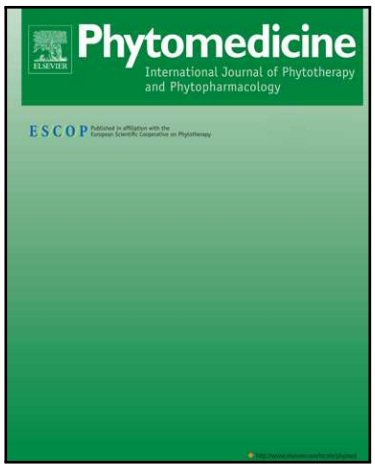

RESPONSE to Gillibert et al. 2019

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RESPONSE to Gillibert et al. 2019

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Introduction

Thank you to the authors for their in-depth analysis of our study. To begin, and as we also did for our recent schistosomiasis trial (Munyangi et al. 2018; data shared on March 18, 2019), we note to the reader that have already shared all of the data obtained in this trial with Dr. Gillibert on March 8, 2019. This was prior to becoming aware of their critique that was submitted January 3, 2019 to Editor Efferth, but only shared with us as of April 3, 2019. Sharing our data shows that we are open and have nothing to hide. Our trial was not a registration study, and further trials, integrating the perspectives and conclusions of the published study, are expected. As with our schistosomiasis trial, we were especially constrained in human and financial resources, thereby limiting what we would have preferred to measure. Nevertheless, the data are as collected, and are the result of all human blood samples being *twice* microscopically analyzed: once during the trial, and then all again after receipt of extensive reviewers' comments after first submission of the manuscript.

Our response to the Argemi et al. 2018 critique is now published (Cornet-Vernet et al. 2019). The drug protocols were only different in that the control drugs, PZQ or ASAQ, were specific to their intended disease. The tea regimen was the same. The study approval date for this study was 2015; 2016 was a typographical error. Along with the data, the Approval Registration documentation also has been provided to the Gillibert team.

We also wish to emphasize that neither *Artemisia annua* nor *A. afra* is an artemisinin monotherapy. Rather each plant species is a potential antimicrobial polytherapy because of its plethora of antimicrobial phytochemicals (see as examples, Efferth 2009; Suberu et al. 2013; Weathers et al. 2014; Penna-Coutinho et al. 2018; Moyo et al. 2019). There is also the long history (>2,000 yr) use of *A. annua* to treat fever diseases with no documented problems of "resistance". This study was justified based not only on the literature that we cited, but also on a growing number of anecdotal observations by local African healthcare providers who had begun using the plant because they were becoming increasingly frustrated with poor ACT outcomes, recently substantiated in a review by Naß and Efferth (2019).

Our study did not report, nor do we support the use of *A. annua* as a prophylaxis. Where ACT and IV artesunate have failed, we counter that it is unethical to withhold use of *A. annua* tea infusions for patients. As we cited, there is also common ethnobotanical use of *A. afra* to treat malaria in Southern Africa, so its use in a trial is not unethical.

We conducted this trial to begin providing the clinical data that WHO in its 2012 whitepaper (WHO 2012) said was lacking. *A. afra* and *A. annua* are not monotherapies. Indeed, the equivalent performance of *A. afra*, lacking much artemisinin, to *A. annua,* replete with artemisinin, and the better performance of both against ASAQ validates this was not an artemisinin monotherapy. Further, past studies using tea infusions may not always have been as rigorously conducted with placebos, with an artemisinin–deficient species, and with better prior knowledge from the subsequent literature on the amount of dried leaves to use, and with precisely prepared infusions. Prior trials also did not measure phytochemical content of the *A. annua* used beyond artemisinin. We did, and considering that at least 10 other phytochemicals having antimalarial activity in *A. annua* are also present in *A. afra*, their presence is important. Tea infusions prepared as described extract nearly 100% of artemisinin (van der Kooy and Verpoorte 2011), which we have also confirmed at >99% recovery (unpublished). Furthermore, and as we cited, we previously addressed the resistance question with *in vivo* animal studies wherein artemisinin-resistant parasites were induced using pure drugs; using dried leaf *A. annua* powder, however, it took at least 3 times longer for any possible artemisinin resistance to develop (Elfawal et al. 2015). In the same study, we also showed that mice infected with artemisinin-resistant *Plasmodium* were treatable with once daily gavage of *A. annua* dried leaf powder, but not with the pure drug. Furthermore, in the case studies described by Daddy et al. (2017) the cure of 18 patients with severe malaria showed that *A. annua* has serious merit; neither ACT nor IV artesunate helped those patients. In the present study, we saw a large number of ASAQ failures; yet in the same localities, *A. afra* and especially *A. annua* were highly effective.

