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Anti-malarial and Immunomodulatory Effects of *Ajuga remota* and *Caesalpinia volkensii* Extracts during *Plasmodium berghei* Infection in Balb/C Mice

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Abstract
Background: *In the control of malaria, artemisinin-based combined therapies have remained the first line treatment for over a decade. Recent reports from Greater Mekong Sub-region on emerging resistance particularly after recrudescing, has however raised concerns on future effectiveness of the drug. This has led to the need to identify alternative antimalarial treatment strategies.*
Objective: *This study evaluated the anti-malarial and immunomodulatory activity of Ajuga remota and Caesalpinia volkensii leaf extracts in Plasmodium berghei infected Balb/C mice as single and combined therapy.*
Materials and methods: *A. remota and C. volkensii leaf extracts were obtained using ethanol and petroleum ether solvents, respectively. To evaluate the impact of individual and a combination of the two plant extracts, five groups of mice (n=15) were inoculated with 200µl of 10^7 P. berghei parasites. Two, twenty four, forty eight and seventy two hours post infection; group one was not treated; the second group received dihydro-artemisinin (DHA); the third received A. remota extract; the fourth C. volkensii while the last group received the combined at a ratio of 1:1. Animals were monitored for 10 days and parasitaemia recorded on day 2, 4, 5 and 6. At days 0, 6, 8 and 10 post-infection, serum and spleens samples were obtained and levels of P. berghei antigen specific immunoglobulin G (IgG) and interferon-γ (IFN- γ) determined.*
Results: *In vivo antimalarial activity assessed using the 4-day suppressive assay, showed that the highest suppression was by 83.66% with the combination of A. remota and C. volkensii. Suppression with A. remota, C. volkensii and DHA was at -23.31%, 4.34% and 54.2% respectively. IgG and IFN-γ levels in the treated groups were compared to the negative control group. IgG and IFN-γ levels varied during different time points, showing significant differences in various groups.*
Conclusion: *A combination of A. remota and C. volkensii plant extracts was effective at suppressing parasite growth in vivo just as the conventional drug DHA. Administration of the extracts had effects on the levels of IgG and IFN-γ when compared to the baseline samples.Administration of the extracts also affected the IgG and IFN-γ levels as we see their production as compared to the baseline samples.*
Keywords: *Plasmodium berghei, Ajugaremota, Caesalpiniavolkensii, IgG, IFN-γ*

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I. Introduction

Malaria is a vector-borne disease caused by the protozoan parasite *Plasmodium.* Species known to affect humans include *P. falciparum, P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*[1]. Globally 3.3 billion people are at risk of malaria infection and development of the disease, with Africa carrying the heaviest burden [2]. The highest incidences of malaria-induced morbidity and mortality is caused by *P. falciparum*[3] with 445 000 number of deaths and infections recorded in 2016 [4].

Chemotherapy remains the most effective way of fighting the disease with drugs such as chloroquine, pyrimethamine, quinine and artemisinin being the most frequently used to treat all human malaria. Artemisinin and its derivatives combined with other anti-malarial (Artemisinin-based Combined Therapies-ACTs) are currently used as the first-line treatment strategy [2]. Intermittent preventive treatment with sulfadoxine-pyrimethamine used during pregnancy has also assisted in protecting women while 15 million children have been protected through seasonal malaria chemoprevention programs in Africa [4]. Despite the success, reduced

sensitivity and drug resistance continue to be recorded in most of the conventionally used malaria drugs including artemisinin.

In the case of artemisinin monotherapy there has been reports of reduced sensitivity in Greater Mekong Sub-region [2]. The use of combined therapy has been a strategy in malaria chemotherapy to increase treatment rates as well as reduce incidences of drug resistance. However, with reduced sensitivity and reported parasite resistance to independent ACT drugs, it is highly likely that the treatment regimens will eventually fail. This highlights the urgent need to come up with new alternative treatment strategies.

