuMaxII, 0.2% CO₃ and 15 µM hypoxanthine. Cultures were kept at 37°C under an atmosphere of 5% CO₂ and 90% N₂ for 72 h. After 72 h, 100 µL lytic buffer (20 mM Tris pH 7.5, 5 mM EDTA, 0.008% saponin and 0.08% Triton X-100) containing SYBR Green 1x final concentration, was added to each well (50 µL final volume). Eight wells were treated as positive control (without drug) and 4 wells as negative controls (without parasite and drug). The plates were read under fluorescence reader at Ex. 485 nm, Em. 535 nm. IC₅₀ was determined on the basis of DNA content of the parasite using MS-Excel template.

Assessment of antimalarial interactions between ART and VET in vitro

In vitro interactions between ART and VET were determined according to Bhattacharya et al. with some modifications. Briefly, 11 combinations of ART-VET (1:1, 1:2, 1:3, 1:4, 1:5, 2:1, 3:1, 4:1, 5:1, 2:3 and 3:2) were prepared in 96-well micro titre plates and serially diluted. Asynchronous culture of P. falciparum (~1% parasitaemia and 2% haematocrit), obtained was concentrated at 45°C in a rotary vacuum evaporator. The concentrated semisolid was then dried in water bath to remove traces of hexane, if any.

Preliminary phytochemical screening

The phytochemical screening of V. zizanioides roots was carried out as described in Ratha et al. adopting generally accepted laboratory technique for qualitative determinations. The result revealed the presence of alkaloids, amino acid, flavonoids, saponins and tannins in the plant roots.
was exposed for 72 h to these drug dilutions (37°C and 5% CO₂). IC₅₀ of both the drugs in combinations were determined by SYBR Green assay as described above. Fractional inhibitory concentration (FIC) was interpreted by the following formula and subsequent isobolograms were plotted.

\[
FIC = \frac{\text{IC}_{50} \text{ of drug in combination}}{\text{IC}_{50} \text{ of drug alone}}
\]

\(\Sigma\text{ FIC} < 0.5\) represents substantial synergism, \(\Sigma\text{ FIC} < 1\) represents synergism, \(\Sigma\text{ FIC} 1\) and \(< 2\) represents additive interaction, \(\Sigma\text{ FIC} \geq 2\) and \(< 4\) represents slight antagonism whereas \(\Sigma\text{ FIC} \geq 4\) represents marked antagonism.

The sum FIC (\(\Sigma\text{ FIC}\)) value for each of the preparations determined by the following formula was used to classify the drug–drug interaction.

\[
\Sigma\text{ FIC} = \frac{\text{IC}_{50} \text{ of drug A in combination}}{\text{IC}_{50} \text{ of drug A alone}} + \frac{\text{IC}_{50} \text{ of drug B in combination}}{\text{IC}_{50} \text{ of drug B alone}}
\]

**Animals and parasites**

Outbred Swiss mice weighing 20±2 g were procured from the animal facilities at the institute and maintained on commercial pellet and water ad libitum under standard housing conditions. The study was conducted in accordance with ethical guidelines for the care and use of animals and approval of Institutional Animal Ethics Committee (IAEC) was obtained for the use of Swiss mice (IAEC/2007/117/Renew 05 dated 16.5.2012). *Plasmodium yoelii nigeriensis* MDR, a rodent malaria parasite, resistant to chloroquine (250 mg/kg ×4 day), mefloquine (128 mg/kg ×4 day) and quinine (400 mg/kg ×4 day) was used for present study. The parasite strain was maintained by serial blood passage from an infected mouse to naive mice.

**In vitro antimalarial activity of VET and ART**

The susceptibility profile of bioactive hexane fraction of VET and ART against both chloroquine sensitive (3D7) and chloroquine resistant strains (K1) of *P. falciparum* was generated before initiating interaction studies. The potency of each partner was indicated by IC₅₀ values calculated by SYBR Green assay. ART exhibited potent antimalarial activity against both chloroquine resistant *Pf K1 IC₅₀ = 1.80 ng/mL and chloroquine sensitive Pf 3D7 IC₅₀ = 0.7 ng/mL. IC₅₀ values of the root extract was calculated as 9.01 µg/mL and 6.3 µg/mL against K1 and 3D7 strains of *P. falciparum*, respectively. Depending upon the CC₅₀ values, ART showed very high safety index while VET’s safety was found to be moderate (Table 1).

