

*Full Length Research Paper*

# **Impact of *Artemisia annua* and *Moringa oleifera* on Viral Load, T Cell Activation, and Exhaustion in Ugandan People Living with HIV/AIDS (PLWH)**

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**Highly active antiretroviral therapy (HAART) effectively controls HIV replication in HIV-positive individuals, but chronic immune activation persists, leading to increased virus replication, T cell depletion, and exhaustion, necessitating lifelong HAART to prevent disease progression. This study explores the potential of supplementing HAART with *Artemisia annua* and *Moringa oleifera* leaf powders as adjuvants to restore immune function and control viral replication in people living with HIV/AIDS (PLWH). A nested cohort study was conducted with 31 PLWH on HAART randomized to the control group (n = 15) and the intervention group (n = 16) that supplemented HAART with *A. annua* and *M. oleifera* leaf powder. Peripheral blood mononuclear cells (PBMCs) were obtained from the study participants to measure the expression of activation markers (IFN- $\gamma$ , IL-2, IL-10, and HLA-DR), inhibitory and degranulation receptors (PD-1 and CD107a, respectively) expressed by CD4+ and CD8+ T cells using flow cytometry at baseline and 12 months. Viral load was also measured using quantitative polymerase chain reaction (qPCR). Compared to the controls on HAART only, patients in the intervention group had increased frequencies of CD4+ T cells (p = 0.003), decreased viral load (p = 0.046), and decreased production of IL-10 (p = 0.010). A non-significant trend showing a decrease in the degranulation marker (CD107a), exhaustion marker (PD-1), and activation markers (HLA-DR, IL-2, and IFN- $\gamma$ ) produced by CD4+ and CD8+ T cells were also observed. The study revealed that HAART supplementation with *M. oleifera* and *A. annua* causes faster viral load suppression and elevation of CD4+ T cells with suppressed IL-10 expression. Co-supplementation also increases the expression of CD4+ T cells associated with the suppression of PD-1 and elevation of IL-2 expression, suggesting virological and immunological recovery among patients on HAART.**

**Key words:** *Artemisia annua*, *Moringa oleifera*, people living with HIV/AIDS (PLWH), T cells, viral load, highly active antiretroviral therapy (HAART), exhaustion.

## INTRODUCTION

The Human immunodeficiency virus (HIV) epidemic remains one of the greatest threats to human health and development (Mugo et al., 2022). HIV is associated with abnormalities of the immune system (Adamu et al., 2021), specifically attacking the T helper cells (or CD4 cells) and inducing abnormal and dysfunctional changes in them (Aprioku et al., 2022). The natural course of untreated HIV infection is characterized by persistent viral and antigen burden driving chronic immune activation and T cell dysfunction or exhaustion (Fenwick et al., 2019; Wang et al., 2020), which coincides with the expression of increased levels of an assortment of immune checkpoint inhibitors (ICIs). Exhausted T cells have a limited prospect of immune-mediated viral control, and in particular, the inability to clear latently infected CD4 T cells that comprise the HIV reservoir, leading to the emergence of non-AIDS morbidities and limiting curative efforts (Martin et al., 2021).

The hallmark of T cell exhaustion is sustained expression of high levels of inhibitory receptors such as PD-1, CTLA-4, CD160, TIGIT, and TIM-3 (Chaudhary et al., 2022). In HIV-1 infection, elevated expression of PD-1 on virus-specific T cells is the primary marker of exhaustion. High PD-1 expression correlates with HIV/AIDS disease progression and with impairment of CD8 T cell functionality, increased viral load, reduced CD4 T-cell counts, loss of proliferative capacity, and restricted IL-2 and IFN- $\gamma$  production. T cell exhaustion is also associated with increased levels of IL-10+, CD38+, and HLA-DR + T cells, which represent an increased immune activation, state (Fenwick et al., 2019).

With the induction of HAART, there is significant immunological recovery in most patients characterized by increased numbers of circulating CD4+ T cell counts, decreased levels of T cell activation, and improvement of other immunoregulatory markers including decreases in PD-1 expression. Despite this, many immune parameters remain abnormal compared to those persons without HIV infection, including higher levels of persistent chronic immune activation, pro-inflammatory cytokine levels, decreased naïve T cell counts, and high proportions of terminally differentiated CD4+ and CD8+ T cells (Chaudhary et al., 2022; Li et al., 2022; Blanch-Lombarte et al., 2023). These aberrations combined contribute to lower treatment response and immune reconstitution, and the development of non-AIDS conditions (Perdomo-Celis et al., 2019; Blanch-Lombarte et al., 2023).

Although they are not effective in eradicating pathogens or tumors, exhausted T cells are not completely unresponsive but retain some effector functions, allowing the host to control the pathogen without deleterious

immunopathology (Li et al., 2023).

There has been considerable interest in avoiding or reversing T cell exhaustion (Wherry and Kurachi, 2015) using traditional Chinese medicine (TCM) compounds and active ingredients by moderating T cell subsets in virus-infected patients (Li et al., 2023). In Uganda, PLWH commonly use medicinal herbs such as *Moringa oleifera* and *Artemisia Annua* L. (Asteraceae), as nutritional supplementation either alone or concurrently with HAART (Gambo et al., 2022) to enhance immunity and manage opportunistic infections (Monera-Penduka et al., 2017). Besides having anti-inflammatory, antioxidant, anti-hyperglycemic, and hepatoprotective activity, *M. oleifera* has been reported to exhibit a positive influence on lymphocytes, neutrophils, erythrocytes, hemoglobin, and packed cell volume (Aprioku et al., 2022), while *A. annua*, an annual herb native to Asia and other continents, has been used for the treatment and prevention of fever, chills, some cancers, and malaria, in traditional and modern medicine (Mirbebahani et al., 2020; Ekiert et al., 2021). It is widely believed that moringa supplement has a positive immune modulating influence and immune enhancement properties. This has encouraged the use of moringa supplements by PLWH; however, the synergistic immunological and/or virological effects of the combined use of *A. annua* and *M. oleifera* by PLWH in Uganda have not been established. Therefore, this study investigated the effect of combined usage of *A. annua* and *M. oleifera* powders on CD4 counts, viral load suppression, and T-cell exhaustion among PLWH on HAART.

