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Synergistic immunostimulation between *Leishmania donovani* antigen and *Carissa edulis* in BALB/c mice with visceral leishmaniasis.

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ABSTRACT

Visceral leishmaniasis (VL) is a significant health issue because of limited administration of chemotherapy treatment, being too toxic and even emerging forms of resistance that necessitates to develop new forms of immunotherapeutic treatment. The research compared the adjuvant (vaccination) capacity of *Carissa edulis* extract combined with soluble *Leishmania donovani* antigen (SLA) in the BALB/c mice. Eighty BALB/c mice received SLA, *C. edulis*, combination, or PBS, then challenged with *L. donovani*; parasite, cytokine, antibodies measured. The highest protection in the mice vaccinated with SLA + *C. edulis* (4.9%-6.0) was observed, parasite burden which was lower as compared to the SLA + *C. edulis* (4.9) and also *C. edulis* (4.9). The biggest cytokine profile accused the best cytokines profile, with significantly higher levels of IFN- γ (217.4 pg/mL ($p = 0.0110$)) as well as postponed levels of IL-10 (35-40 pg/mL). The combination group showed robust activation of humoral response given that IgG2a performed as high as (3 mg/mL ($p = 0.0245$)) markedly higher as compared to either treatment, whereas IgG1 increases were smaller and SLA-dependent. It was further ascertained that the level of parasite burden is positively correlated with IL-10 ($r = 0.97$, $p = 0.0061$) and IgG1 ($r = 0.92$, $p = 0.0278$), but negatively with IgG2a, which supports the protective nature of Th1-related immunities. The results have shown that the extract of *C. edulis* is synergistically antigenic stimulating the Th1-biased response with increased IFN- γ and IgG2a, and decreased IL-10, as well as enhanced the parasite clearance effect.

Keywords: Immunomodulation, visceral leishmaniasis, *Carissa edulis*, IgG, Cytokines

INTRODUCTION

Visceral leishmaniasis (VL) or kala-azar is the most severe form of leishmaniasis and incurs significant morbidity and mortality in the tropical and subtropical belts (Wamai et al., 2020). It is induced mainly by *Leishmania donovani* in East Africa and the Indian subcontinent and by *Leishmania infantum* in the Mediterranean and Latin America (Alvar et al., 2012). Acquired through bites of an infected female phlebotomine sandfly, VL as an epidemic impacts internal organs, including the liver, spleen and bone marrow. Untreated, the disease is highly lethal, and case fatality estimates are up to 100 percent within two years of infection (Steverding, 2017). Worldwide, countries such as India, Bangladesh, Sudan, Ethiopia, Brazil, and Kenya are known to report more than 90 percent of all new VL cases (Sundar, 2015).

The available treatment regimens of VL currently consist of pentavalent antimonials, amphotericin B, paromomycin, and miltefosine (Ponte-Sucre et al., 2017; Singh et al., 2016). Although these drugs may be useful, their use is limited by several negatives: the drug becomes toxic, prolongs treatment, costs a lot, and new resistance to the drug is being encountered. This is the case with amphotericin B, which is the most effective but also nephrotoxic and thus cannot be administered (Barragán et al., 2010). This resistance to antimonials has already made it obsolete in parts of the world, most of them in Bihar, India. In addition to this, the unavailability of drugs in the endemic areas, lack of patient

compliance, and lack of pediatric formulations also contribute to the poor disease management (Pacheco-Fernandez et al., 2023a, 2023b). These restrictions highlight why preventive methods such as effective vaccines and adjunct therapy are increasingly needed.

The results of clinical development of *Leishmania* infection are the compound effect between the host and the parasite (Volpedo et al., 2021). The progression or control of VL hinges on the balance between protective Th1-type immune responses and disease-promoting Th2 or regulatory pathways. Macrophage-mediated Th1-cell-mediated interferon-gamma (IFN- γ)-mediated activation of macrophages is the main mediator of protective immunity against *L. donovani* (Samant et al., 2021). This cytokine stimulates the generation of nitric oxide, which is an important leishmanicidal agent. Interleukin-10 (IL-10), on the other hand, is the key to the maintenance of the parasites, since it inhibits the activation of the macrophages and the ability of the immune system to evade.

This polarization toward Th1 (cell-mediated) or Th2 (humoral) skewing of immune responses has shown important implications in controlling or worsening disease. High levels of IL-10 are linked with progression of the disease, whereas high levels of IFN- γ and IgG2a are linked to parasite clearance (Bogdan et al., 2024; Costa-Da-silva et al., 2022). Therefore, the cytokine-antibody interplay can be modified by the knowledge of this interaction, which is an important approach to VL vaccine design and immunotherapy. This study aimed to assess the adjuvant capacity of *C. edulis* root extracts administered with soluble *Leishmania donovani* antigen (SLA) in the BALB/c mice predisposed to visceral leishmaniasis.

METHODOLOGY

Plant collection and identification

Carissa edulis was gathered with the assistance of traditional medical practitioners in the Loitokitok region, Kajiado County, Kenya. A specimen was sufficiently prepared so it could be recognized at the National Museums of Kenya (NMK) and a voucher specimen was placed at the museum so that it could be referred to in the future.

Preparation of *C. edulis* extract

For *C. edulis*, the bark of the roots was cut into small pieces and dried in the shade for 72 hours. These were reduced into a fine powder in an electrical mill and weighed. It was prepared by placing the ground material in 1g/5ml of Methanol and then soaking for 48 hrs followed by filtering using the Whatman No.1 filter paper in a conical flask. The filtration was again passed through Whatman filter paper No.1 twice every 24hours and the final crude extract filtrate was concentrated through a rotary evaporator and the weight was taken. This powder was kept under -20°C in an airtight container until it was used.

Culture of *Leishmania donovani* promastigotes

The strain of *Leishmania donovani* NLB-065, which was isolated in the spleen of an infected individual in Baringo County, Kenya, and propagated by intracardiac passage between hamsters at the Kenya Institute of Primate Research (KIPRE), was used in this study. The infected Hamster spleen was used to obtain cellular material that was then cultured in Schneider's Drosophila Complete media (Sigma USA) and monitored to grow. The promastigotes were centrifuged at 2500 rpm to harvest stationary phase promastigotes in 15 minutes at room temperature. The subsequent product (pellets) underwent three centrifuge-based washes of sterile Phosphate Buffered Saline (PBS) followed by counting of promastigotes using a hemocytometer.