**In vitro interaction between VET and ART**

Antimalarial activity of ART was investigated in combination with VET against both CQS *Pf 3D7 and
CQR Pf K1 strains of *P. falciparum* employing isobologram method and data were analyzed at the IC$_{50}$ level. IC$_{50}$ values of combined treatment were analyzed in relation to their individual IC$_{50}$ and Σ FIC’s (sum FIC of VET+ART) calculated for the VET-ART interactions. Interactions were classified with Σ FIC’s <1 as synergistic, ≥ 1< 2 as additive and ≥ 2 is antagonistic. Combinations of VET and ART showed synergism in 1:1, 1:2, 1:3, 1:4 and 2:1 ratios while other tested ratios showed additive interaction against Pf 3D7 strain. When the same ratios of VET and ART were tested against Pf K1 strain, two combinations (1:1 and 3:2) were found to be synergistic against *P. falciparum* K1 whereas nine combinations (1:2, 1:3, 1:4, 1:5, 2:1, 3:1, 4:1, 5:1, 2:3) showed additive pattern. The isobolograms of VET and ART suggest that these two partners have concentration dependent antimalarial interactions (Fig. 1). The mean sum FIC values for all the tested ratios were found to be 1.02±0.24 against 3D7 and 1.12±0.32 against K1 strain.

**In vivo antimalarial activity of ART with VET by oral administration**

The MDR strain of *P. yoelii nigeriensis* used in this study showed a high level of resistance to chloroquine, quinine, quinidine, amodiaquine, mepacrine, mefloquine and halofantrine. ART at doses of 5 and 10 mg/kg ingested orally imparted 5% and 30% cure, respectively. Antimalarial potential of VET was evaluated against MDR *P. yoelii nigeriensis* in Swiss mice. VET administered at 1000 mg and 500 mg/kg × 5 days (Day 0-4) were not curative for infected mice but when VET (1000 mg/kg × 5 days) was given in combination with ART at 10 mg/kg × 5 days to the infected mice, it was found to be 100% curative (*P* <0.0001) with mean survival time (MST) of ≥28 days (*P* <0.0001). VET alone protected none of the treated mice and ART at 10 mg/kg dose gave only 30% cure with 16.70±7.44 days of MST. Further, reducing the dose of ART to 5 mg/kg × 5 days with combination of VET 1000 mg/kg × 5 days resulted in slightly reduced antimalarial potential (89.47% cure; *P* <0.0001)). ART alone at 5 mg/kg × 5 days dose to

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**Table 1**—Mean IC$_{50}$ values of α/β arteether (ART) and vetiver root extract (VET) against K1 and 3D7 strains of *P. falciparum*

<table>
<thead>
<tr>
<th>Drug/Extract</th>
<th>50% Inhibitory concentration (IC$_{50}$) Mean±SD</th>
<th>50% Cytotoxic conc. (CC$_{50}$, µg/mL)</th>
<th>Safety Index (CC$<em>{50}$/IC$</em>{50}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P/K1</td>
<td>P/3D7</td>
<td>Vero cells</td>
</tr>
<tr>
<td>ART</td>
<td>1.80±0.44</td>
<td>0.77± 0.73</td>
<td>374.49</td>
</tr>
<tr>
<td>VET</td>
<td>9.01±0.28</td>
<td>6.37±4.16</td>
<td>114.07</td>
</tr>
</tbody>
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ART IC$_{50}$ is given in ng/mL while VET IC$_{50}$ is in µg/mL.

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**Fig. 1**—The isobolograms representing *in vitro* antimalarial interactions (mean values of two experiments). The interaction between VET and ART against (A) CQR strain; and (B) CQS strain of *P. falciparum*.

*P. yoelii* infected mice, cured only 5% mice. Our results clearly establish synergistic effect of ART and VET combinations against MDR *P. yoelii nigeriensis* (Table 2). Curative dose of ART was reduced up to 4 times with the combination of VET. Data of the mean survival time of infected and treated group of mice with ART and VET combination has shown synergism as evidenced by their extended survival in combination group than alone treated groups (Fig. 2).

The present study assessed the antimalarial property of Indian traditional plant *Vetiver zizanioides* (VET) individually and in combination with artemisinin derivative α/β arteether (ART). Both chloroquine sensitive (Pf3D7) and resistant (Pf/K1) strains treated with VET exhibited arrested growth of parasites, with IC$_{50}$ values of 6.3 and 9.01 µg/mL,
respectively. This study demonstrated the fair antimalarial potential of VET in vitro. Fujisaki et al. reported IC\textsubscript{50} 1000 \(\mu\)g/mL bicyclovetivenol 10% fraction for vetiver oil constituent against \( Pf \) FCR3 in in vitro studies. The antimalarial activity in the whole plant against \( P. falciparum \) was also reported\textsuperscript{30} High IC\textsubscript{50} values (56 \(\mu\)g/mL) reported in literature may be due to the use of whole plant oil constituent while we have used only hexane root extract which may contain the active ingredients.

\textit{Vetiver zizanioides} is known to contain alkaloids flavonoids, tannins, saponins and amino acid\textsuperscript{24,32}. Previous reports of Vetiver extract showed its effective antioxidant and antimicrobial property\textsuperscript{28,39}. 