## MATERIALS AND METHODS

### Study design and population

This study was a cohort nested in a Randomized Controlled Clinical Trial (RCCT03366922) at Mbarara Regional Referral Hospital (MRRH) in, Uganda. The parent trial included 267 HIV-positive, 18-year-old participants on HAART, with normal hematological and biochemical indices, excluding those using other herbal medicines, pregnant, or suffering from opportunistic illnesses. The nested study involved 31 participants from the parent study, divided into two groups: 16 in the intervention group, using *M. oleifera* and *A. annua*, and 15 in the control group on ART only.

### Preparation of plant materials

A botanist from Mbarara University of Science and Technology (MUST) authenticated young leaves of *A. annua* and *M. oleifera* from Kabale district, Western Uganda. The leaves were air-dried, mechanically pulverized, and packaged in 4 and 10 g of *A. annua* and *M. oleifera* respectively, following study-specific procedures and good compounding practices.

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### Administration of treatments and measurement of study parameters

Participants in the intervention arm were given 10 and 4 g of *M. oleifera* and *A. annua* powders respectively to mix and take in their drink of choice (cassava, millet, or posho porridges) every morning at 8 a.m. for 12 months. Each parcel supplied to the participant contained *M. oleifera* and *A. annua* packs labeled with participants' study numbers to last one month. Parcels also contained dosing instructions as well as information on storage and safety. To ensure adherence to the *M. oleifera* and *A. annua* and HAART treatments, every participant received a reminder SMS every morning between 7 and 8 a.m. Participants were requested to respond with the message "Taken" or a call prompt as a proxy to confirm adherence/compliance to the intervention. The study team worked closely with the HIV clinic to ensure that participants were reviewed and accessed routine HIV care as prescribed, including HAART. During the pre-consenting, and throughout the study period, staff emphasized to the participants that *M. oleifera* and *A. annua* herbal medicines were not replacements for HAART in the treatment of HIV, and as such the study participants remained in the study only while enrolled on HAART as prescribed. The following phrase was also added in the consent form: "Please note that these herbal medicines are not being given to you for treatment of HIV, and as such you MUST keep taking your prescribed ARVs as usual and correctly".

A blood sample for the isolation of peripheral blood mononuclear cells (PBMCs) was drawn immediately after enrolment but before initiation of *M. oleifera* and *A. annua* treatments, and then at 12 months, while for the determination of viral load, blood was drawn at 'baseline, 6 and 12 months following the initiation of treatment.

### PBMC Isolation and cryopreservation

PBMCs were isolated from blood by density centrifugation using Ficoll gradient method. Cells were suspended in fetal bovine serum (FBS) supplemented with 10% dimethyl sulfoxide (DMSO), kept overnight at -80°C in a controlled freezing container (Mr. Frosty™, Nalgene), and transferred to liquid nitrogen (-197°C) for long-term storage. Flow cytometry was performed on the stored PBMC samples.

### PBMC thawing and stimulation

Cryopreserved PBMCs were retrieved from liquid nitrogen and thawed in R20 (RPMI with 20% FBS, 1% Pen/Strep, 2 mM Glutamine, 15 mM HEPES) in a water bath at 37°C. The cells were rinsed and rested in R10 (RPMI with 10% FBS, 1% Pen/Strep, 2 mM Glutamine, 15 mM HEPES) in a humidified incubator at 37°C and 5% CO<sub>2</sub> for 4 h. PBMCs were counted manually in a Neubauer chamber using a microscope and stimulated for 5 h in R10 supplemented with premixed PMA (50 ng/ml) plus Ionomycin (1 µg/ml) and Brefeldin A (BioLegend) in a humidified incubator at 37°C and 5% CO<sub>2</sub>. A negative control was also set up using R10 medium without a stimulant for comparison for each sample (Ssekamatte et al., 2021).

### Antibodies used in the study

For surface staining, the following anti-human antibodies were used: Pacific Blue CD3 (Clone SK7), PerCP CD4 (Clone RPA-T4), Alexa Fluor® 700 CD8 (Clone SK1), BV 650 CD107a (Clone H4A3), PE/Cy7 PD-1 (Clone NAT105), and APC HLA-DR (Clone L243). For intracellular cytokine staining, the following anti-human antibodies were used: BV 785 IFN-γ (Clone 4S.B3), BV 605 IL-2

(Clone MQ1-17H12), and PE IL-10 (Clone JES3-19F1), all obtained from BioLegend USA.

### Cell surface staining

PBMCs (about 1.0 × 10<sup>6</sup> cells/mL) were washed with 1X PBS and then stained with the fixable viability dye, zombie aqua for 30 min in the dark at 4°C. These PBMCs were suspended in cell staining buffer before staining them with 50 µL of the appropriate surface antibody panel for 30 min in the dark at 4°C. All antibodies and reagents were obtained from BioLegend, USA.

### Intracellular staining

Following surface staining, PBMCs were fixed by adding 0.5 mL of 4% paraformaldehyde to each sample tube, incubated in the dark for 30 min at room temperature, and then centrifuged at 1800 rpm for 5 min and the supernatant was discarded. The fixed cells were permeabilized using a working concentration (1X in deionized water) of intracellular staining perm wash buffer. Intracellular cytokine antibodies were prepared in 1X intracellular staining perm wash buffer, and 50 µL of the intracellular antibody cocktail was added to each tube and incubated for 30 min in the dark at 4°C. After incubation, cells were washed with 1X intracellular staining perm wash buffer thrice and finally suspended in 100 µL cell staining buffer and samples were acquired on the Cytoflex LX flow cytometer. Compensations and fluorescence minus ones (FMOs, for PD-1, CD107a, and intracellular markers) controls for the antibody fluorochromes were run together with the optimization gains for every channel using unstained cells. Compensation calculations were done and applied to the samples. A minimum of one hundred thousand events were acquired and recorded for every sample. Data was exported for analysis using FlowJo software (Ssekamatte et al., 2021).