The use of plant based drugs for the treatment of malaria has been practiced since ancient times and has proven to be highly effective and safe[5].Current malaria drugs such as quinine and artemisinin that have high potency are plant based [6]. In Brazil for example, *Eucalyptus globulus*, *Citrus aurantiifolia* among others are popularly used as anti-malarial plants [7]. In Kenya, a study carried out in the Central region, identified 58 species in 54 genera and 33 families as anti-malarial herbal remedies [8]. The most common species are *Caesalpinia volkensii*, *Strychno shenningsii*, *Ajuga remota*, *Warbugia ugandensis* and *Olea europaea*. *Ajuga remota* and *C. volkensii* are widely used in ethno-medicine to treat malaria [8] This verifies the use of traditional herbal plants as a valuable and alternative source of antimalarial agents since they have been shown to be safe and efficient in treating malaria. Most of these plants however, require further scientific verification on their antimalarial effects and also their effects on the immune responses [9]

Natural plant extracts have been shown to have diverse mode of action including stimulation of the immune system. Studies by Zhu and colleagues [10] found that pretreatment with *Achyranthes bidentata* polysaccharides selectively enhanced ThI immune response as the number of macrophages, IFN-γ, TNF-α and nitric oxide levels were higher than the control group. IFN-γ contributes to the immunity against blood stage infection rendering protective immune responses and further induces IgG blood stage specific antibodies [11]. A study conducted by Ma and colleagues 200[12] showed that administration of artesunate and chloroquine did not show any impact on the immune system compared to the untreated group.

Though plants can exhibit strong antimalarial activities, there is need to evaluate immunomodulation effects of their extracts on the host during their administration. This study, therefore, analyzed antimalarial activity, IgG and IFN-γ immune responses of *A. remota* and *C. volkensii* crude extracts in Balb/c mice infected with *P. berghei* parasites as single or combined therapy.

II. Materials And Methods

Parasites:*Plasmodium berghei ANKA* parasites cryopreserved in liquid nitrogen was thawed and washed with 0.9% NaCl followed by intravenous inoculation into naive recipient donor mice (n=6) at 0.2ml each. Animals were monitored for signs of acute malaria with parasitaemia in peripheral blood recorded from day 4 post infection. At parasitaemia of ≥10%, donor mice were euthanized and blood collected for subsequent infection in experimental and control mice.

Study animals: Male and female Balb/C mice aged 6-8 weeks were acquired from the Institute of Primate Research (IPR), Kenya. The mice were housed at the IPR according to institutional standards and guidelines for murine welfare and housing (Association for Assessment and Accreditation of Laboratory Animal Care International (AALAC) accredited). The room was air-conditioned at 22°C and 50-70% relative humidity. The animals were subjected to day and night cycles and were fed on nutritional pellets (Unga Farm Care, Ltd, Nairobi, Kenya) and water was given *ad libitum*.

Extracts preparation: Plant leaves were collected from Sagana, Kirinyaga County, Kenya and identified as *A. remota* and *C. volkensii* at the National Museum of Kenya (NMK) herbarium where voucher specimens were preserved. To obtain extracts that were used as antimalarial treatment, leaves obtained from *A. remota* and *C. volkensii* plants were air dried under a shade at Jomo Kenyatta University of Agriculture and Technology (JKUAT), Kenya and ground into a fine powder using a hammer grinder. 300g of each of *A. remota* and *C. volkensii* powder was added to 1.5 liters of absolute ethanol (Sigma, US) and petroleum ether (Sigma, US) respectively. The mixture was allowed to settle for 48hrs and supernatant decanted and filtered through a whatman filter (Sigma, US) paper. The extract was concentrated using a Büchi rotary evaporator R-900 and stored at 4°C. For treatment purposes, extracts were dissolved in 2.5% ethanol (Sigma, US) and 1X phosphate buffered saline (PBS) (IPR protocol) added to a final concentration of 80mg/kg body weight. For the combined extract *A. remota* and *C. volkensii* was constituted as in the above step in the ratio of 1:1. DHA that was used as a control was dissolved in PBS only to a final concentration of 10mg/kg body weight.

