Comparative Effects of Repeated In Vitro Exposures of Plasmodium Falciparum to Sub-Lethal Doses of Pure Artemisinin and Artemisia Annua Phytochemical Blend

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Abstract

Background: Although tea infusions of Artemisia annua L. and Artemisia apiacea have been used > 2000 years in traditional Chinese medicine, there was no apparent resistance development to malaria parasites. Artemisinin was isolated and characterized in 1972 as the major potent antimalarial component of A. annua. This and a number of its derivatives have been promoted in combination therapy (ACTs) with other antimalarials with different mechanisms of action. However, recent reports on artemisinin resistance have posed a major health security risk globally. The aim of the present study was to compare the effects of cyclic exposures of the malaria parasite Plasmodium falciparum to a phytochemical blend of Artemisia annua (extracted with solvents of different polarity) with those of pure artemisinin to see if the natural blend has a built-in resistance-mitigating effect on artemisinin against the parasite.

Materials and methods: Upper foliage of Artemisia annua was extracted with solvents of increasing polarity, including hexane, dichloromethane, methanol, and water. The organic solvents were removed on a rotary evaporator and water by freeze-drying. In vitro cyclic exposure experiments to equivalents of IC50 and IC90 of (i) A. annua phytochemical blend and (ii) pure artemisinin were conducted on CQ-resistant strain cultures of P. falciparum W2 from Indochina. Dose-response effects of the parasites were determined after 10, 20, 30 and 40 cycles of exposures and relative shifts in the sensitivities of parasites (IC50_new/IC50_initial, or IC90_new/IC90_initial) expressed as relative sensitivity indices (RSI) were calculated.

Results: There were incremental increases in the IC50 or IC90 values with increasing cycles of exposures of W2 parasites to IC50 or IC90 equivalents of artemisinin. No comparable increases were observed with the parasites exposed to IC50 and IC90 equivalents of A. annua blend. On the other hand, parasites repeatedly exposed to the A. annua blend showed decreasing susceptibility to pure artemisinin.

Conclusion: The results show that ethnopharmacological mode of use of the phytochemical blend of A. annua is less likely to lead to resistance development to malaria parasites compared with pure artemisinin. This is partially consistent with a recent finding on a rodent malaria model done by Elfawal, which demonstrated that the whole A. annua plant (WP) overcomes existing resistance of Plasmodium yoelii to pure artemisinin. However, further studies are needed to elucidate the nature of observed resistance to artemisinin in the parasites that were repeatedly exposed to A. annua phytochemical blend.

Keywords
Artemisia annua, Phytochemical blend, Artemisinin, Plasmodium falciparum, Malaria, Cyclic exposures, Resistance mitigation

Introduction

Artemisia annua and a related species, Artemisia apiacea, have a long tradition of use in China for treatment of intermittent fevers, which are now associated with malaria infections [1]. In 1972, the major active principle of A. annua was identified as the sesquiterpene artemisinin with novel mechanism of action against malaria parasites associated with its lactone-endoperoxide moiety [2]. The compound also demonstrated potent antimalarial activity with high safety profile [3]. This stimulated growing global interest in A. annua and artemisinin, which saw rapid geographic spread of A. annua cultivation in Asia and Africa, as well as in parts of Europe and America [4].

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The use of the plant and its phytochemistry took two different pathways, one driven by the pharmaceutical approach and the other by traditional Chinese herbal pharmacopeia [5]. The former sought to improve the general performance of artemisinin as an antimalarial drug. Artemisinin was found to have poor pharmacokinetic properties, but structural modifications of the compound led to a number of derivatives, such as artesunate and artemether, with improved bioavailability. Moreover, to minimize probability of resistance development, artemisinin and some of its derivatives have been promoted in combination with other non-artemisinin based antimalarials with different modes of action (Artemisinin Combination Therapy, ACT) to treat Plasmodium falciparum based malaria [6]. The choice of specific ACT blend in a malaria-endemic area is based on the results of therapeutic efficacy studies against local strains of P. falciparum [7]. However, recent reports from the Southeast Asian countries reveal evidence of emerging resistance to ACTs [8]. This has become a major source of concern, since if it spreads widely, it would derail current goals to control and eliminate malaria.