### Statistical data analysis

Flow cytometry data were analyzed using FlowJo version 10.7.1 software (Becton Dickinson, New Jersey, USA). Gating was standardized and set using Fluorescence Minus One (FMO) controls, and subset cellular frequencies were expressed as percentages of parent gates. Statistical data were analyzed using GraphPad Prism software version 8. Paired t-test was used to determine the difference between treatment and control group mean frequencies of CD4+ and CD8+ T cells, expressing HLA-DR, CD107a, PD-1, IFN-γ, IL-2, and IL-10 while linear regression was used to determine the association between CD4 count and the above surface and intracellular markers. HIV Viral load data were analyzed using STATAv15. The Mann-Whitney (Rank sum) test was used to check for viral load median differences between the test and control groups at the three time points, while the Kruskal-Wallis followed by Dunn's multiple comparison tests were used to detect any median differences between the baseline and later concentrations. p-values less than 0.05 were considered statistically significant and hence concluded that the means and medians were different.

## RESULTS

### Baseline characteristics of study participants

Baseline demographics, CD4, and viral load

**Table 1.** Baseline Characteristics of Study Participants in the Two Study Groups.

Characteristic	Control	Artemisia + Moringa	p-value
	Group (n=15)	Group (n =16)	
	Mean ( $\pm$ SD) or n (%)	Mean ( $\pm$ SD) or n (%)	
Age (years)	40	43	0.489
<b>Gender</b>			
Male	5 (33)	5 (31)	0.901
Female	10 (48)	11 (52)	
Mean CD4 (cells/ $\mu$ L)	258 (66)	247 (57)	0.630
Mean CD8 (%)	49 (14)	54 (10)	0.233
VL (copies /mL)	9684 (36117)	48 (63)	0.310
<b>HAART regimen</b>			
AZT +3TC +EFV	6 (40)	4 (40)	0.375
3TC + EFV + TDF	6 (40)	5 (31)	
3TC + EFV + others	3 (20)	7 (44)	

HAART: Highly active antiretroviral therapy; AZT: Zidovudine; 3TC: Lamivudine; EFV: Efavirenz; NVP: Nevirapine; TDF: Tenofovir; VL: Viral load.

characteristics of the participants in the control and intervention groups were comparable at enrollment. Similarly, at baseline, the number of patients on the three categories of HAART regimens; AZT + 3TC + EFV, 3TC + EFV + TDF, and 3TC + EFV + others, was statistically similar for both the intervention and control groups (Table 1). A gradual decline in viral load was observed from baseline through the 6 months and up to the 12 months of participant follow-up. However, the viral load was only significantly different ( $p = 0.046$ ) between the intervention and control groups at the 12 months' follow-up time point.

#### Effect of *A. annua* and *M. oleifera* on CD4+ and CD8+ T cell frequencies, activation and exhaustion

All participants in this study underwent CD4 and CD8 counts at enrollment, with no statistically significant differences observed in CD4 ( $p = 0.630$ ) and CD8 ( $p = 0.233$ ) counts (Table 1). However, after 12 months of *A. annua* and *M. oleifera* supplementation of HAART, the intervention arm exhibited significantly higher CD4 count frequency ( $p = 0.003$ ), but not CD8 count frequency, compared to the control group. Further examination of the frequencies of HLA-DR and PD-1 as surrogate surface markers for activation and exhaustion, respectively, expressed by activated CD4+ and CD8+ T cells, revealed that both the intervention and control groups showed some reduction in the expression of HLA-DR and PD-1 after 12 months of patient follow-up; however, the within-group changes were not statistically significant (Figure 1).

#### Effect of *A. annua* and *M. oleifera* on CD4+ CD8+ T cells expression of CD107a

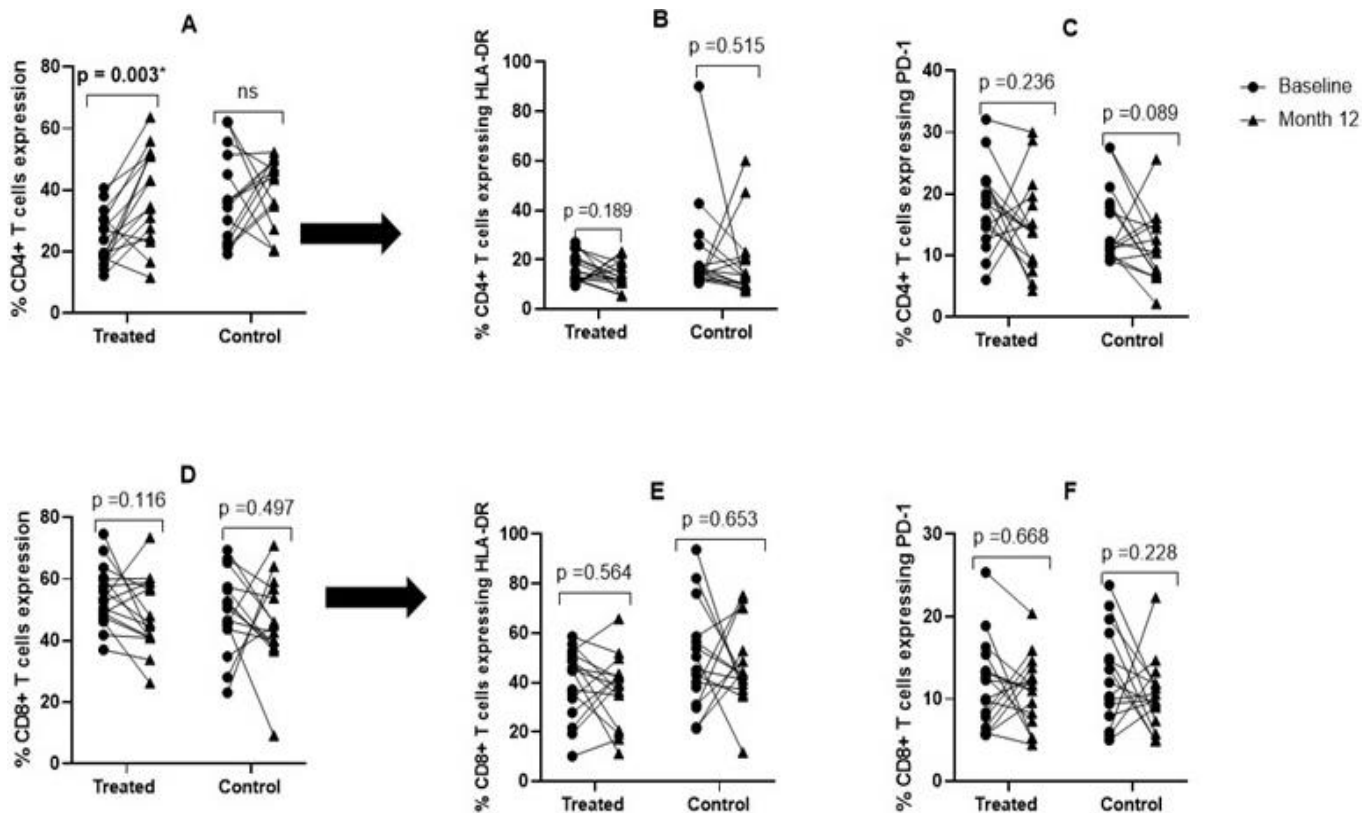
The effect of *A. annua* and *M. oleifera* supplementation of HAART on T cell cytotoxicity was investigated. After 12 months of follow-up, compared to the baseline time point, no significant difference was found in the frequencies of CD4+ T cells (Figure 2A) and CD8+ T cells (Figure 2B) expressing CD107a in both the *A. annua* and *M. oleifera* intervention and control group.