Study design and experimental procedure: Completely randomized block design which involves the principles of replication and randomization was used in the study. Seventy-five Balb/C mice were randomly selected into five groups (n=15) and were all inoculated intraperitoneally with 200µl of 10^7*P. berghei* infected erythrocytes (figure 1). While group one was infected with malaria parasite only, groups two, three, four and five were subsequently subjected to a four-day suppressive treatment regimen. Group 2 was treated with DHA; group 3 *A. remota* (*Ar*E); group 4 *C. volkensii* (*Cv*PE) and group5 a combination of *A. remota* and C. *volkensii* (*Ar*E/*Cv*PE) (figure 1). The extracts and drugs were given at 2, 24, 48 and 72hr post infection as previously

mentioned[13]. On days 2, 4, 5 and 6 blood was taken to determine parasitaemia from every group by microscopy (Zeizz, West Germany). At day 0, 6, 8 and 10th day post infection, three mice per group were euthanized, spleen cells were collected and peripheral blood was collected by cardiac puncture for immunological assays. Serum was separated from the blood and stored at -20°C until use.

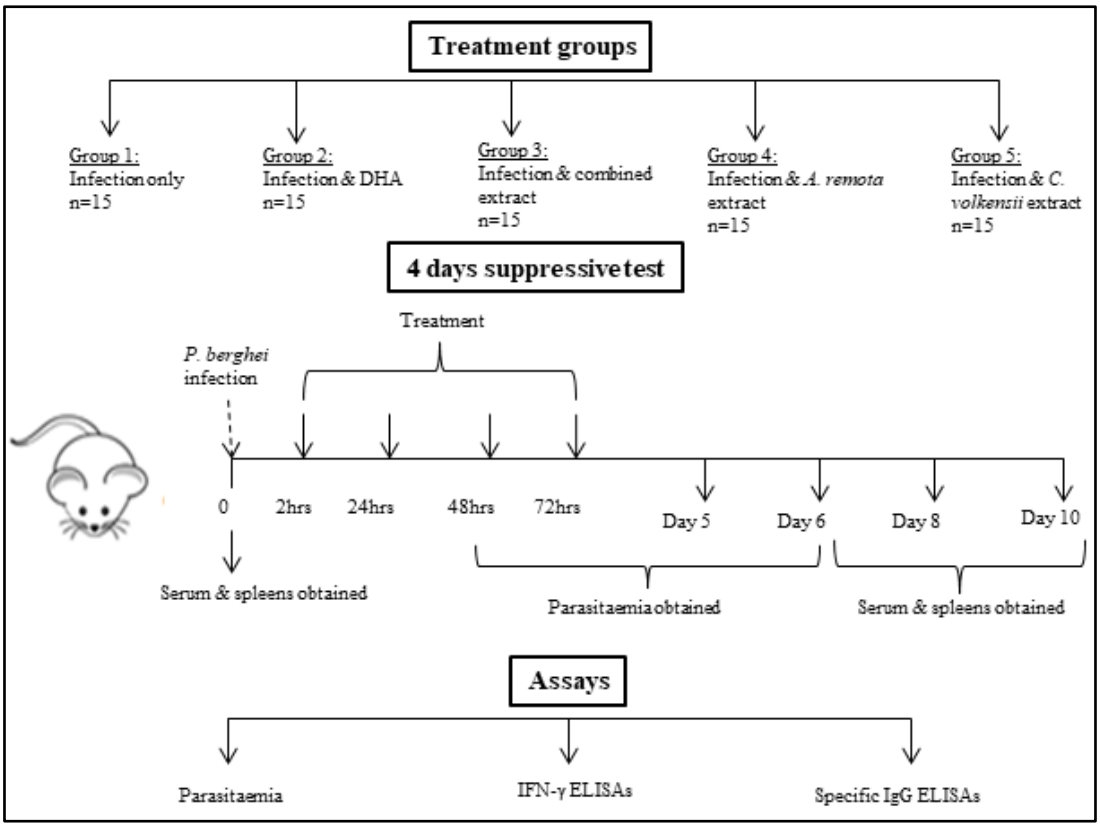

Figure 1: Experimental design. Five groups were treated using the following plant extracts *A. remota*, ethanol extract and *C. volkensii*, petroleum ether extract, combined extract while DHA (dihydroartemisinin) was used as a control drug. *P. berghei* was the parasite used for infection.