Preparation of Soluble *Leishmania* Antigen (SLA)

The promastigotes obtained were three times washed in sterile phosphate-buffered saline (PBS). The parasites were sonicated at 20 kHz at four intervals of 40 seconds on ice, spaced with periods of one minute. Whole soluble protein was extracted by the rapid freezing and thawing of the sonicated material three times in the presence of liquid nitrogen. The suspension of the parasites was centrifuged at 10,000 rpm, 30 min, 4°C. Supernatants were concentrated to the

level of protein with the Bio Rad protein Assay kit (Bio Rad) according to the manufacturer-provided method and stored at -70°C.

Vaccine preparation

The soluble *Leishmania donovani* antigen in combination with *C. edulis* extract was constituted in sterile PBS at a ratio of 1:1 before use for vaccination.

Experimental design

Eighty BALB/c inbred mice of both sexes of aged between 8-10 weeks, were purchased from the animal house, Department of Zoological Sciences, Kenyatta University. The mice were housed in the animal house at Kenyatta University and were fed with commercially prepared feed (Kenya Unga Ltd) and water was given *ad libitum*. The mice were allocated into different groups of 12 mice each. Three mice in each group were sacrificed and blood taken to measurement of immune system parameters before any vaccinations. Three groups were vaccinated as follows: Group 1 was injected with the soluble *Leishmania donovani* antigen only, Group 2 was injected with the *C. edulis* extract only, and Group 3 was injected with a 1: 1 mixture of the *C. edulis* extract and the soluble *Leishmania donovani* antigen. The fourth group was exposed to sterile phosphate-buffered saline (PBS) and served as a negative control group. The vaccinations were made into the tail base with a needle and a syringe subcutaneously. Each of the respective groups received two booster injections of the same vaccine component and dose as at the time of the initial vaccination at week 0 and week 2. Two weeks following the second vaccination, the rest of the mice were inoculated with virulent *Leishmania donovani* promastigotes. Each group of the 4 mice was sacrificed at weeks 0, 2, 4, 6 and 8 and blood was taken to measure cytokine, antibody levels and parasite burden.

Quantification of IgG antibodies

Total IgG, IgG1 and IgG2a antibodies of parasite-specific response were quantified by enzyme-linked immunosorbent assay (ELISA) (Mutiso et al., 2012). Overnight, the polystyrene micro-ELISA plates were coated by adding 100ml of *Leishmania donovani* soluble antigen at a concentration of 10µg/mL, which was in bicarbonate buffer (pH 9.6). The rest of the coating buffer was rinsed off and non-specific binding sites were blocked using 3% bovine serum (BSA) for one hour at 37°C. The Unbound BSA was then rinsed 6 times with 0.005% Tween 20 in PBS followed by the addition of 100 ml of the diluted serum samples (1:10) of both the experimental and control groups and incubation for 2 hours at 37°C. Free serum was washed as above. One hundred microliters of 1:4000 horse radish-conjugated sheep anti-mouse IgG, IgG1 or IgG2a (Rockland Immunochemicals) were incubated in the wells at 37°C for one hour. Unbound conjugate solution was washed as above and Tetramethylbenzidine horseradish peroxidase substrate was added to the wells. The plates were incubated at 37°C in darkness for 30 minutes to enable the colour to develop before the optical densities of the plates at 630nm are read in a microplate reader.

Quantification of cytokine levels

The levels of IFN-γ and IL-10 were determined by enzyme-linked immunosorbent assays. Briefly, micro-ELISA plates made of polystyrene were coated overnight with 100 µl of a cytokine-specific monoclonal antibody. Bovine Serum Albumin (BSA) (3% in PBS/0.05% washing buffer) was used to block non-specific binding sites for 1 hour. The plates were washed six times with the washing buffer and subsequently, 100 ml of the samples and standards were added and incubated for 2hours after which 100 µl of specific monoclonal detecting antibody were added per well and incubated 1 hour at room temperature. The wells were then incubated with streptavidin-HRP dilution and then 100 µL/well of Tetramethylbenzidine (TMB) microwell peroxidase substrate 1:1000. Plates were incubated at 37°C and optical densities of the IFN-γ were read at 630nm in a microplate reader, whereas optical densities of IL-10 were read at 405 nm. The quantities of the cytokines were determined through comparison with the generated standard curve.

Determination of parasite burden

Splenic impression smears from terminated mice were made for the parasite *L. donovani*. The process of fixing and staining was similar to that of parasitemia. Under a microscope, the number of parasites per 500 splenic cell nuclei was determined.

Statistical analysis

The normality of all of the continuous variables was assessed using Kolmogorov-Smirnov. Frequency and descriptive statistics were used to analyse the categorical variables. Means and standard deviations were used to describe variables that were in the Gaussian distribution. Where required, the one-way analysis of variance (ANOVA), Kruskal-Wallis, Pearson tests or the unpaired t-tests were used to compare groups. GraphPad Prism 10.2® (GraphPad Software, La Jolla, CA, USA) was used in statistical analysis. Any p-value below 0.05 will be regarded as significant.

RESULTS

Monitoring of the burden of the parasites experienced was done weekly following inoculation with *L. donovani* virulent promastigotes in all groups. In PBS control reared the parasite load increased abruptly by week 6 and maintained an increasing trend through week 8 resulting in a mean parasite load per over 30 percent ($p < 0.001$ relative to the vaccinated cohorts). Conversely, mice vaccinated with soluble *Leishmania* antigen (SLA) revealed a moderate rise in parasite load with the highest intensity reaching about 12-15 percent at week 8, which was moderate compared with PBS controls, yet notably higher as compared to the SLA + *C. edulis* group ($p = 0.0012$).

Mice treated with *Carissa edulis* extract alone demonstrated a parasite burden profile similar to the SLA group, with parasite loads stabilizing at ~10–12% by week 8. The difference was statistically significant between this reduction and the PBS group ($p = 0.0024$) but not significantly different to the SLA-only group ($p = 0.0724$). The SLA + *C. edulis* presented the lowest parasite load during the course of the study, and leveled off at approximately 6-8% in week 8. This burden reduction in this group was very large compared to that in PBS ($p = 0.0031$), SLA alone ($p < 0.01$), and *C. edulis* extract alone ($p = 0.0011$).