#### Effect of *A. annua* and *M. oleifera* on CD4+ CD8+ T cells expression of IFN- $\gamma$

The effect of *A. annua* and *M. oleifera* supplementation of HAART on the recovery of exhausted T cells was investigated. Following 12 months of patients' follow-up, no significant difference was found in the frequencies of CD4+ T cells (Figure 3A) and CD8+ T cells (Figure 3B) expressing IFN- $\gamma$  in both the *A. annua* and *M. oleifera* and control groups.

#### Effect of *A. annua* and *M. oleifera* on CD4+ CD8+ T cells expressing IL-2

We also investigated the effect of *A. annua* and *M. oleifera* supplementation of HAART on the recovery and activation of exhausted T cells. After 12 months of patients' follow-up, no significant difference was found in the frequencies of CD4+ T cells (Figure 4A) and CD8+ T



**Figure 1.** CD4 and CD8 T cell frequencies and HLA-DR and PD-1 expression on CD4+ and CD8+ T cells. Comparison of baseline and 12 months' time-point frequencies of CD4 T cells [A], CD8 T cells [D], expression of HLA-DR on CD4 [B] and CD8 [E] T cells. Changes in PD-1 expression between baseline and 12 months' time-points are shown in C [CD4+ T cells] and F [CD8 T Cells] while the percentage of CD8+ T cells [D] expressing HLA-DR and PD-1 is shown in E and F for the participants in the intervention group (n = 16) and on HAART only group (n = 15). The difference between the intervention and control group mean frequencies above was determined using a paired t-test and p-values less than 0.05 were taken to be statistically significant.

cells (Figure 4B) expressing IL-2 in both the *A. annua* and *M. oleifera* and control groups.

#### Effect of *A. annua* and *M. oleifera* on CD4+ CD8+ T cells expressing IL-10

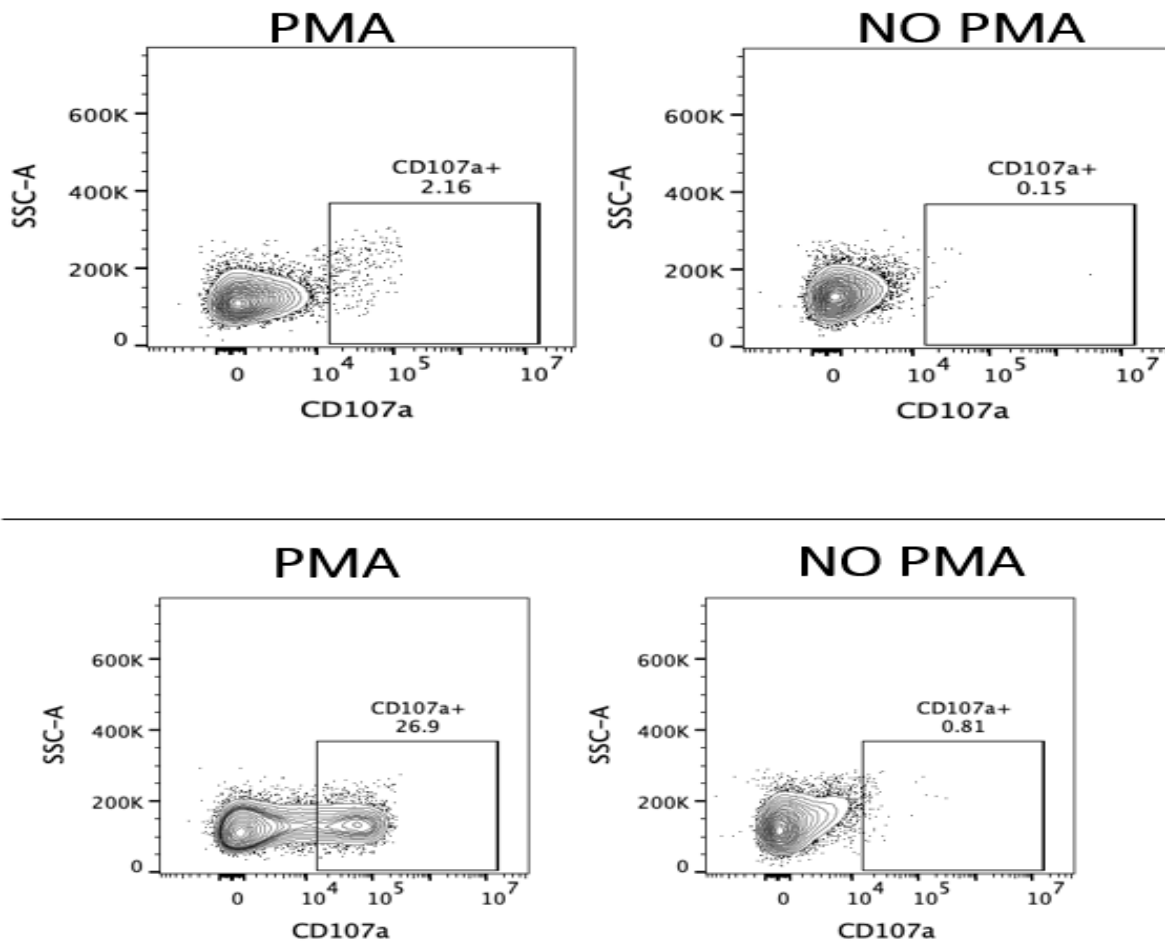
Finally, the effect of *A. annua* and *M. oleifera* supplementation of HAART on the reduction of chronic inflammation in exhausted T cells was investigated. After 12 months of follow-up, compared to the baseline time point, no significant change or difference was found in the mean frequency of CD8+ T cells expressing IL-10 in both the *A. annua* and *M. oleifera* and control groups (Figure 5B). The control group similarly did not show a statistically significant change in the mean frequency of CD4+ T cells expressing IL-10 at the 12 months of follow-up when compared to baseline. However, there was a significant reduction in the mean frequency of IL-10 in the *A. annua* and *M. oleifera* treatment group when the 12 months' time point was compared to the baseline visit (Figure 5A).