We agree more leaves were in 5 vs. 0.2 g, but we followed suggestions of WHO in how to do this. It also would have been better to have an identically prepared placebo tablet rather than what was essentially a sugar tablet. We fully informed the reader how we did the trial, and the trial was conducted in a manner consistent with WHO recommendations for testing an herbal and within our limited financial resources. Without interviewing the participating clinicians, those administering the drugs would not know which plant species was delivered to a patient, nor if there was a dosing study, so we disagree with the assertion that they could identify the group and the patient. Furthermore, here is how the tea infusion for each plant was anonymized, prepared, and then delivered: the patient selected a sealed, opaque envelope; that envelope was taken by another worker out of the room where the patient remained and into the kitchen where the envelope was opened and the tea was brewed; the resulting tea was brought back to the patient to drink. Thus, neither the administering clinician nor the patient knew the amount of leaves by which the tea was brewed. Blindness was not broken.

In this region of the DRC, hospitals in the Western sense are not common and trained trial clinicians are limited. Rather these were local health clinics where patients resided during treatment. We regret the confusion; stating that the subject patients remained in hospital would have been better stated as “remained in clinic”. Exclusion of patients with persistent vomiting could not cause an attrition bias because all groups of patients were similarly treated.

Eligible patients were randomized in a 1: 1 ratio per treatment group using block randomization with varying block sizes of 4, 6 and 8 across sites. The randomization sequences were computer generated. Blindness and treatment allocation procedures consisted of three stages. First, an independent person assigned the patients to the groups using the randomization sequence. Secondly, placebo and treatment each had a unique code. Third, another independent person allocated the treatments or placebo based on the unique code associated with the randomization sequence. Finally, to blind clinicians and patients to any serious adverse events that may occur, we arranged for an independent researcher with no prior

contact with patients to assess patient response and determine any treatment allowance for the individual.

Response to other comments

As Gillibert et al. already stated, the primary outcome was provided in the Results, so the comment that it was not included in the Methods section is "splitting hairs". Furthermore, the values are interspersed within the Results text where *p*-values are also provided. Regarding D0 vs. D1, we regret the confusion as D1 is the day of enrollment and treatment, and patients did not have to wait 24 h to be treated. In the Methods section, it specifically states that "After testing, patients were administered their first drug dose", and "From D0-D7, patients were treated in hospital to ensure therapeutic compliance." For cases of treatment failure at follow-up, patients were treated again with the same dose of treatment. For example, in the ASAQ arm especially, where there were many failures, patients with onset or persistence of parasitemia on D7 or later were supported by another dose of ASAQ.

There are three treatment comparisons. The groups are *A. afra* vs. *A. annua*, *A. afra* vs. ASAQ, and *A. annua* vs ASAQ, however we did compare *Artemisia* arms (*A. afra* + *A. annua*) together with ASAQ. We used a post-hoc test (Bonferroni multiple comparison test) to compare three treatments. One-way ANOVA showed overall statistically significant differences in group means. We continued to run one post hoc test (Bonferroni) using SPSS to examine data and then summarized the results. Based on the Bonferroni multiple comparison test, the temperature decline rates between *A. annua* and *A. afra* from D1 to D0, D2 to D0, D7 to D0, D14 to D0, D21 to D0, D28 to D0 are all not statistically significant ($p > 0.05$). In contrast, temperature decline rates between *A. annua* and ASAQ from D1 to D0 and from D2 to D0 are statistically significant at $p < 0.05$, 95%CI: (0.928%, 1.717%), and $p = 0.027 < 0.05$, 95%CI: (0.036%, 0.837%). The temperature decline rate between *A. afra* and ASAQ from D1 to D0 is also significant ($p < 0.05$, 95%CI: (0.890%, 1.707%)).