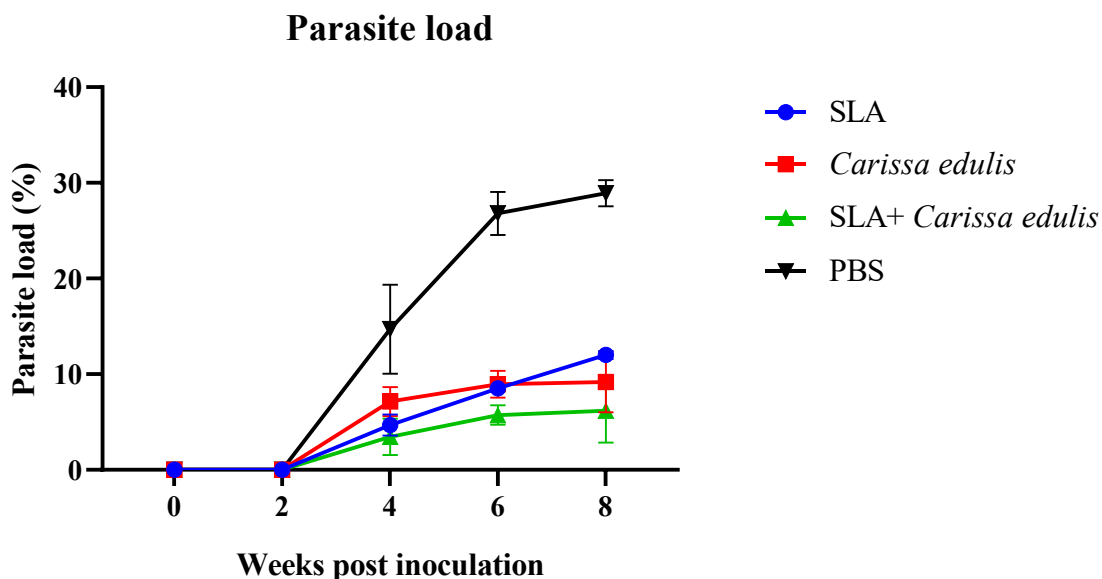


Figure 1. Parasite load following different treatment interventions. Parasite burden (%) was assessed at each timepoint following inoculation in four treatment groups: SLA alone (blue circles), *C. edulis* extract alone (red squares), combined SLA + *C. edulis* treatment (green triangles), and PBS control (black triangles). Data points represent mean values $\pm$ standard error of the mean (SEM) for n=4 per group at the week 8.

The IFN- γ levels show significantly divergent patterns of cytokine responses relative to the IL-10 and the combination treatment exhibits robust immunostimulatory responses. The group of SLA + *C. edulis* treatment showed the strongest IFN- γ response with an acute, progressive rise in response between baseline and about 300 pg/mL at week 8 (Figure 2). This is almost a sixfold increment over the period of study, implying the effect of synergy between the two treatment elements. The overall effect of SLA monotherapy was an upward trend of moderate but steady increase to about 160 pg/mL at the end point, and this suggests a strong level of immunomodulatory function. A one-sample t-test was used to test the hypothesis that the mean immune response of each treatment group was significantly different from a given hypothesis. The findings demonstrated that all the treatment groups, i.e., SLA ($p = 0.0059$), *C. edulis* ($p = 0.0009$), SLA + *C. edulis* ($p = 0.0110$), and PBS ($p < 0.0001$), were statistically significant. The outcome of the ANOVA ($F = 8.486$, $p = 0.0013$) shows that the inter-group variability is highly significant, where the combination therapy registered the highest mean concentration (217.4 pg/mL), SLA alone (113.2 pg/mL), and *C. edulis* alone (67.40 pg/mL).

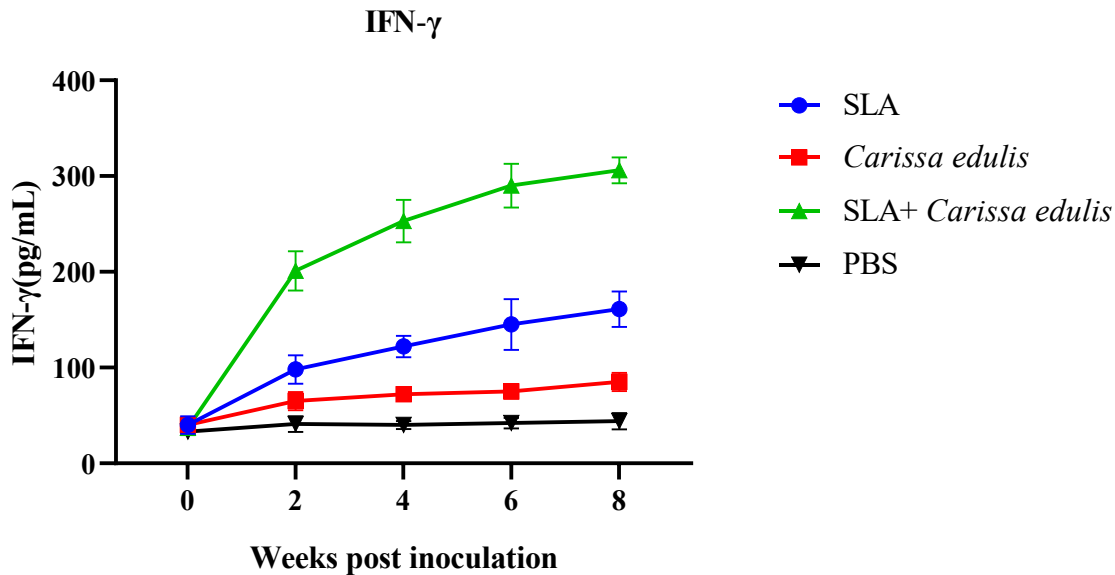


Figure 2. IFN- γ cytokine production following different treatment interventions over a 8-week post-inoculation period. Plasma IFN- γ concentrations (pg/mL) were measured at 0, 2, 4, 6, and 8 following inoculation in four treatment groups: SLA alone (blue circles), *C. edulis* extract alone (red squares), combined SLA + *C. edulis* treatment (green triangles), and PBS control (black triangles). Data points represent the mean values $\pm$ standard deviation of the mean (SD) for n = 5 per group.