#### Association of CD4+ and CD8+ T cell recovery with PD-1, HLA-DR, CD107a and IL-2

A linear regression analysis was performed to determine the association of CD4+ and CD8+ T cell recovery with PD-1, HLA-DR, CD107a, and IL-2. It was observed that an increase in IL-2 resulted in a corresponding increase in the mean frequencies of the CD4+ and CD8+ T cells, while the decrease in PD-1 was associated with an increase in CD4+ but not CD8+ T cells. No association was observed between CD4+ and CD8+ T cells and HLA-DR and CD107a.

#### Ethical approvals

The study was approved by the MUST Research Ethics Committee with registration number 12/08-19. Written informed consent was obtained from each participant and all the procedures used were per the ethical standards of the responsible committee on human experimentation according to the Helsinki Declaration. Participants'



**Figure 2.** Production of CD107a by CD4+ and CD8+ T cells. PBMCs were stimulated and incubated at 37°C for 6 hours with PMA/ionomycin or unstimulated (US) plus Brefeldin A and stained for CD107a. A and B show representative gating of CD4+ and CD8+ T cells expressing CD107a respectively, following flow cytometry data acquisition. The percentage of T cells expressing CD107a is shown in A (CD4+ T cells) and B (CD8+ T cells) for the participants in the intervention group (n = 16) and HAART-only group (n = 15). The difference between the intervention and control group mean frequencies of CD107a was determined using a paired t-test and p-values less than 0.05 were taken to be statistically significant.

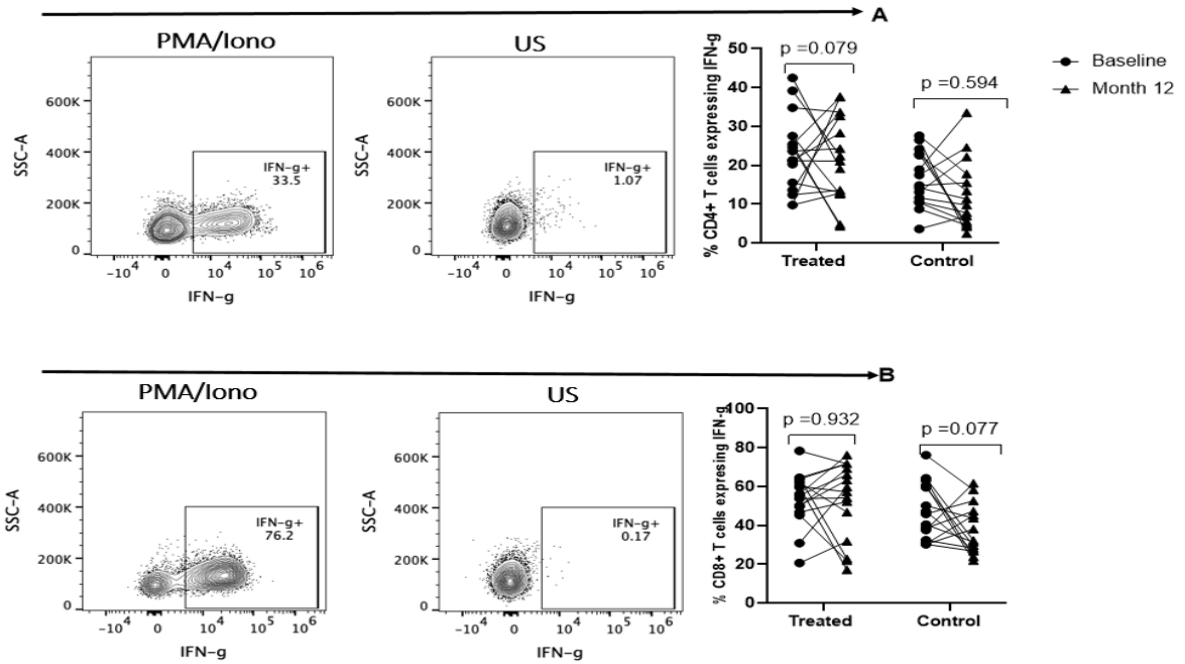
identities were kept anonymous throughout the study process.