***Plasmodium berghei* antigen preparation:** Crude *P. berghei* antigen from late blood stage parasites was prepared as previously described by Deelder and his colleagues [14]. The pellet of the antigen obtained was re-suspended in 4X PBS that contained protease inhibitor cocktail (Thermo Fisher Scientific, US). The suspension was sonicated at an output 6 for 10 seconds with 1-minute intervals in ice to lyse parasite cell wall. The bursts were repeated 6 times. The lysate was centrifuged at 14000 rpm for 60min at 4^0C and the supernatant (antigen) collected. The antigen was filter sterilized using 0.45um syringe filter (Thermo Fisher Scientific, US), concentration determined and aliquots stored at -20 until use.

Spleen cells preparation and cultures: At euthanasia, mice spleens were collected under sterile conditions. The spleens were placed in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) media, crushed through a wire mesh and sieved to release the spleen cells. The suspension was washed twice using 1X PBS (washing buffer) and centrifugation at 1500 rpm for 10 min at 4°C. RBCs in the pellet were lysed using 2.5mls of ACK lysing buffer (Gibco, USA) for 2 minutes at room temperature. The lysing buffer was washed off and spleen cells counted using a haemocytometer. The spleen cells were frozen in liquid nitrogen until use.

Spleen cells from liquid nitrogen were thawed and transferred to 5ml 1X PBS + 10% fetal bovine serum (FBS, Gibco, Canada). This was followed by centrifugation at 1500rpm for 10 minutes at room temperature. The supernatant was discarded and the pellet containing spleen cells placed in complete media (RPMI-1640; 10% FBS, Gibco, Canada; 1% HEPES, Sigma-Aldrich, Dorset, UK; 1% Gentamycin, Sigma-Aldrich, Dorset, UK; 1% L-glutamine, Sterillin, England). The cells were acclimatized for 2 hours at 37^0C, 5% CO_2. The cells were counted and 106 cells (in 1 ml of culture media) cultured in the presence of 1.25µg/ml *P. berghei* antigen and the mitogen 5µg/ml Concanavalin A. The supernatant was collected after 72hrs and stored at -20ºC.

Antibody assay: NUNC Maxisorp® 96-well ELISA plates were coated with 5µg/ml *P. berghei* antigendiluted in 1X PBS and incubated overnight at 4°C. The plates were washed twice with PBS and were blocked with 200µl PBS-Tween (Fisher Biotech, USA) (0.05% Tween 20 + 5% BSA) and incubated for two hours at 37^0C. The wells were washed 5 times with PBS-Tween 20 (PBS-T) and 100µl of serum samplesand standards diluted

in blocking buffer at a ratio of 1:200 was added to each well. The plates were incubated for two hours at 37^0C and washed 5 times with PBS-T. 100µl of horseradish peroxidase (HRP) conjugated goat anti-mouse IgG antibodies (Santa Cruz Biotech) diluted 1:1000 in blocking buffer was added into the wells and incubated for two hours at 37^0C. The plates were washed 5 times with PBS-T and 100ul of 3,3',5,5'-Tetramethylbenzidine (TMB KPL, USA) substrate was added into each well. Antibody measurement was recorded as optical density obtained using an ELISA reader (Biotek Elx808) at a wavelength of 630nm.

Cytokine assay: NUNC Maxisorp® 96-well ELISA plates were coated with 100µl of 1µg/ml of anti-mouse IFN-γmAb AN18 (Mabtech) and incubated overnight at 4°C. The plates were washed twice with PBS and blocked with 200µl of incubation buffer (PBS with 0.05% Tween 20 + 0.1% BSA) after which they were incubated for one hour at room temperature. Washed the plates 5 times and added 100ul of spleen cells culture supernatants.The rest of the assay was conducted as specified in the manufacturer's provided protocol. 100µl of TMB substrate was added, and the optical density readings obtained using ELISA reader at 630nm wavelength.

Ethics approval and consent to participate: Institutional Science and Ethics Committee (IRC) of IPR approved this study (study IRC/05/16). The membership of IRC is established based on the World Health Organization for committees that review biomedical research guidelines. The Primate Vaccine Evaluation Network (PVEN), National Institutes of Health (NIH) and the Helsinki Convention on the Humane Treatment of Animals for Scientific Purposes guidelines are observed as well. The Institutional Science and Ethics Review Committee/Animal Care and Use Committee (IRC-ACUC) is registered by the National Commission for Science, Technology and Innovation, Kenya.