The findings indicate that there is a specific IL-10 production pattern among various treatment groups in an 8-week post-inoculation time. *C. edulis* extract alone gave the highest levels of IL-10 of about 60 pg/mL at week 8, which can be seen to show a steady rise over the entire time of the study (Figure 3). The SLA treatment group followed this same pattern except that the highest concentrations were lower, with the maximum concentration reaching approximately 50 pg/mL as of the final timepoint ($p = 0.0006$) and *C. edulis* alone had a mean of 46.80, which was also significantly different compared to the baseline values ($p = 0.0010$). Contrary, IL-10 values in the combined SLA + *C. edulis* treatment were comparatively stable at 35-40 pg/mL ($p < 0.0001$), whereas those in the PBS control group were the lowest and least variable at about 30 pg/mL ($p < 0.0001$). ANOVA analysis revealed that there were significant differences between treatment groups ($F = 4.306$, $p = 0.0209$).

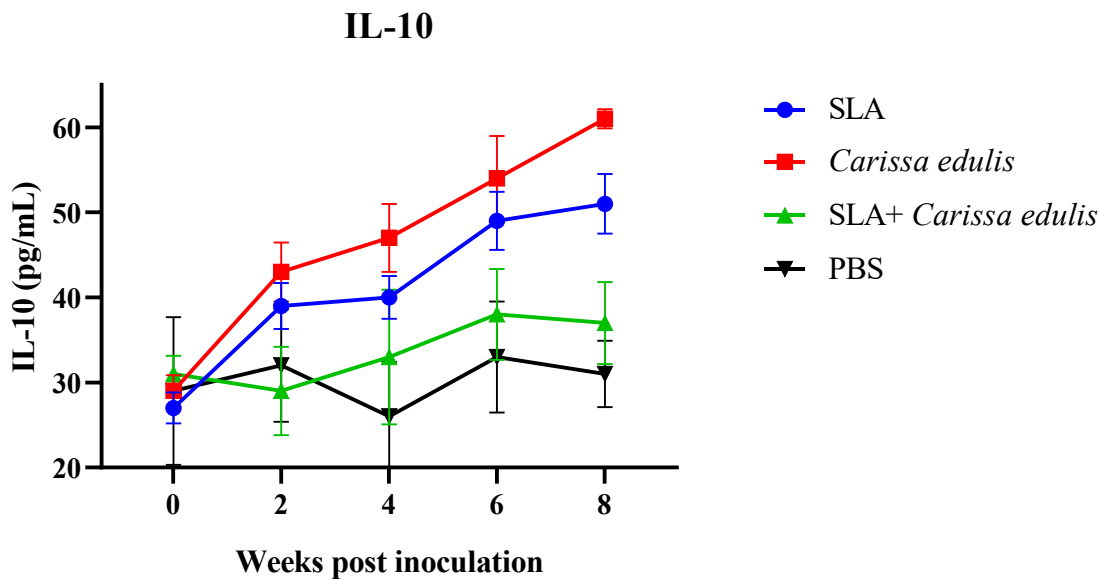


Figure 3. IL-10 cytokine production following different treatment interventions over a 8-week post-inoculation period. Serum IL-10 concentrations (pg/mL) were measured at 0, 2, 4, 6, and 8 following inoculations in four treatment groups: SLA alone (blue circles), *C. edulis* extract alone (red squares), combined SLA + *C. edulis* treatment (green triangles), and PBS control (black triangles). Data points represent the mean values $\pm$ standard deviation of the mean (SD) for $n = 5$ per group.

The data shown by the IgG antibody response indicate a distinct pecking order of humoral immune activation among the treatment groups, where the combination therapy gave the strongest antibody production (Figure 4). The SLA + *C. edulis* treatment group showed a gradual increase in IgG levels over the course of the study and reached a maximum of about 4 $\mu\text{g/mL}$ near the conclusion of the 8th week, with their highest average antibody of 2.576 $\mu\text{g/mL}$, which was the highest across all groups. This response profile indicates early B-cell stimulation and antibody production when the two immunomodulatory agents are used concomitantly. SLA monotherapy yielded a continuous moderate enhancement of IgG production peaking at about 2 $\mu\text{g/mL}$ at the endpoint and a mean concentration of 1.362 $\mu\text{g/mL}$, which was not as significant but was sufficiently humoral immune stimulation. *C. edulis* alone extract did not produce significant antibody responses, but instead showed comparably stable levels of about 0.7340 $\mu\text{g/mL}$ at all points

throughout the observation period (mean: 0.7340 $\mu\text{g/mL}$, $p = 0.0119$), indicating that it does not have a great impact on humoral immunity when used singly. Another statistically significant increase in immune response ($p = 0.0185$) was indicated by the combination group, SLA + *C. edulis*. PBS group showed no IgG production and the levels were always close to baseline with minimal changes (mean: 0.2240 $\mu\text{g/mL}$, $p = 0.0006$). Highly significant differences among treatment groups as indicated by the ANOVA ($F = 6.939$, $p = 0.0033$) support the hierarchy of immune activation observed and also indicate that the different interventions tested have different methods of humoral immune modulation.