## DISCUSSION

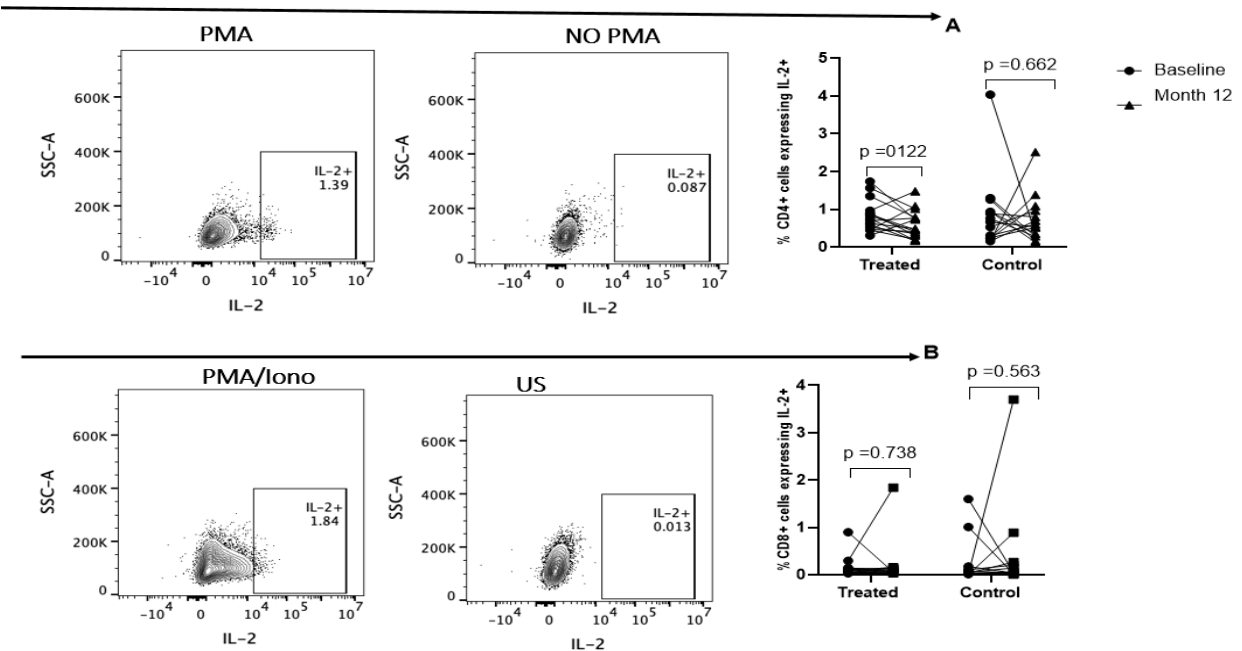
The effect of twelve months' consumption of a combination of *A. annua* and *M. oleifera* leaf powder together with standard HAART on viral load, CD4, and CD8 T cell degranulation, activation, and exhaustion among PLWH on HAART in South-western Uganda was investigated. In the study, the average age of the participants was 41 years (Table 1). This finding is supported by The Uganda Population-based HIV Impact Assessment (UPHIA 2020-2021), which stated that HIV prevalence is highest in the 45-49 age group (12.1%) followed by the 50-54 age group (11.8%), compared to

the national average of 5.8%, highlighting the fact that HIV in Uganda is most prevalent among people aged 35 years and older compared to younger age groups. These findings are in agreement with studies carried out in Tanzania that observed a higher HIV prevalence for both men and women aged 25-55 years than younger ages of less than 25 years (Negin et al., 2016; Mosha et al., 2022). This high HIV prevalence is attributed to the widespread availability and usage of HAART by PLWH in Uganda, which increases their chances of surveillance.

Although there was a significant reduction in viral load in the intervention group after 12 months of patients' follow-up compared to the control, this reduction was gradual and was not observed at month 6 (Table 2) following initiation of the intervention. This could have been because of the compromised immune systems of the patients included in this study. To participate in this



**Figure 3.** Production of IFN- $\gamma$  by CD4+ and CD8+ T cells. PBMCs were stimulated and incubated at 37°C for 6 hours with PMA/ionomycin or unstimulated (US) plus Brefeldin A and stained intracellularly for IFN- $\gamma$ . A and B show representative gating of CD4+ and CD8+ T cells expressing IFN- $\gamma$  respectively, following flow cytometry data acquisition. The percentage of T cells expressing IFN- $\gamma$  is shown in A (CD4+ T cells) and B (CD8+ T cells) for the participants in the intervention group (n = 16) and the HAART-only group (n = 15). The difference between the intervention and control group mean frequencies of IFN- $\gamma$  was determined using a paired t-test and p-values less than 0.05 were taken to be statistically significant.



**Figure 4.** Expression of IL-2 by activated CD4+ and CD8+ T cells. PBMCs were stimulated and incubated at 37°C for 6 hours with PMA/ionomycin or unstimulated (US) plus Brefeldin A and stained intracellularly for IL-2. A and B show representative gating of CD4+ and CD8+ T cells expressing IL-2 respectively, following flow cytometry data acquisition. The percentage of T cells expressing IL-2 is shown in A (CD4+ T cells) and B (CD8+ T cells) for the participants in the intervention group (n = 16) and HAART-only group (n = 15). The difference between the intervention and control group mean frequencies of IL-2 was determined using a paired t-test and p-values less than 0.05 were taken to be statistically significant.

**Table 2.** Effect of *Artemisia annua* and *Moringa oleifera* on Viral Load in PLWH on HAART.

Variable	Median (IQR)		P value (Mann-Whitney test)
	Treatment (%)	Control (%)	
Baseline Viral Load	20 (0 - 87)	67 (0 - 502)	0.115
Viral Load at 6 months	0 (0 - 20)	0 (20 -100)	0.117
Viral Load at 12 months	0 (0 - 20)	50 (0 -183)	0.046*

IQR, inter quartile range. The median viral load readings in the treatment and control groups were compared at the baseline, six, and twelve months.

study, patients should have been on HAART for at least one year with a CD4 count of less than 350 cells/ $\mu$ L, which could have led to the delayed rebound of viral load to normal. These results are in agreement with the findings of Hussen et al. (2019), who in their study among PLWH reported that the median duration to suppress the viral load from the initiation of HAART to suppression of the viral load below 1000 copies/mL is 1 month to 7 months (Sultan Hussen et al., 2019).