Data analysis: Graphs were obtained to present the means and the standard error of mean (SEM) of the data. The IFN-γ cytokine and IgG concentrations were determined by interpolating the optical density values with the standard curve. Parasite load and the mean concentrations were analyzed using Kruskal-Wallis followed by uncorrected Dunn's post-hoc analysis. Linear regression was used to determine the parasitaemia slopes differences as the days increased. The analysis was done using Graph-Pad Prism version 7.00 for windows. Differences with pvalues of <0.05 were considered significant at 95% level of confidence.

III. Result

A combination of *A. remota* and *C. volkensii* leaves extract suppress *P. berghei* infection: Parasitaemia was monitored during and after the four-day suppression test till day 6 as per Fidock *et al*., [13]. During the four-day suppression test, *P. berghei* growth was suppressed by 83.7% on day five when a combination(*Ar*E +*Cv*PE)of plant extracts was used (figure 2). Whereas, *Ar*Eand *Cv*PE extracts suppressed growth by-23.3%, 4.3% respectively,whileDHAsuppressed by 54.17%. The parasitaemia on day 5 was highest in the group treated with *Ar*E with a mean peak of 48.6±22.4ml^3 X10^6/ml (mean±SEM) of parasitized red blood cells (pRBCs). The lowest parasitaemia was recorded in the group treated with the combined extract having a mean of 6.4±0.7ml^{3}X10^6 pRBC/ml. On day 6, *Ar*E, *Cv*PE, DHA and the combined extract had suppressed *P. berghei* by -15.5%, -43.1%, 90.5%, and 66.7% respectively. The group that had the highest parasitaemia was the group treated with *Cv*PE with a mean of 90.3±23.2ml^3 X10^6/ml and the lowest was the group treated with DHA with a mean of 5.99±0.5ml^3 X10^6/ml while the one treated with the combined extract had 21.0±3.1ml^3 X10^6/ml on day 6. The medians were statistically significant (p=0.0018) where post-hoc analysis showed significance among means (table 1). There are two groups that showed a significant difference at different time point other than with day 2 which are *Cv*PE day 6 vs. DHA day 5 (p=0.0496) and DHA day 6 vs. *Cv*PE day 6 (p=0.0496) indicating that *Cv*PE had a high parasitaemia as compared to DHA. The five slopes showed a significance difference of p value <0.05 (p=0.0231).

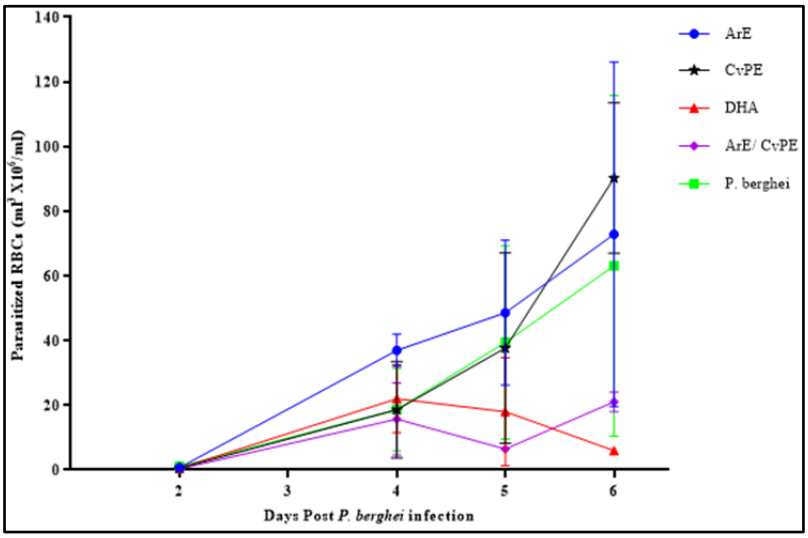

Figure 2: Impact of individual and combined plant extracts on *P. berghei*parasitaemia levels in mice. Plant extracts used: *Ar*E (*A. remota*, ethanol extract) and *Cv*PE (*C. volkensii*, petroleum ether extract) while DHA (dihydroartemisinin) was used as a control drug. *P. berghei* signifies the infected untreated group.