Table 2—Chemotherapeutic response of arteether, vetiver and their combinations against multi drug resistant \( P. yoelii nigeriensis \) in Swiss mice

<table>
<thead>
<tr>
<th>Drug/Extract</th>
<th>Mean % parasitaemia ± SD (No. of infected mice/ No. of total live mice)*</th>
<th>% Cure</th>
<th>MST±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>ART + VET</td>
<td>48.60±12 (10/10)</td>
<td>0</td>
<td>6.3±0.45</td>
</tr>
<tr>
<td>- 500</td>
<td>42.17±17 (20/20)</td>
<td>0</td>
<td>6.8±2.48</td>
</tr>
<tr>
<td>- 1000</td>
<td>16.16±13 (3/3)</td>
<td>0</td>
<td>13.2±5.35</td>
</tr>
<tr>
<td>5 -</td>
<td>9.8±15 (1/1)</td>
<td>5</td>
<td>16.7±7.44</td>
</tr>
<tr>
<td>10 -</td>
<td>5.1±26 (5/6)</td>
<td>30</td>
<td>17.4±7.0***</td>
</tr>
<tr>
<td>5 500</td>
<td>34.45±22 (1/1)</td>
<td>0</td>
<td>26.4±4.8***</td>
</tr>
<tr>
<td>10 500</td>
<td>27.57±30 (1/1)</td>
<td>0</td>
<td>26.3±4.75***</td>
</tr>
<tr>
<td>5 1000</td>
<td>18.25±25 (1/1)</td>
<td>0</td>
<td>89.47***</td>
</tr>
<tr>
<td>10 1000</td>
<td>15.3±3 (1/1)</td>
<td>100***</td>
<td>&gt;28***</td>
</tr>
<tr>
<td>Control</td>
<td>30.67±20 (20/20)</td>
<td>0</td>
<td>6.65±0.56</td>
</tr>
</tbody>
</table>

\( P \) values of combination groups are calculated against their one partner drug i.e. arteether.

\( *P = 0.09 ; **P = 0.0052 ; ***P \leq 0.0001 \); \( ^* \) Data is pooled from 2-4 different experiments; 5 mice/group are used in each experiment.

Fig. 2—Survival record of treated and control mice infected with \( P. yoelii nigeriensis \) MDR
Presence of flavonoids and tannins could be responsible for free radical scavenging or antioxidant nature\textsuperscript{23,40}. Oxidative stress has been shown to play an important role in the development of anaemia in malaria. Increase in total antioxidant status is important role in the development of anaemia in malaria. Increase in total antioxidant status is important in recovery from malaria\textsuperscript{41}. Vetiver plant has been reported to stimulate the production of red blood cells (RBCs) and, thus beneficial for anaemia treatment\textsuperscript{25}. This antioxidant activity can counteract the oxidative damage induced by the malaria parasite. The antioxidant activity of this extract appears to contribute to their antimalarial property.

In the present study vetiver extract has shown in vitro antimalarial property with IC\textsubscript{50} 6.30 µg/mL and safety index (SI) 17.9, and therefore, its combination with ART was assessed both in vitro and in vivo.

Interaction studies for VET and ART against CQS \textit{P. falciparum} 3D7 were found to be synergistic (\Sigma FIC value <1) at five ratios (1:1,1:2,1:3,1:4,2:1) while at other combination (1:5,3:1,4:1,5:1,2:3,3:2 ) it was additive. Similarly, in vitro combination studies of VET and ART against CQR P/K1 strain revealed the synergistic activity in two ratios (1:1 and 3:1). In vitro data against two \textit{P. falciparum} lab strains demonstrate that both combinations show a tendency towards synergism/additiveness for VET and ART. In vivo combination studies performed in the murine \textit{P. yoelii nigeriensis} MDR model also showed additive to synergistic effect. No gross sign of toxicity was observed for any combination tested in vivo.

Mice treated with the combination of ART and VET showed better survival compared to the groups that were treated individually with ART or VET. VET could enhance the antimalarial activity of ART. VET at a dose of 1000 mg/kg for 5 days in combination with ART 10 mg/kg for 5 days, produced 100% cure, while single drug ART groups showed a maximum 30% cure of treated animals. At lower doses also, both the combinations produced higher cure rates with prolonged survival time as compared to either treatment. These data clearly indicate that both combinations have additive to synergistic antimalarial potential against multidrug resistant malaria parasites in mice. Antimalarial potential of ART was also enhanced with the use of an anti fungal ketoconazole and antibacterial azithromycin\textsuperscript{37,42}.

In conclusion, the synergism/additiveness found between VET and ART is reported for the first time. To the best of our knowledge, the vetiver root hexane extract has not been reported for its antimalarial activity against chloroquine sensitive/resistant \textit{P. falciparum} so far. The curative dose of ART in the combination was reduced to its one fourth, and this lead has indicated the enhancing effect of VET with artemisinins but action mechanism of VET needs to be elucidated in detail. Extension of similar studies on other species of vetiver may throw some light on their antimalarial potential.

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**References**