The observed significant decrease in viral load at 12 months ( $p = 0.046$ ) after the initiation of the intervention was a result of the effectiveness of supplementation of HAART with a combination of *M. oleifera* and *A. annua* leaf powder in preventing the replication of HIV since the patients were on HAART for at least a year before the intervention but still had virological and immunological failure. This finding, similar to that in the study by Yang et al. (2018), is very important because effective HAART has been proven to reduce the viral loads of infected individuals and might reduce the risk of sexual transmission to uninfected persons (He et al., 2018). Contrary to our findings, Gambo et al. (2021) did not observe a significant difference in viral load after 12 months of supplementing HAART with a combination of *M. oleifera* with *A. annua* between their study groups (Gambo et al., 2021). This can be attributed to the fact that in our study, immunocompromised patients (CD4 count of  $\leq 300$  cells/ $\mu$ L) on HAART were recruited and administered a combination of *M. oleifera* with *A. annua*, while the patients (CD4 count of  $\leq 500$  cells/ $\mu$ L) in their study were administered *M. oleifera* in addition to HAART.

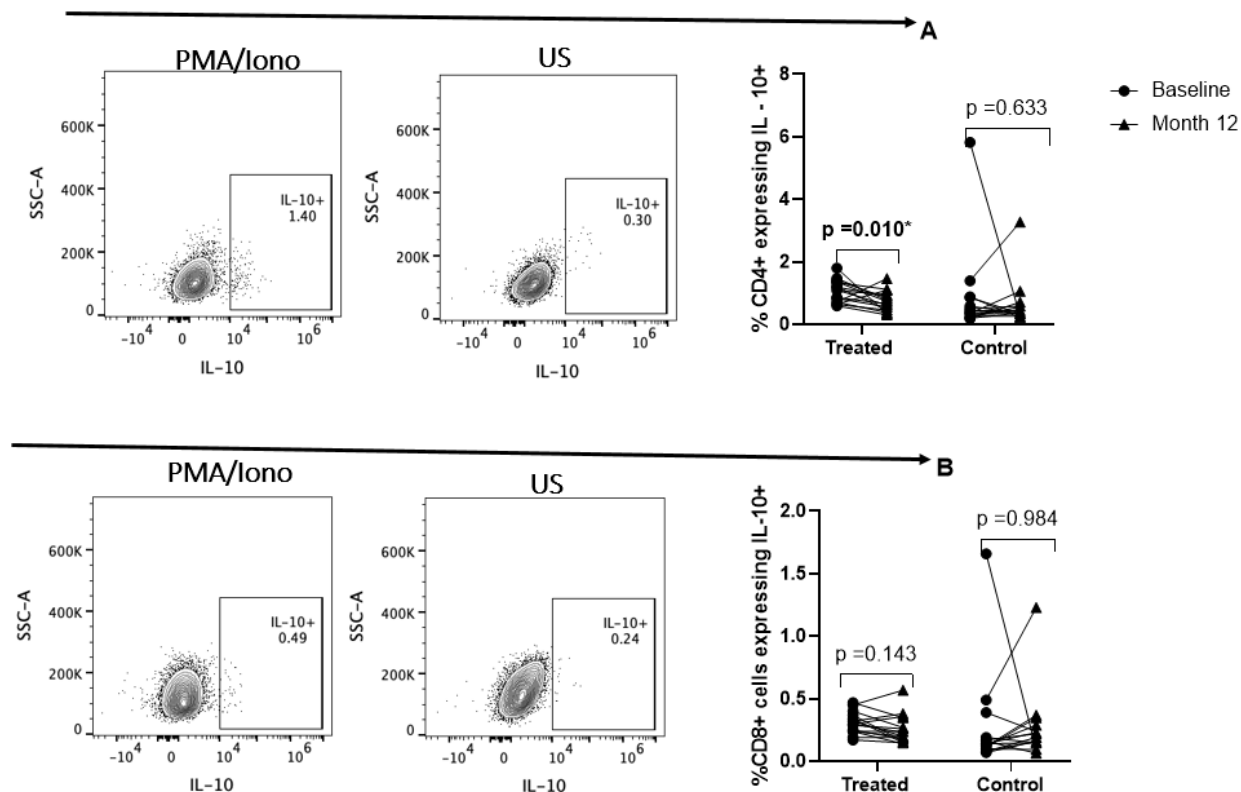
After 12 months of patients' follow-up, a significant increase in CD4+ T cell frequency (Figure 1A) coupled with a significant decrease in the expression of IL-10 (Figure 5A) by these cells was observed in the intervention group but not in the control group, suggesting suppression of T cell exhaustion and increased T cell functionality among PLWH receiving the intervention treatment. These findings led to the hypothesizing of associations between CD4+ and CD8+ T cells and the expression of functional markers (CD107a, PD-1, HLA-DR, IFN- $\gamma$ , and IL-2) on these cells. Linear regression analysis was performed, revealing that an increase in CD4+ T cell frequency was associated with an increase

in IL-2 and a decrease in PD-1, while an increase in CD8+ T frequency was only positively associated with IL-2. However, in the intervention group, no correlation was found between the expression of PD-1, CD107a, and HLA-DR by CD8+ T cells (Table 3).

This increase in CD4+ T cells and the decrease in IL-10 expression suggest that a combination of *A. annua* and *M. oleifera* leaf powder leads to immunologic recovery and decreases T cell exhaustion among PLWH on HAART, which is often associated with uncontrolled HIV replication. Similar findings of increases in CD4 count were also observed in other studies by Gambo et al. (2021) and Ndlovu et al. (2022), where higher CD4 counts were reported in the intervention group compared to the control group (Gambo et al., 2021; Ndlovu et al., 2022). Moreover, the positive associations between T cell frequencies and IL-2 expression and the negative association with the expression of PD-1 also suggest that the intervention was able to decrease T cell exhaustion resulting in the recovery of T cell function coupled with virological recovery. Similarly, Fenwick et al. (2019) observed that the functionality of HIV-specific T cells improves with effective HAART with both CD4 and CD8 T cells producing increased levels of IL-2 following stimulation, as observed in our study.