Effects of *A. remota* and *C. volkensii* on IgG responses during *P. berghei* infection:IgG levels wereevaluated in mice infected with *P. berghei* and treated with plant extracts (figure 3). Apart from the baseline readings (2580±119.1pg/ml) the rest of the concentrations were as follows: on day 6 the group treated with *A. remota*the concentration of IgG was3206±1369pg/ml followed by an increment on day 8(10168±5582pg/ml) while on day 10 the levels reduced to 9317±2667pg/ml. For the group treated with *C. volkensii* the concentration on day 6 was 3455±615.5pgml that increased gradually from day 8 (8390±3506pgml) to day 10 (21472±17198pg/ml). A combination of plant extracts recorded a concentration of 4673±2112pg/ml on day 6 that decreased on day 8 (3905±808.6pg/ml) and a slight increase was obtained on day 10 (7586±2327pg/ml).On day 6, the untreated group had the highest mean of 24975±21549pg/ml lowered on day 8 (7803±2467pg/ml) while on day 10 increased to 22236±6562pg/ml. On day 6 the group treated with DHA had the lowest concentration (4284±2639pg/ml) as compared to day 8 which had the highest IgG concentration of 41251±17276pg/ml which lowed on day 10 to 6916±932.8pg/ml.

There was a significant difference in mediansof IgG concentrations (p=0.0052). The significance was between all the groups with the baseline samples while others included *Ar*E day 6 vs. DHA day 8 (p=0.0145),*Ar*E day 6 vs. *P. berghei* (infected untreated group) day 10 (p=0.0296),*Ar*E/*Cv*PE day 6 vs. DHA day 8 (p=0.0145),*Ar*E/*Cv*PE day 6 vs. *P. bergheiо*nly day 10 (p=0.0296),*Cv*PE day 6 vs. DHA day 8 (p=0.017),*Cv*PE day 6 vs. *P. bergheiо*nlyday 10 (p=0.0342) and DHA day 6 vs. DHA day 8 (p=0.0287).

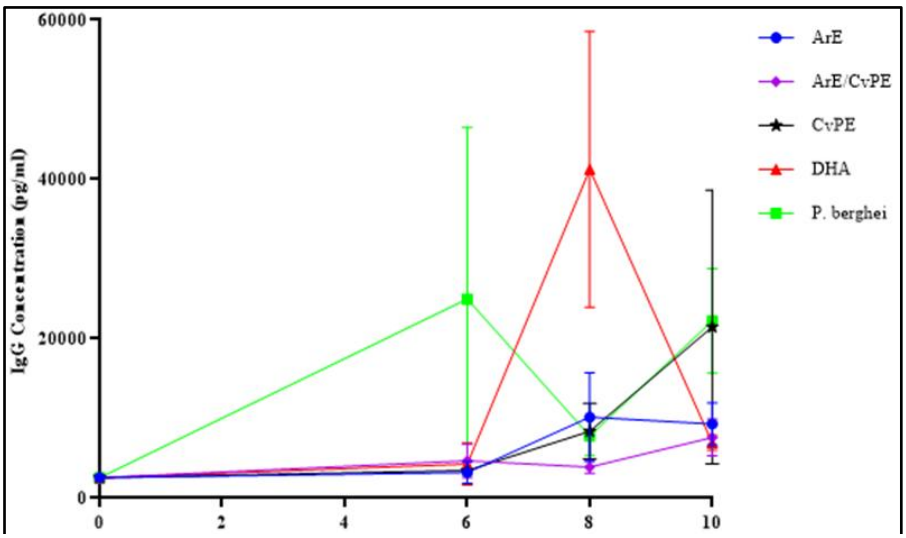

Figure 3: Impact of individual and combined plant extracts on total IgG levels in mice. Plant extracts used: *Ar*E (*A. remota*, ethanol extract) and *Cv*PE (*C. volkensii*, petroleum ether extract) while DHA (dihydroartemisinin) was used as a control drug. *P. berghei* signifies the infected untreated group.