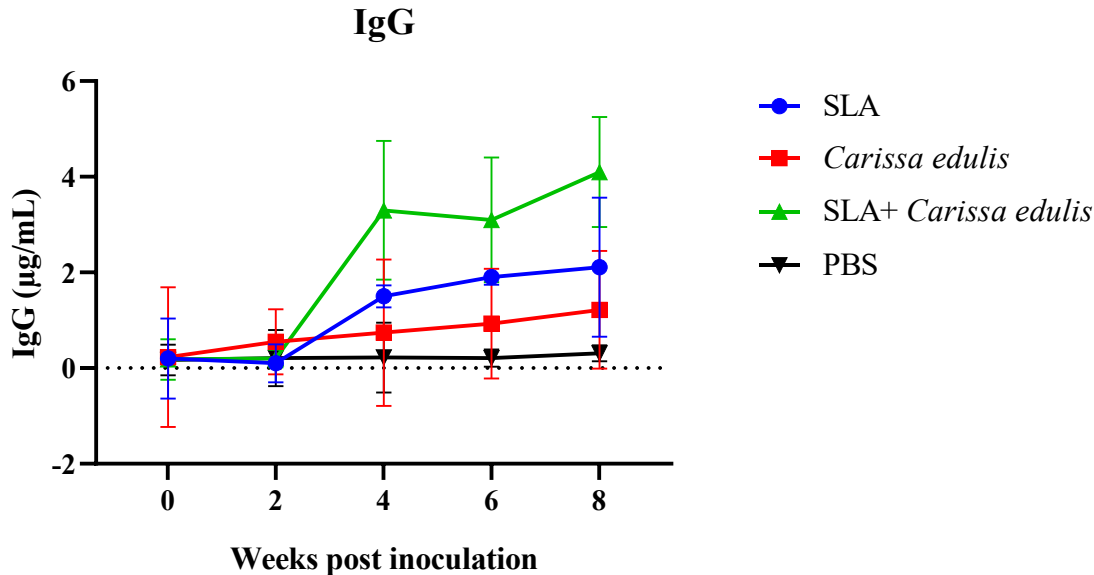


Figure 4. IgG antibody production following different treatment interventions over a 8-week post-inoculation period. Serum IgG concentrations ($\mu\text{g/mL}$) were measured at weeks 0, 2, 4, 6, and 8 following inoculation in four treatment groups: SLA alone (blue circles), *C. edulis* extract alone (red squares), combined SLA + *C. edulis* treatment (green triangles), and PBS control (black triangles). Data points represent mean values $\pm$ standard deviation of the mean (SEM) for $n=5$ per group.

The pattern of IgG1 subclass antibody responses shows sharp differences in relation to the overall IgG production, with SLA-containing treatment showing strikingly similar efficacy independent of combination therapy. The response trajectories of both the SLA monotherapy ($p = 0.0169$) and SLA + *C. edulis* ($p = 0.0139$) groups were virtually the same, as the groups reached a comparable peak concentration of about 1.7 $\mu\text{g/mL}$ at week 8, with mean concentrations of 1.062 $\mu\text{g/mL}$ and 1.202 $\mu\text{g/mL}$, respectively (Figure 5). The isolated use of *C. edulis* extract yielded modest IgG1 concentrations, with a reasonably constant concentration between 0.8 mg/mL and 0.63 $\mu\text{g/mL}$ over the period, (mean: 0.5080 $\mu\text{g/mL}$, $p = 0.0082$), which indicates that it is a poor inducer of IgG1 on its own. The control group of PBS displayed almost no antibody production, and it remained continuously close to the baseline (mean: 0.1340 $\mu\text{g/mL}$, $p = 0.0004$). It was statistically confirmed that all the treatment groups were significantly higher than theoretical baselines ($p = 0.0169$), and ANOVA analysis ($F = 5.920$, $p = 0.0065$) showed that there were significant inter-group differences. These results indicate that, although SLA causes an excellent stimulation of IgG1-mediated Th2-type

humoral responses, the increased total antibody output in combination therapy is probably due to activation of other antibody subclasses or isotypes.

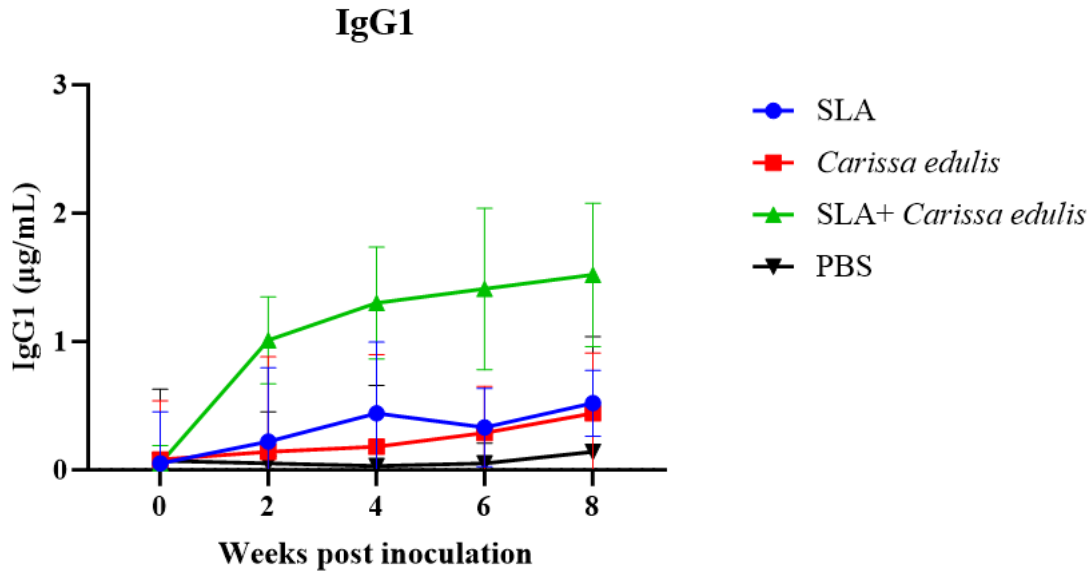


Figure 5. IgG1 antibody subclass production following different treatment interventions over a 8-week post-inoculation period. Serum IgG1 concentrations (µg/mL) were measured at weeks 0, 2, 4, 6, and 8 following inoculation in four treatment groups: SLA alone (blue circles), *C. edulis* extract alone (red squares), combined SLA + *C. edulis* treatment (green triangles), and PBS control (black triangles). Data points represent the mean values $\pm$ standard deviation of the mean (SD) for n = 5 per group.

The IgG2a subclass responses show the most remarkable treatment effect in all of the measured immune parameters, and combination therapy gives rise to significantly higher levels of antibody far beyond the sum of the individual treatment effects (Figure 6). The SLA + *C. edulis* combination group was found to have a high level of IgG2a production, with the highest level of about 3 µg/mL at week 8, which is about 6-7-fold higher than either of the treatments in isolation ($p = 0.0245$). Interestingly, the two individual treatments yielded almost comparable, small responses of IgG2a, with SLA alone ($p = 0.0196$) and *C. edulis* alone ($p = 0.0236$) retaining similar levels of 0.4-0.5 µg/mL over the duration of the study. The PBS control group was found to have a negligible production of anti-*Leishmania* antibody, and fluctuations were maintained close to the baseline of the control group through the entire period of observation ($p = 0.0078$). According to statistical analysis, all treatment groups were significantly raised above the theoretical baseline values ($p = 0.0245$), and the results of ANOVA ($F = 8.443$, $p = 0.0014$) demonstrated highly significant inter-group differences.