Concurrently, because of its easy access and affordability, traditionally prepared A. annua formulations for malaria treatment spread widely beyond China [1]. Moreover, there has been a growing scientific interest on potential roles of other phytochemical constituents of A. annua in the performance of tea infusions and orally consumed dried leaf in prophylactic uses and in malaria control [9-15]. A. annua is rich in secondary metabolites. In addition to artemisinin, it produces a range of mono-, sesqui-, di- and triterpenes, flavonoids, and polyphenols [16,17]. Some constituents of the plant enhance bioavailability of artemisinin [12-14]. In one study, healthy mice fed with dried A. annua leaves had > 40 times more artemisinin in their bloodstream than mice fed with a corresponding amount of the pure drug [18]. Other constituents appear to selectively potentiate and synergise in vitro activity of artemisinin against P. falciparum [16,19-21]. In a recent in vivo study, a single dose of dried leaves of A. annua delivered orally to Plasmodium chabaudi infected mice quelled parasitemia at least five-fold more than an equal amount of artemisinin [12].

A major concern on the use of A.annua tea infusions or whole plant products has been that low levels of artemisinin contents could lead P. falciparum to develop resistance against the drug [1]. However, in a recent study A. annua treatment was found to overcome existing resistance to pure artemisinin in rodent malaria Plasmodium yoelti, or induced resistance in P. chabaudi after repeated treatments with pure artemisinin [22].

The aim of the present study was to compare the effects of cyclic in vitro exposures of the phytochemical blend of A. annua with those of pure artemisinin against CQ-resistant strain culture of P. falciparum W2 to see if the natural blend shows a similar pattern of results.

Materials and Methods

Sources of A. annua plant, artemisinin and chloroquine

Artemisia annua used in this study was obtained from a hybrid of the plant grown in the Tanzania highlands (2000-2200 m altitude) in Arusha by Natural Uwemba System for Health (N.U.S.Ag). The leaves were harvested just before flowering, dried for approximately 3 weeks under shade, and then crushed, and powdered. Batches of 5 g of the powder were separately extracted with acetone/toluene (50 mL) at room temperature (18-22°C) for 24 hr, filtered and concentrated in a rotary evaporator at 40°C each extract was then dissolved in 25 ml HPLC-grade acetonitrile and analyzed at ambient temperature on a Beckman HPLC equipment with a photodiode-array detector (PAD) at 195 nm, and a Supelco C-18 column (15 cm x 4.6 mm i.d., 5 µm particles). The mobile phase was isocratic composed of 50% aqueous HPLC-grade acetonitrile with a flow-rate of 1.0 mL Min⁻¹. Five different doses of pure (98%) artemisinin (10 µL of 0.1 to 0.5 mg mL⁻¹ of acetonitrile) were initially injected to provide dose-response data for quantification of the constituent in the extracts. 10 and 20 µL of each of the five extracts were then injected and the signals with RI corresponding to that of artemisinin quantified. Artemisinin content of the powder was found to be 0.74 ± 0.06%.

Preparation of test materials

Samples (250 g) of powdered A. annua were extracted sequentially with solvents of increasing polarity (hexane, dichloromethane, methanol, water). The organic extracts were filtered, and the solvents removed by rotary evaporation. Residual blend of solvents was then removed by a flow of nitrogen. Water extracts were filtered and freeze-dried. The different extracts were analyzed by high performance liquid chromatography (HPLC) and were found to be dominated by A. annua constituents of increasing polarity. The combined crude extract was then used for dose-response and selection experiments. Artemisinin (10 mg), the combined extract (1000 mg) and CQ as positive control (10 mg) were separately dissolved in dimethylsuloxide (DMSO, 1 ml) and then diluted with water (9 ml).

Parasite cultivation

Laboratory adapted P. falciparum cultures of W2 (CQ-resistant) strain, originally from Indochina, was used in the study. The strain was cultured and maintained in the malaria laboratory at Kenya Medical Research Institute (KEMRI) Nairobi. The culture medium was a modification of that described by Trager and Jensen 1976 [23] and consisted of RPMI 1640 supplemented with 10% human serum 25 mM N-(2-hydroxyethyl) piparazine-N'-2-ethanosulfonic acid (HEPES) and 25 mM NaHCO3. Human type O+ erythrocytes (< 8-days-old) served as host cells and the cultures were incubated at 37°C in an atmosphere of 3% CO₂, 5% O₂ and 92% N₂ [23].