Effects of *A. remota* and *C. volkensii* on IFN-γ responses during *P. berghei* infection:IFN-γ levels were evaluated in mice infected with *P. berghei* and treated with plant extracts. Similar to IgG, IFN-γ levels varied for each plant extract and DHA (figure 4). The negative control group had concentration of IFN-γ lower on day 6 (7116±3193pg/ml) compared to day 8 (16291±10189pg/ml) which decreased on day 10 (9544±341.4pg/ml). The group treated with *A. remota*, had the concentrations lower on day 8 (5610±108.6pg/ml) compared to day 6 (6542±3272pg/ml) and increased on day 10 (10121±691.3pg/ml). *C. volkensii* had a concentration of 7306±1999pg/ml on day 6, registered a slight decrease on day 8 (7257±602.9pgml) and an increase on day 10 with 11756±1093pg/ml. The groups treated with the combined extract (6792±1923pg/ml, 7300±1673pg/ml and 9957±257.7pg/ml), and DHA (4712±2534pg/ml, 7297±1639pg/ml and 9585±198.5pg/ml) had the concentration increasing from day 6 throughout till day 10 respectively. The naive group had a concentration of 2601±2601pg/ml.

The results showed that there was a significant difference among medians (p=0.0331). There was a significance difference between baseline and the other days 6, 8 and 10 samples as well as other time points as follows:DHA day 6 vs. *Ar*E day 10 (p=0.0473), DHA day 6 vs. *Ar*E/*Cv*PE day 10 (p=0.0435), DHA day 6 vs. *Cv*PE day 10 (p=0.0172), *Ar*E day 8 vs. *Ar*E day10 (p=0.0447), *Ar*E day 8 vs. *Ar*E/*Cv*PE day 10 (p=0.0411) and *Ar*E day 8 vs. *Cv*PE day 10 (p=0.0161).

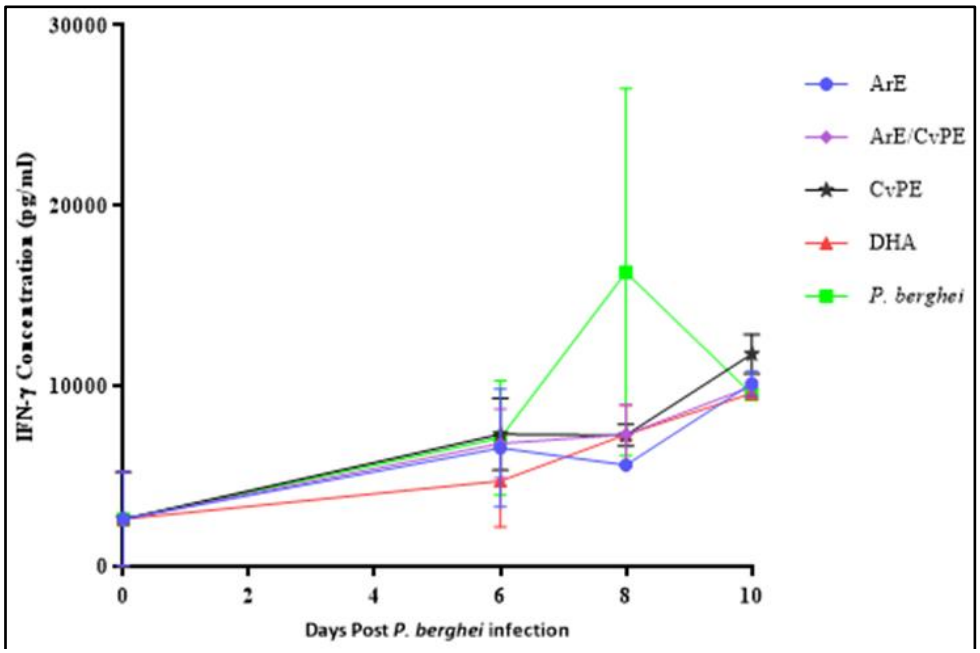

Figure 4: Impact of individual and combined plant extracts on IFN-γ levels in mice for days 6, 8 and 10 post-infection. Plant extracts used: *Ar*E (*A. remota*, ethanol extract) and *Cv*PE (*C. volkensii*, petroleum ether extract) while DHA (dihydroartemisinin) was used as a positive control drug. *P. berghei* signifies the infected untreated group.

IV. Discussion:

Herbal preparations for *A. remota* and *C. volkensii* are widely used in ethnomedicine to manage malarial illnesses in Central Kenya[15]. Investigations on both plants *in vitro* and *in vivo* indicated that the two plants have antimalarial effects[16],[17]. However, there is no data available on the efficacy and the impact of a combination of these two plants on the immune system during malaria infection.