The decline rate of temperature between *A. annua* and ASAQ from D1 to D0 is significant ($p < 0.05$, 95%CI: (16.780%, 21.219)), and from D2 to D0 is significant ($p < 0.05$, 95%CI: (8.882%, 10.578%)). This indicates that the fever decline rates for *A. annua* are higher than for ASAQ at D1 and D2; from D7 to D0 is significant ($p < 0.05$, 95%CI: (3.146%, 3.869%)), from D14 to D0 is significant ($p < 0.05$, 95%CI: (0.185%, 0.247%)), from D21 to D0 is significant ($p < 0.05$, 95%CI: (0.969%, 3.003%)), and from D21 to D0 is significant ($p < 0.05$, 95%CI: (1.504%, 3.205%)).

The decline rate of temperature between *A. afra* and ASAQ from D1 to D0 is statistically significant ($p < 0.05$, 95%CI: (7.567%, 12.164%)), from D2 to D0 is significant ($p < 0.05$, 95%CI: (7.279%, 9.036%)). This indicates that the fever decline rates for *A. afra* are higher than those for ASAQ at D1 and D2; from D7 to D0 is significant ($p < 0.05$, 95%CI: (2.586%, 3.336%)), from D14 to D0 is significant ($p < 0.05$, 95%CI: (0.147%, 0.211%)), from D21 to D0 is significant ($p < 0.05$, 95%CI: (1.296%, 3.404%)), and from D21 to D0 is significant ($p < 0.05$, 95%CI: (1.471%, 3.233%)).

Based on Bonferroni multiple comparison test, the decline rates of parasites between *A. annua* and *A. afra* from D1 to D0 ($p < 0.05$, 95%CI: (6.521%, 11.744%)), and D2 to D0 ($p < 0.05$, 95%CI: (0.575%, 2.570%)) are statistically significant. The parasite decline rates between *A. annua* and *A. afra* from D7 to D0, D14 to D0, D21 to D0, D28 to D0 are all not significant ($p > 0.05$).

The numbers in Figure 1 are correct and based on microscopic reanalysis of all blood smears by Dr. Munyangi post original reviewers' comments. However, Table 5 data inadvertently were not updated upon final submission. We regret our error and below is the corrected Table 5:

Table 5. Level of microscopically determined gametocyte carriage decrease D14-D28 within age groups			
Age (yrs)	*A. annua*	*A. afra*	ASAQ
Children (5-15)	102/102 (100%)	51/51 (100%)	102/105 (97.1%)
Adult (16-65)	146/146 (100%)	172/172 (100%)	360/367 (98.1%)
Overall	248/248 (100%)	223/223 (100%)	462/472 (97.9%)
N, total within age group; n, number of patients with microscopically undetectable gametocytes.			

We address patient numbers as follows: total patients in the trial = 957; patients in the data analysis = 943; patients that left the *A. annua* arm = 2/250 (0.80%); patients that left the *A. afra* arm = 6/229 (2.62%); patients that left the ASAQ arm = 6/478 (1.26%). The number of patients in the Lubile site was originally 300, but after the second recount of all patient slides, there was a group of patients who had not received a complete follow-up. Secondly, there were inconsistencies between temperature and parasitemia. Thirdly, the control slides at Kalima gave parasitemia rates that were lower than the inclusion threshold criteria for the study. Thus, 43 of the Lubile patients were excluded from the study.

Gillibert et al. misstated the Abstract Results statement of fever clearance as being "evaluated at 48 h for ASAQ and at 24 h for Artemisia groups." Fever was recorded (evaluated) for every patient, every day for the first 4 days and then D7, 14, and 28 thereafter. In the upper panel, the average temperature of *A. afra* is 37.4°C, not below 37°C. Our definition of fever in patients was a temperature ≥37.5°C.