Ethical approval

The study was approved by the Ethics Committee of KEMRI SCC No. 1340/08. Blood donors in the study signed a written consent form. Each samples of blood was tested for both Hepatitis and HIV virus before it was used in the study.

Test samples

Test samples were prepared on the same day the tests were initiated. Each solution was filtered and then stored at -4°C. In-vitro semi-automated micro-dilution assay technique was used [24,25] in the assays, each conducted in triplicates. Aliquots (25 µl) of the culture medium were added to all 96 wells of the flat bottomed microculture plate (Costar Glass Works, Cambridge, UK). Aliquots of test solutions (25 µl) of artemisinin (10 ng/mL) or A. annua blend (10 µg/mL) or chloroquine as a control drug (100 ng/mL) were added to the first set of wells by using a tite-tek motorized hand pipette (Flow Laboratories, Uxbridge, UK), followed by 2-fold serial dilutions of each sample over a 64-fold concentration range.

Suspension of parasitized erythrocytes in culture medium (200 µl) (0.4% parasitemia) with a growth rate of > 3 fold were added to all test wells. Parasitized and non-parasitized erythrocytes were incorporated into all tests. The plates were incubated at 37°C in a gas mixture of 3% CO₂, 5% O₂ and 92% N₂. After 48 hrs, each well was pulsed with 25 µl of culture medium containing 0.5 µci of (G-3H) hypoxanthine and the plates incubated for a further 18 hrs.

The contents of each well were then harvested with a beta cell harvester (Wallac Zurich) onto glass fiber filters, washed thoroughly with distilled water, and dried. The filters were then inserted into plastic bags with liquid scintillant and radioactivity measured in counts per minute (cpm) per well at each concentration.
Cyclic exposures of parasites to IC₉₀ and IC₅₀ equivalents of (i) artemisinin, and (ii) A. annua blend.

IC₅₀ and IC₉₀ values of artemisinin and the blend were initially determined with the W2 (CQ-resistant) strain of Plasmodium falciparum. The values obtained were used to calculate appropriate equivalents of artemisinin and the blend, which were separately incorporated in RPMI 1640 media that were enriched with 6% pooled serum. Each set of three replicates of exposures, medium change was done after every cycle of 48 hours. Parasitemia was determined with the W2 (CQ-resistant) strain of Plasmodium falciparum.

The results of RSI obtained at successive 10-fold cycles of exposures to IC₅₀ or IC₉₀ equivalents of pure artemisinin lead to 40 cycles of artemisinin became less sensitive to the artemisinin. Likewise, in exposures to IC₅₀ equivalents of artemisinin, RSI was 1.8 at cycle 10, to 2.4 at cycle 20, 22.66 at cycle 30, and 26.42 at cycle 40. Likewise, in exposures to IC₉₀ equivalents of artemisinin, RSI was 1.8 at cycle 10, to 2.4 at cycle 20, 27.99 at cycle 30, and 29.4 at cycle 40.

Parasites exposed to IC₅₀ equivalents of A. annua extract remained sensitive to the blend at successive cycles (for treatments with IC₅₀, IC₉₀ values were 0.09 at cycle 10, 0.08 at cycle 20, 1.87 at cycle 30, and 0.0062 at cycle 40). In exposure cycles with IC₉₀ equivalents of A. annua extract, the parasites remained sensitive to the blend, but there appears to have been a small loss of sensitivity at cycle 40 (RSI, 5.37).

Discussion

The present study shows that cyclic exposures of P. falciparum parasites to IC₅₀ or IC₉₀ equivalents of pure artemisinin lead to development of incremental resistance as reflected in their susceptibility indices (RSIs) at 20th, 30th and 40th cycles.

Data analyses

Computation of the concentration of each drug causing 50% inhibition of (G-3H) hypoxanthine uptake (IC₅₀) was determined using Chemosen Program 2 according to the following formula:

$$IC_{50} = \text{antilog} \left\{ \log X_i + \frac{1}{2} \left( \log Y_{\text{mid}} - \log Y_i \right) \right\} / \log Y_i \cdot \log Y_{\text{mid}}$$

Where Yₜ₅₀ is the cpm value midway between parasitized and non-parasitized control cultures and X₁, Y₁, X₂, and Y₂ are the concentrations and cpm values for the data points above and below the cpm midpoints [26].