Although a statistically significant difference in T cells expressing HLA-DR (Figure 1B and E), CD107a (Figure 2A and B), and IFN- $\gamma$  (Figure 3A and B) markers was not observed, the findings show an increase in CD107a and IFN- $\gamma$  and a decrease in HLA-DR expressed by CD4+ and CD8+ T cells. Previous studies have shown that the increased activation and inflammatory state among PLWH cause alterations in CD8+ T-cells, including persistently increased absolute counts, high expression of the activation markers (HLA-DR and CD38), and the exhaustion marker (PD-1), and low cytotoxic ability. The intervention was able to reverse some of these defects, as seen in the reduction in the expression of HLA-DR coupled with increased CD4 + cells and reduced expression of viral load. As established in previous studies, CD8+ T-cells are critical in the control of HIV infection and are characterized by a high cytotoxic capacity, which depends on both the lytic granule contents (particularly granzymes and perforin) and the degranulation capacity, widely evaluated by the





**Figure 5.** Expression of IL-10 by activated CD4+ and CD8+ T cells. PBMCs were stimulated and incubated at 37°C for 6 hours with PMA/ionomycin or unstimulated (US) plus Brefeldin A and stained intracellularly for IL-10. A and B show representative gating of CD4+ and CD8+ T cells expressing IL-10 respectively, following flow cytometry data acquisition. The percentage of T cells expressing IL-10 is shown in A (CD4+ T cells) and B (CD8+ T cells) for the participants in the intervention group (n = 16) and the HAART-only group (n = 15). The difference between the intervention and control group mean frequencies of IL-10+ was determined using paired t-test and p-values less than 0.05 were taken to be statistically significant.

**Table 3.** Association of CD4+ and CD8+ T cells and CD107a+, PDL-1+ and IL-2+.

Variable	CD4+ T cells			CD8+ T cells		
	Coefficient	P-value	95% CI	Coefficient	P-value	95% CI
HLA- DR	-1.48	0.177	-3.75 - 0.78	-0.12	0.708	-0.82 - 0.58
PD-1	-2.91	0.026*	-3.43 - -0.25	-1.21	0.124	-5.40 - 0.42
CD107a	3.79	0.070	-0.279 - 7.85	0.28	0.419	-0.45 - 1.01
IL-2	48.04	0.031*	5.20 - 90.88	15.57	0.031*	1.71 - 29.43
Constant	53.37	0.000	36.95 - 69.79	53.28	0.000	37.43 - 69.13

Linear regression was used to determine the association between CD4+ and CD8+ T cells and CD107a+, PDL-1+, and IL-2+. The association was taken to be statistically significant at p-values less than 0.05.

expression of CD107a (Perdomo-Celis et al., 2019). Similarly, the intervention was able to improve the cytotoxic activity of CD4+ T cells. Recent studies have shown that CD4+ T cells can also elaborate cytotoxic responses, inducing apoptosis of target cells. Cytotoxic CD4+ T cells (CD4+ CTLs) exhibit cytolytic functions that resemble those of CD8+ T-cells; in fact, there is evidence suggesting that they may have a role in the control of

viral infections (Juno et al., 2017; Sanchez-Martinez et al., 2019; Cenerenti et al., 2022).

The findings collectively show that in chronic diseases like HIV and AIDS, adequate micro and macronutrients are vital for normal body functioning, maintaining optimal immunological function, and improving the efficacy of HAART taken by PLWH (Gambo et al., 2022). *M. oleifera* leaves, which are a rich source of both macro-and

micronutrients, are often taken as a supplement by PLWH, especially in developing countries, to enhance immunity and manage opportunistic infections (Monera-Penduka et al., 2017). The observed viral load suppression and concurrent increase in CD4+ T cell frequency can be attributed to the combined effect of the phytochemicals found in the powdered leaves of *M. oleifera* and *A. annua*, which were used to enhance HAART among PLWH. Some limitations of this study must be noted. Compliance with HAART and the interventional treatments was monitored by the self-reporting of the study participants to the investigators and was prone to patient bias. Additionally, PMA/Ionomycin was used instead of HIV-specific peptides.

## CONCLUSIONS AND RECOMMENDATION

The study demonstrated that supplementation of HAART with *M. oleifera* and *A. annua* leads to reduced viral load, increased CD4+ T cell frequencies, and inhibition of IL-10 production. Moreover, it elevates the expression of CD4+ T cell frequencies associated with PD-1 suppression and IL-2 elevation, indicating both virological and immunological recovery among PLWH on HAART. Further investigations in settings with rigorous monitoring of adherence to HAART and intervention treatments are recommended. Additionally, studies utilizing HIV-specific peptides to identify T cell responses specific to HIV would be beneficial. Moreover, it is suggested to conduct research aimed at identifying and purifying phytochemicals exhibiting observed activity as potential adjunctive therapies for HIV/AIDS management.

## ABBREVIATIONS

**AIDS:** Acquired immune deficiency syndrome; **BL:** Baseline; **CD4:** Cluster of differentiation 4; **CTLs:** cytotoxic T-lymphocytes; **FACS:** Fluorescence-activated cell sorting; **DMSO:** dimethyl sulfoxide; **HAART:** Highly active antiretroviral therapy; **HEPES:** N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; **HIV:** Human immunodeficiency virus; **HLA-DR:** Human Leukocyte Antigen – DR; **ICIs:** immune checkpoint inhibitors; **IFN- $\gamma$ :** *Interferon gamma*; **IL:** interleukin; **MUST:** Mbarara University of Science and Technology; **PCR:** Polymerase chain reaction; **PBMCs:** Peripheral blood mononuclear cells; **PBS:** phosphate buffered saline; **PLWH:** People living with HIV; **PMA:** phorbol myristate acetate; **RPMI:** Roswell Park Memorial Institute; **SD:** Standard deviation; **UNCST:** Uganda National Council for Science and Technology; **US:** unstimulated; **VL:** viral load; **WHO:** World health organization.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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