In the upper panel of Figure 3, the magnitude of the number of parasites is 10^4. At D0, the mean parasites for each of the three groups was high (*A. annua*: 4.24 x 10^4/μL, *A. afra*: 3.39 x 10^4/μL and ASAQ: 4.30 x 10^4/μL), but after 1 treatment day (D1), the number of parasites in *Artemisia* groups decreased significantly. The mean parasites for *A. annua* was 0.017 x 10^4/μL, so it seemed very close to zero in the figure's upper panel. The *A. afra* mean was 0.16 x 10^4/μL and the ASAQ mean was 0.81 x 10^4/μL. In the survival analysis, however, we treated 0 parasites as an event. That is, the patient was considered cured when parasites were undetectable, so in the lower panel, at D1 and D2, the proportion of patients was still 100%, but actually, the number of parasites in the patients in both *Artemisia* groups was very small.

We reported our observed measurements. However, without more analysis (e.g. PCR) we cannot and should not speculate how or why. Dr. Munyangi had great difficulties implementing this *Artemisia* tea trial against malaria. Despite having received all appropriate approvals, several academics and ministers subsequently attempted to obstruct the trial. There were also attempts to sabotage his work by stealing his laptop. There were even efforts to poison him … he almost died. Nevertheless, Dr. Munyangi persisted and this manuscript is the summary of that study. As we stated and much to our dismay, the dried blood samples needed for PCR analysis were not properly stored, so most of them were degraded. Given our limited resources, it was not possible to analyze all samples as might easily be done in a well-endowed lab. Considering there are concerns and caveats about making PCR "corrections" (Juliano et al. 2010), we did not feel it appropriate to insert any of the sparse PCR data we may have gleaned from this study into the manuscript. Future studies will measure reinfection vs. recrudescence using PCR.

We agree there is discrepancy with Wit et al. (2016), but the trophozoite counts are as reported by twice analyzed microscopic counts. Final values used in our report were shared with the Gillibert team.

For hemoglobin, we tested the differences between hemoglobin levels among all three groups, $\Delta_{12}= |h_{annua} - h_{afra}|$, $\Delta_{13}= |h_{annua} - h_{ASAQ}|$, $\Delta_{23}= |h_{afra} - h_{ASAQ}|$. H_0: $\Delta_{12}= \Delta_{13}= \Delta_{23}$ H_a: not all are equals. First, we ran ANOVA among all three groups (p <0.05) and then a Bonferroni multiple comparison test was conducted and the difference in hemoglobin levels between *A. annua* and ASAQ were not statistically significant. The error bars are ± 1SE. Comparing the means of all three groups, they are actually significantly different at D0. If we compared the differences of the means, then the difference between *A. annua* and ASAQ are not significantly different. We did not run the hypothesis test on the mean hemoglobin levels h_{annua}, h_{afra}, h_{ASAQ}.

To maintain blindness throughout the trial, two physicians were assigned to follow patients: a physician investigator or evaluator was responsible for evaluating the efficacy endpoints of the treatment and the other physician or attending physician was responsible for managing patients: monitoring of tolerance, good compliance with treatment ... the aim of this follow-up strategy was to prevent the patient's assessment doctor from being aware of the treatment allocated with respect to specific side effects. This is how adverse effects were assessed without the removal of blindness.

In contrast to authors' statements, Table 2 does provide average ages of patients for all sites where a treatment was administered. In two sites we remind authors that either *A. afra* (Kamundala) or *A. annua* (Lubile), but not both, were administered, so that is why there is a "not measured" (nm) shown in Table 2. In contrast, ASAQ was administered to patients at all five sites and in the other three sites both plant species were tested. All patients that were administered drugs in this trial were measured.

The conclusion is that as we and others (see recent review by Naß and Efferth 2019) observe mounting failures of ACTs in Africa, *A. annua* in particular could provide an effective and rapid alternative for holding the parasite at bay. This is a completely safe therapeutic that could be implemented now.

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