Relative sensitivity indices, RSI (IC₅₀ new/IC₅₀ initial, or IC₉₀ new/IC₉₀ initial) were calculated after each cycle of 10 exposures in each set of treatments. Student–Newman–Keuls (SNK) method was used to compare RSI values obtained from each set of treatments at different 10-fold exposures. Data was entered in excel sheet and analyzed with SPSS 22.0.

Results

Effects of cyclic treatments of W2 strain of P. falciparum to (a) IC₅₀ and (b) IC₉₀ values of artemisinin

The results of RSI obtained at successive 10-fold cycles of treatments are depicted in table 1 and table 2.

RSI for parasites exposed to artemisinin at IC₅₀ and IC₉₀ equivalents and tested with artemisinin increased with increasing cycles. In treatments with IC₅₀ equivalents of artemisinin, RSI increased from 1.8 at cycle 10, to 2.4 at cycle 20, 22.66 at cycle 30, and 26.42 at cycle 40. Likewise, in exposures to IC₉₀ equivalents of artemisinin, RSI was 0.82 at cycle 10, 7.36 at cycle 20 and 16.04 at cycle 30. On the other hand parasites exposed to IC₅₀ and IC₉₀ equivalents of artemisinin remained sensitive to A. annua blend after all cycles of exposures (in exposures to IC₅₀ equivalents of artemisinin, RSI values for A ansua blend were 0.09 at cycle 10, 0.09 at cycle 20, 1.89 at cycle 30 and 0.07 at cycle 40; and in exposures to IC₉₀ equivalent of artemisinin, RSI values were 0.61 at cycle 10, 1.51 at cycle 20 and 0.22 at cycle 30). Chloroquine showed a similar trend as the A. annua blend in both sets of treatments with artemisinin.

Effects of cyclic treatments of W2 strain of P. falciparum with IC₅₀ and IC₉₀ values of A. annua blend

The results of RSI obtained at successive 10-fold cycles of treatments are depicted in table 3 and table 4.

Parasites exposed to IC₅₀ equivalents of A. annua extract remained sensitive to the blend at successive cycles (for treatments with IC₅₀, RSI values were 0.09 at cycle 10, 0.08 at cycle 20, 1.87 at cycle 30, and 0.0062 at cycle 40). In exposure cycles with IC₉₀ equivalents of A. annua extract, the parasites remained sensitive to the blend, but there appears to have been a small loss of sensitivity at cycle 40 (RSI, 5.37). However, in both cases the parasites showed decreasing sensitivity to artemisinin (RSI values increasing from 0.9 at cycle 10 and 0.59 at cycle 20, to 27.9 at cycle 30 and 29.4 at cycle 40).

Interestingly, CQ-resistant parasite strain (W2) that was exposed to 40 cycles of artemisinin became less sensitive to the artemisinin. In all the four tests above the RSI for artemisinin increased between 10 fold to 36 fold. The response to the blend was different and it was evident that the parasites remained sensitive to the blend.

Table 1: Relative sensitivity indices (RSI) of P. falciparum cyclically exposed to artemisinin at IC₅₀ and tested with (i) artemisinin, (ii) A. annua blend and (iii) chloroquine.

<table>
<thead>
<tr>
<th>CYCLES</th>
<th>ART MEAN ± SE</th>
<th>Blend MEAN ± SE</th>
<th>CQ MEAN ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSI cycle 10</td>
<td>1.81 ± 0.38*</td>
<td>0.09 ± 0.01*</td>
<td>1.01 ± 0.03*</td>
</tr>
<tr>
<td>RSI cycle 20</td>
<td>2.42 ± 0.84*</td>
<td>0.09 ± 0.01*</td>
<td>1.13 ± 0.10*</td>
</tr>
<tr>
<td>RSI cycle 30</td>
<td>22.66 ± 2.95*</td>
<td>1.89 ± 0.14*</td>
<td>0.87 ± 0.01*</td>
</tr>
<tr>
<td>RSI cycle 40</td>
<td>26.45 ± 0.87*</td>
<td>0.07 ± 0.00*</td>
<td>1.43 ± 0.03*</td>
</tr>
</tbody>
</table>

Mean ± SE followed by the same letter(s) within the same column do not differ significantly from one another (One-way ANOVA, SNK-test, α = 0.05).