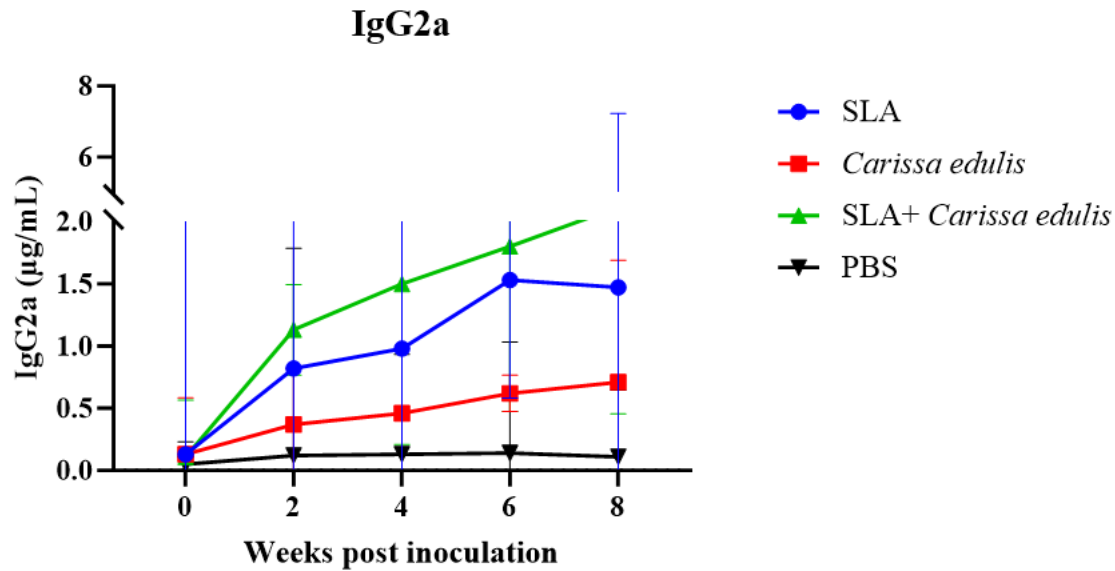


Figure 6. IgG2a antibody subclass production following different treatment interventions over a 8-week post-inoculation period. Serum IgG2a concentrations (µg/mL) were measured at weeks 0, 2, 4, 6, and 8 following inoculation in four treatment groups: SLA alone (blue circles), *C. edulis* extract alone (red squares), combined SLA + *C. edulis* treatment (green triangles), and PBS control (black triangles). Data points represent mean values $\pm$ standard deviation of the mean (SD) for $n=5$ per group. Note the axis break between 0.5 and 2.0 µg/mL to accommodate the dramatically elevated response in the combination therapy group.

The correlation analysis demonstrated that parasite load was significantly positively associated with all measured immunological parameters, which is the most important information about the immune-pathogen interaction in this vaccination model. The highest correlation was found between the parasite burden and the levels of IL-10 ($r = 0.9702$, $P = 0.0061$, $R^2 = 0.9413$), which suggested that the high parasite loads were strongly correlated with the large production of the anti-inflammatory cytokine. This observation is in line with the known pathogenesis of leishmaniasis in which IL-10 upregulation is one of the major processes through which *Leishmania* parasites circumvent host immune defenses and acquire chronic infection.

The parasite load versus IFN- γ production ($r = 0.8989$, $P = 0.0380$, $R^2 = 0.8081$) correlation gives a more complicated immunological scenario that should be carefully interpreted during the leishmaniasis immune regulation. Although traditionally IFN- γ is regarded as the main protection cytokine against *Leishmania* infection, the positive correlation to parasite burden may indicate that high levels of IFN- γ are evidence of continuous immune response to persistent infection as opposed to effective clearance of the parasites. Correlations of the antibody responses shed more light on the association between humoral immunity and the outcome of infection. The positive correlation between the parasite load and total IgG level ($r = 0.9261$, $P = 0.0239$, $R^2 = 0.8576$) indicates that increased antibody level can be counterintuitive to poor control of the infection, in line with previous reports that leishmaniasis is often characterized by a strong but ineffective humoral response. The analysis of IgG subclass shows differentiation as IgG1 was significantly positively correlated with parasite burden ($r = 0.9182$, $P = 0.0278$, $R^2 = 0.8430$), whereas IgG2a exhibited an even stronger relationship ($r = 0.9587$, $P = 0.0100$, $R^2 = 0.9190$).

DISCUSSION

Parasite burden is an important endpoint in vaccine testing against *Leishmania donovani* because it directly relates to the ability of an immunization approach to prevent replication, dissemination and pathology of the parasite. All treatment groups had dissimilar levels of parasite control in the given study, as the combination treatment (SLA + *C. edulis*) was the most effective in reducing parasitemia.

The lowest parasite burden was observed in the group provided SLA + *C. edulis*, with a mean burden of 3.460% and end-parasitemia of approximately 6%. There was also little improvement in the group between weeks 6 and 8, indicating that not only did the combination therapy clear parasites, but it was also effective in inhibiting parasite growth during late-infection phases. This observation is a strong indicator in favor of the use of this combination as a prophylaxis intervention and perhaps as a therapeutic intervention. The very close efficacy between the individual therapies at week 8 in considering the better performance that combination therapy exhibited, is an indication of additive antiparasitic effects, which differ from the dramatic synergistic effects of immune response regarding production of IgG2a during the course of study.

Although there is a synergistic immunologic response in this group, especially increased IFN- γ and IgG2a-responses, the antiparasitic effect is additive, not synergistic. However, its ability to control parasites is considerably greater than that of either monotherapy; it fails to exhibit the exponential reduction which would be anticipated in the case of immunological synergy. Such a difference underlines the complexity of immune activation to pathogen clearance, indicating that more than antibody subclass switching is involved in parasite elimination.