In this study, the combined extract (80mg/kg) was the best in *P. berghei* suppression at 83.66% on day five post infection followed by Dihydroartemisin (DHA) (10mg/ml) with 54.17% suppression, *C. volkensii*suppressed by 4.3% while *A. remota* was the poorest giving a negative suppression of -23.31% at 80mg/ml. On day 6 the individual extracts (*A. remota* = -15.5% and *C. volkensii* = -43.1%) showed no suppression with the parasitaemia higher than that of the untreated group (negative control group). DHA and the combined extract however had a suppression of 90.5%, and 66.7% respectively on day 6. This showed that individual extracts were not as active in parasite suppression as the combined. This concurs with a study on *Vernonia amygdalina*leaf extract that showed a higher efficacy when used in combination with chloroquine as opposed to individual treatment in clearing *P. berghei*[18].

During malaria infection, antibodies have been linked with partial clearance of the parasites. Studies by Nunesand colleagues showed that antibody secretion decreases parasitaemia by opsonizing infected red blood

cells to facilitate active uptake by macrophages or by activating the complement cascade[19]. Studies in humans have revealed the role of IgG1 and IgG3 in protection against severe disease [20]. These two isotypes have been shown to play a role in facilitating opsonization [21]. In this study, high levels of total IgG antibody production in all groups were recorded throughout the study as compared to baseline samples. There were significant differences between the highest and lowest levels of protection as show in table 2. Based on these results, the role of IgG was evident in protecting the animals against high parasitaemia especially on day 8 and 10. The significant difference occurred between the individual plant extracts and the conventional drug or the combined extract which showed a tremendous suppression at the end of 4-days suppressive test.

The pro-inflammatory cytokine IFN-γ has been shown to play a role in facilitating killing of malaria parasites. This study recorded high levels of IFN-γat days 6-10 compared to the day 0 (before infection) in all groups of animals. These levels were however significant across all groups compared with the baseline levels and some groups on days 6, 8 and 10. A correlation between IFN-γ responses and protectionwas observed.

The study proved that during the *P. berghei* infection the spleen is triggered to release IFN-γ into the blood circulation. IFN-γ is a macrophage activating factor involved in the immune response to malaria [22]. It is produced by CD8+ and CD4+ T lymphocytes in specific responses to antigen and by natural killer cells (NK cells) in a nonspecific approach and are important in controlling blood stage parasites early in the infection [22]. The earlyinflammatory response is essential to control parasite replication in order to promote the clearance of infected erythrocytes [23].

The study revealed that between 6^{th} to 10^{th} days post infection IFN-γ was released indicating secretion at later days as well. IFN-γ contributes to the resolution of primary infection by restricting parasite replication in the early phase of the infection [24]. High parasite suppression in the group treated with the combined extract was attributed to the stimulation caused by the extract leading to IFN-γ production in mice during *P. berghei* infection. Results of this study concur with those of Dey and Chaudhuri[25]wherethe Th1 cytokine (IFN-γ) expression on administration of *Dioscorea alata*was observed to increase in mice during *P. berghei* infection.In this study, the treatment of the infected mice with a combination of *A. remota* and *C. volkensii* improved the inflammatory effect of the *P. berghei* parasite.The results of this study confirm that IFN-γ is significant in parasite clearance though insufficient alone.

V. Conclusion:

In conclusion, individual plant extracts were not as effective as the combined extract in suppressing parasitaemia. Therefore, *A. remota* and *C. volkensii* extracts showed the ability to kill and inhibit parasite growth in mouse models when used in combination unlike when used individually. A combination of *A. remota* and *C. volkensii* plant extracts proved more effective though complete parasite clearance was not achieved. This indicated a synergistic and or additive effect of the combined extracts of *A. remota* and *C. volkensii* unlike that of the individual extracts. IgG and IFN-γ levels varied during different time points showing significant difference. This is evidence that administration of the extracts did affect the IgG and IFN-γ responses. Further studies to investigate the effect of the combined extracts on other inflammatory cytokine responses during murine malaria would be crucial.

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