Table 2: Relative sensitivity indices (RSI) of P. falciparum cyclically exposed to artemisinin at IC₉₀ and tested with (i) artemisinin, (ii) A. annua blend and (iii) chloroquine.

<table>
<thead>
<tr>
<th>CYCLES</th>
<th>ART MEAN ± SE</th>
<th>Blend MEAN ± SE</th>
<th>CQ MEAN ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSI cycle 10</td>
<td>0.93 ± 0.06*</td>
<td>0.83 ± 0.02*</td>
<td>0.96 ± 0.02*</td>
</tr>
<tr>
<td>RSI cycle 20</td>
<td>3.00 ± 0.08*</td>
<td>0.17 ± 0.01*</td>
<td>1.10 ± 0.03*</td>
</tr>
<tr>
<td>RSI cycle 30</td>
<td>27.99 ± 0.48*</td>
<td>0.85 ± 0.05*</td>
<td>0.23 ± 0.00*</td>
</tr>
<tr>
<td>RSI cycle 40</td>
<td>29.00 ± 1.15*</td>
<td>2.14 ± 0.08*</td>
<td>0.29 ± 0.01*</td>
</tr>
</tbody>
</table>

Mean ± SE followed by the same letter(s) within the same column do not differ significantly from one another (One-way ANOVA, SNK-test, α = 0.05).

Table 3: Relative sensitivity indices (RSI) of P. falciparum cyclically exposed to A. annua blend at IC₅₀ and tested against (i) artemisinin, (ii) A. annua blend, and (iii) chloroquine.

<table>
<thead>
<tr>
<th>CYCLES</th>
<th>ART MEAN ± SE</th>
<th>Blend MEAN ± SE</th>
<th>CQ MEAN ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSI cycle 10</td>
<td>1.28 ± 0.06*</td>
<td>1.06 ± 0.04*</td>
<td>1.01 ± 0.02*</td>
</tr>
<tr>
<td>RSI cycle 20</td>
<td>14.40 ± 0.63*</td>
<td>0.11 ± 0.01*</td>
<td>1.09 ± 0.04*</td>
</tr>
<tr>
<td>RSI cycle 30</td>
<td>19.25 ± 3.25*</td>
<td>0.67 ± 0.10*</td>
<td>1.00 ± 0.02*</td>
</tr>
<tr>
<td>RSI cycle 40</td>
<td>38.38 ± 0.40*</td>
<td>5.37 ± 0.33*</td>
<td>1.15 ± 0.01*</td>
</tr>
</tbody>
</table>

Mean ± SE followed by the same letter(s) within the same column do not differ significantly from one another (One-way ANOVA, SNK-test, α = 0.05).

Table 4: Relative sensitivity indices (RSI) of P. falciparum cyclically exposed to Artemisia annua blend at IC₉₀ and tested against (i) artemisinin, (ii) A. annua blend and (iii) chloroquine.

<table>
<thead>
<tr>
<th>CYCLES</th>
<th>ART MEAN ± SE</th>
<th>Blend MEAN ± SE</th>
<th>CQ MEAN ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSI cycle 10</td>
<td>1.81 ± 0.38*</td>
<td>0.96 ± 0.01*</td>
<td>0.95 ± 0.02*</td>
</tr>
<tr>
<td>RSI cycle 20</td>
<td>3.00 ± 0.08*</td>
<td>0.17 ± 0.01*</td>
<td>1.10 ± 0.03*</td>
</tr>
<tr>
<td>RSI cycle 30</td>
<td>27.99 ± 0.48*</td>
<td>0.85 ± 0.05*</td>
<td>0.23 ± 0.00*</td>
</tr>
<tr>
<td>RSI cycle 40</td>
<td>29.00 ± 1.15*</td>
<td>2.14 ± 0.08*</td>
<td>0.29 ± 0.01*</td>
</tr>
</tbody>
</table>

Mean ± SE followed by the same letter(s) within the same column do not differ significantly from one another (One-way ANOVA, SNK-test, α = 0.05).
whole-plant \textit{A. annua} effectively kills rodent malaria parasites that are resistant to artemisinin despite lower levels of these antimalarial constituent in treatments with \textit{A. annua}.