The performance of both SLA and *C. edulis* as monotherapies in the context of parasite control was moderate, with SLA having a mean parasite burden of 5.588% and *C. edulis* having 7.313%. By week 10, these groups had obtained about 12% parasite load with some protective effect, but not enough to prevent progressive parasitemia. The partial efficacy of the SLA group is consistent with the earlier studies of soluble leishmanial antigens provoking protective immunity in BALB/c mice, but which also need a strong adjuvant to induce optimal efficacy (Agallou et al., 2017; Raman et al., 2012a). Of particular interest is the similar efficacy of *C. edulis* based on its novelty as an immunomodulator in leishmanial infection. Even though *C. edulis* did not cause a strong response in IgG2a or IFN- γ with its own exposure, it probably has mild direct antiparasitic effects or can modulate early innate immune responses, which agrees with reports of triterpenoid and flavonoid-mediated antimicrobial activity in phytotransplantations (Ejazi et al., 2020; Hojatizade et al., 2019).

As anticipated, progressively increasing parasitemia was observed in the PBS-treated mice, reaching the peak parasite burden (15.51%), and almost 30 percent at week 10, indicating the natural progression of untreated *L. donovani* infection in BALB/c mice. This control result confirms the infection model and underscores the protective effect of the experimental treatments. The present study shows that soluble *Leishmania donovani* antigen (SLA) in combination with *C. edulis* extract causes a much greater IFN- γ response in BALB/c mice in comparison with the individual agents or the phosphate-buffered saline (PBS) control.

The progressive and persistent rise in the levels of IFN- γ to about 300 pg/mL in week 10 indicates a polarized Th1-type immune response, which is essential in the protection against *L. donovani*. The role of IFN- γ in leishmaniasis is consistent with this observation. Th1 cells produce IFN- γ , which plays a central role in the activation of macrophages to destroy intracellular *Leishmania* amastigotes through the production of nitric oxide (Flores-García et al., 2011). Previously, SLA-based vaccines have been demonstrated to induce moderate IFN- γ response, and provide partial immunity in mouse models (Caras et al., 2025; Chawla et al., 2019). The results of our study support this tendency because the SLA-only group began to show a steady rise in levels of IFN- γ , reaching its maximum at 160 pg/mL.

It is worth noting that the extract of the *C. edulis* provided a modest IFN- γ response (around 120 pg/mL in week 10), but it showed a statistically significant immunostimulatory response. This observation confirms previous ethnopharmacological studies suggesting that *C. edulis* has immune-modulatory effects (Al-Youssef & Hassan, 2012; Muruthi et al., 2023). Nevertheless, the role of the same in anti-leishmanial immunity has not been investigated in

many studies previously. The present study is so far the first report of an experiment to show that, when used as an immunotherapeutic or vaccine adjuvant, *C. edulis* can increase IFN- γ in vivo.

The most pronounced observation refers to the synergistic effect that was observed in the SLA + *C. edulis* group that demonstrated the highest level of IFN- γ concentration (mean 217.4pg/mL, maximum 300pg/mL). This suggests a high level of immunological synergy between the phytochemical content of SLA and the phytochemical content of *C. edulis*. Although past studies have examined natural adjuvants, including saponins, alum, or monophosphoryl lipid A in the leishmanial vaccine (Raman et al., 2012b; Sinani & Şenel, 2025), the use of *C. edulis* is a new botanical option. The enhanced IFN- γ response in the combination group can be explained by immunopotentiating compounds, which are known to regulate the expression of cytokines: flavonoids and triterpenoids (Alqathama, 2024).

Our findings agree with research in which the combination of antigens and plant-based immunomodulators improved protective immunity. As an example, a previous study showed that co-administration of *Nigella sativa* extract with SLA substantially improved the production of IFN- γ and the elimination of parasites in mice (Hosseinzade et al., 2019). Likewise, administration of *Azadirachta indica* extract has been reported to stimulate Th1 cytokines in concert with recombinant leishmanial proteins (Chouhan et al., 2015). Nevertheless, the scale of the IFN- γ rise in the current study using *C. edulis* is significantly greater than what has been found in such models, indicating its potential as a new and practical immunological adjuvant.

Furthermore, the type of IFN- γ differences among groups could serve as a useful source of information on the effectiveness of the vaccine. (Fang & Zhu, 2019) noted that a strong Th1 response (high IFN- γ , low IL-10) is related to resistance and represents a strong response, whereas a Th2-biased response (low IFN- γ , high IL-10) is related to susceptibility. Hence, the upsurge in IFN- γ synthesis that was observed in the SLA + *C. edulis* group indicates the possibility of a protective immunodulatory profile. These cytokine patterns can also be useful as vaccine efficacy predictors pending parasitological results (e.g., parasite burden after a challenge).

It can be seen in the current study that differences can be identified in the various patterns of IL-10 production in the various treatment groups after 10 weeks. The highest concentration of IL-10 was observed in the group that was in contact with *C. edulis* extract only, and it increased steadily to about 60 pg /ml by week 10. The SLA-only group showed an intermediate increase in IL-10 to about 50 pg/mL after the study. This is in agreement with prior findings that reported that SLA-vaccine could stimulate both pro- and anti-inflammatory cytokines in an immune response cascade (Srivastava et al., 2016a; Sundar & Singh, 2014). The IL-10 we have observed could have been the feedback suppressing inflammation after Th1 activation, which is well documented in models of leishmaniasis.

Interestingly, the combination group (SLA + *C. edulis*) retained rather low levels of IL-10 (35-40 pg/mL) in comparison with monotherapies. Possibly, this is a regulatory balancing effect, perhaps as a result of a synergistic switch to a Th1-biased immune response, which inhibits the production of IL-10. It is preferable to keep IL-10 low and IFN- γ high whenever it comes to the immunology of vaccines, where the activation of macrophages is desirable in addition to their ability to roll out parasites (Maksoud & El Hokayem, 2023). The much lower IL-10 in the combination group might, therefore, be a superior immune spectrum to resist *L. donovani*.

The most notable and new detail of this experiment is the clear cytokine balance obtained with an SLA + *C. edulis*. Not only were the highest levels of IFN- γ induced, but the levels of IL-10 produced during this group were comparatively low, so that the ratio of IFN- γ to IL-10 is high. This Th1-type profile with a low degree of regulatory suppression is typical of a protective immune response against the parasite *Leishmania donovani* and correlates with successful resistance to the disease. Thus, in the case of murine models, the successful vaccine candidates usually exhibit high levels of IFN- γ coupled with subdued IL-10 responses (Del Río et al., 2024). By contrast, the *C. edulis*-alone group, though immunostimulatory in a sense, may stimulate an immunoregulatory or tolerogenic phenotype, with a high level of IL-10, which can restrain immunoprotective action as a monotherapy. Likewise, SLA triggered IFN- γ and IL-10 by itself, which demonstrated the activation of immunity to some extent, with the regulation returning.

The gradual and progressive increase in antibody titers over the 10-week experiment represents a synergistic activity between SLA and *C. edulis* to improve B cell activation and resultant production of antibodies. This fact correlates with the fact that effective vaccine preparations must induce not only a robust cellular (Th1-type) response, but also a complementary humoral response, particularly against the extracellular promastigote stage and in early infection (Srivastava et al., 2016b). The elevated IgG level in the combination group could be explained by the adjuvant effects of phytochemicals present in *C. edulis* that may increase the level of antigen presentation and the activation of T-helpers, stimulating the growth and differentiation of B-cells (Joshi et al., 2014; Oliveira et al., 2024).

The production of IgG alone in response to SLA only was moderately but significantly greater than baseline (mean = 1.362 mg/mL; $p = 0.0158$), reaching about 2 mg/mL by week 10. This correlates with previous research that demonstrated SLA, though immunogenic, must have an adjuvant to prime the production of optimal humour (Lopes et al., 2021; Raman et al., 2012a). The results confirm the inherent immunostimulatory capabilities of SLA, but do not warrant any with respect to the induction of potent IgG reactions on its own.

The SLA monotherapy, as well as the SLA + *C. edulis* combination, showed almost the same trajectories of IgG1 with a peak of around 1.7 mg/mL over a period of one week and averages of 1.062 and 1.202 mg/mL, respectively. These findings demonstrate that SLA was the leading cause of IgG1 follow-up, and the immunomodulator *C. edulis* does not considerably affect the Th2-type humoral pattern evaluated as IgG1 only. This result is in contrast to total IgG patterns, where combination therapy resulted in much greater overall antibody titers, implying that the exponential effect of SLA + *C. edulis* appears to occur exclusively through non-IgG1 isotypes.

It is expected that, given the historic SLA data of balanced responses in Th1/Th2 in the absence of adjuvant activation, the observed IgG1 responses can be characterized as balanced reactions (Lopes et al., 2021; Raman et al., 2012a). SLA may elicit a mixed immune response in the absence of a robust Th1-biased adjuvant, which explains the intermediate induction of IgG1.

The group that received *C. edulis* extract alone did not show significant changes in the level of IgG1, but additional titers were about 0.8 mg/mL (mean: 0.5080 mg/mL, $p = 0.0082$). This small IgG1 result, albeit statistically significant relative to baseline, confirms the finding that *C. edulis* has little ability to activate robust humoral immunity on its own. These findings can be compared to the previous results because *C. edulis* alone causes low total IgG and low levels of IFN- γ , which supports a weakly antigenic appearance in immune responses unless combined with an antigen.

Notably, moderate IgG1 induction can be an indication of a regulatory or tolerogenic response, considering the elevated levels of IL-10 in the moderate group. The strong association of Th2-type responses with IL-10 class-switching in mice to IgG1 (Rasquinha et al., 2021) is perhaps the cause of the modest but persistent IgG1 production. As anticipated, the negative control group showed insignificant production of the IgG1 type (mean = 0.1340 mg/mL, $p = 0.0004$), which confirms the specificity of the antigen-mediated antibody response and supports the validity of the experimental model.

The significantly positive relationship between IL-10 and the load of parasites ($R^2 = 0.9413$) suggests that the outcome of infection may be controlled by the regulatory immune reactions that may be greater than the protective responses produced by inoculation. The magnitude of the correlation seen in this experiment is notable when compared to other prior publications and may indicate that the *C. edulis* system of adjuvants is potentially modulating immune control in a manner whose mechanisms remain unexplored in greater detail.

The results of the present study must be framed within this safety paradigm, recognizing simultaneously the possible therapeutic value and the strict safety assessment that occurs in any prospective development route. This experiment reported the largest amounts of IFN- γ and IgG2a--indicators of Th1-type immunity which indicates that concomitant treatment with SLA and *C. edulis* stimulates cell-mediated immunity in a strong manner needed to fight the parasites. In both SLA and combination groups, IgG1 levels indicated that SLA causes a Th2-related antibody response despite adjuvant pairing. Nevertheless, the high IgG2a with decreased IgG1 on the combination lot was biased towards a protective Th1 reaction. Comparatively lower levels of IL-10 in the SLA + *C. edulis* group were associated with increased levels of parasite control, demonstrating the importance of regulating anti-inflammatory cytokines in the

establishment of defence immunity. The high immunogenicity and effectiveness of the combination therapy against parasites warrant the progression of the combination therapy to further preclinical trials and more extended follow-up.

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AUTHOR CONTRIBUTIONS

Judith Njeri: Conceptualization, Methodology, Investigation, Writing original draft

Joshua M. Mutiso: Conceptualization, Supervision, Review and editing manuscript

Rebecca M. Ayako: Methodology, Data analysis, Visualization, Writing original manuscript

Michael M. Gicheru: Conceptualization, Supervision, Resources, Project administration, Review and editing manuscript

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Competing interests

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Author contributions

The design and conception of the study included all the authors. Preparation of material, data collection and analysis were completed by Judith Njeri, Rebecca M. Ayako and Joshua M. Mutiso. Rebecca M. Ayako authored the first draft of the manuscript with comments offered on earlier versions being made by all the authors including Michael M. Gicheru. The specific roles were: Judith Njeri (conceptualization, methodology, investigation, data curation, formal analysis); Joshua M. Mutiso (supervision, validation, writing - review and editing); Rebecca M. Ayako (data analysis, visualization, writing - original draft, review and editing, project administration); and Michael M. Gicheru (conceptualization, supervision, resources, funding acquisition). All authors have approved the final manuscript.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethical approval

Study approval was sought from Kenyatta University. Animal tests were identified with utmost caution and protocol to be very stringent in the Institute Animal Care and Use Committee (IACUC), and encompassed the internationally set standards of carrying out animal research. The course of the study had some humane endpoints which had to minimize suffering in the animals.