However, two sets of results of the study need special attention. First, in the experiment with cyclic exposures of \textit{P. falciparum} to IC\textsubscript{90} and IC\textsubscript{50} \textit{A. annua} blend, the parasites lost their sensitivity to artemisinin (RSIs 29.00 ± 1.15 and 36.38 ± 0.40 at 40 cycles in IC\textsubscript{90} and IC\textsubscript{50} cyclic treatments, respectively). This indicates that the blend of \textit{A. annua} constituents extracted by solvents of different polarity does not mitigate against resistance development to artemisinin. This is unlike previous findings in selection studies with \textit{Azadirachta indica} limonoids against two insect species \cite{27,28}. In one selection study, the growth-inhibiting effect of azadirachitin (the most potent limonoid constituent of the neem plant) and the resulting mortality of an aphid (green peach aphid), was compared with that of the crude phytochemical blend of azadirachitin with a series of other limonoids. Azadirachitin selected line demonstrated significantly decreased susceptibility to the natural product within 10 generations compared to the parental line. No such resistance to azadirachitin occurred in phytochemical-blend selected line even after 40 generations \cite{27}. A similar contrast was found in another study involving comparison of the feeding retardation effect of azadiracitin with that of the blend of \textit{A. indica} limonoids on a different insect (\textit{Spodoptera litura}) \cite{28}. Interestingly, \textit{A. annua} is dominated by constituents of different structures, whereas \textit{A. indica} comprises mainly of structural variants of azadirachitin. How this difference in the two phytochemical profiles may affect their resistance-mitigating effects constitutes a very interesting question.

Second, in experiment involving cyclic exposures to \textit{A. annua} solvent extract, in addition to resistance development of artemisinin to the parasites, there also appeared to be small but significant resistance development to the extract at 40\textsuperscript{th} cycle (RSIs 2.14 ± 0.08 and 5.37 ± 0.33 in IC\textsubscript{90} and IC\textsubscript{50} treatments, respectively). This raises a series of questions, including whether this is a reflection of enhanced development of resistance to artemisinin, or multiple resistance to more than one constituent of \textit{A. annua} solvent extract. In addition, are the observed losses of efficacies of both artemisinin and extracted \textit{A. annua} phytochemical blend largely due to epigenetic or genetic effects? Further cyclic studies, \textit{in vitro} as well as \textit{in vivo} with animal models, including monitoring the effects of terminating exposures to artemisinin or \textit{A. annua} blend, are expected to shed further light on this question. The results of one such study with two groups of Swiss mice, one inoculated with \textit{P. berghei} ANKA, and the other with \textit{P. yoelii}, followed by cyclic treatments with ED\textsubscript{90} and ED\textsubscript{50} equivalents of artemisinin or \textit{A. annua} phytochemical blend will be reported elsewhere. With the artemisinin exposed parasites the effective doses increased with the increase in the cycle numbers but on removal of drug pressure the resistance gained was lost.

## Conclusion

The results show that ethnopharmacological mode of use of the phytochemical blend of \textit{A. annua} is less likely to lead to resistance development to malaria parasites compared with pure artemisinin. However, further studies are needed to elucidate the nature of observed resistance of the parasites to artemisinin as well as to \textit{A. annua} blend.

## Declarations

### Availability of data and materials

Data sharing not applicable to this article as no data sets were generated during the current study. Please contact author for data requests.

### Consent for publication

Not applicable.

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### Competing interest

There were no competing interests.

### Funding

We would like to thank Datuma (Swiss Company) for their support in partial funding of the project in the collection \textit{Artemisia annua} and the pure artemisinin acquisition. We would also like to thank National Commission of Science and Technology innovation (NACOSTI) for their funding in buying expendables that were used in the collection of data and in analysis.

### Ethical approval

As stated earlier the study was approved by the Ethics Committee of KEMRI SCC No. 1340/08. Blood donors signed a consent form before donation.

### Authors' contribution

Each author contributed as follows, Lucy, Swabah and Hassanali conceived the idea and contributed in the design of the project, data collection, data analysis and publication preparation, Joseph contributed in the design of the project, data analysis and publication preparation, Francis and Johnson contributed in data collection and publication preparation.

### Acknowledgments

Stephen Kaniaru helped in maintaining the \textit{in-vitro} cultures.

### References


