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Original Article

Human Peripheral Blood Mononuclear Cells Proliferation and Secretion of Type I Interferon after Treatment with Polyherbal Formulations Fractions

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ABSTRACT

Background and aims*. Human immunodeficiency virus (HIV) infections are becoming resistant to therapy, despite extensive discoveries. The use of natural remedies for HIV treatment is becoming global. This study evaluates the in-vitro effect of aqueous extract of polyherbal formulation (PHF) fractions on peripheral blood mononuclear cells (PBMCs) proliferation and secretion level of type 1 interferons.* ***Methods****. Column chromatography of the PHF yielded four fractions (A, B, C, and D). Different concentrations (25 µg/ml, 50 µg/ml, 100 µg/ml, and 200 µg/ml) of each fraction were prepared. A blood sample from a newly diagnosed HIV-positive volunteer was used. The percentage viability, cytotoxicity, and proliferation of the PBMCs were determined using the trypan blue dye exclusion method and WST-8 assay respectively.* ***Results****. The viability of the PBMC significantly decreased with increasing concentrations of each PHF fraction (P=0.0001). While fraction D exhibits the highest percentage of cytotoxicity, 16.17% at 200 g/ml, fraction C exhibits the lowest percentage of cytotoxicity at 25 g/ml concentration and upregulate interferons expression (IFN-α, IFN-β). Fractions A, B, and C induces significant PBMC proliferation (P 0.0001).* ***Conclusion****. The fractions have proliferative potential and enhance interferon production. In-depth PHF fractions and structural studies of the bioactive compounds are recommended.*

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INTRODUCTION

The use of plant compounds for pharmaceutical purposes has gradually increased [1], and according to the World Health Organization medicinal plants are probably the best source of a variety of drugs [2]. Plant-related pharmacological formulations are highly ranked for viral infections [3]. A variety of medicinal plants demonstrated a tremendous antiviral effect at various stages of viral growth [4]. The majority of the world's population uses herbs for the management of human immunodeficiency virus (HIV) related symptoms [4]. Various medicinal plants are used in the treatment of HIV/AIDS such as *Withania somnifera* [5], *Tinospora cordifolia* [6], *Moringa oleifera* [7], *Hypericumper foratum* [8], *Silybum marianum* [4], and *Allium sativum* [9]. About 36.9 million people are living with HIV/AIDS (PLHIV) globally, with more than two-thirds living in sub-Saharan Africa (SSA) [10]. Acquired immunodeficiency syndrome (AIDS), the

advanced stage of the human immunodeficiency virus (HIV) infection is a major public health problem [11]. Due to the high prevalence of HIV/AIDS and the emergence of drug-resistant strains, the demand for new antiviral drugs against AIDS is rising [12]. Management with antiviral agents is frequently yielding unsatisfactory clinical outcomes [13]. Refractory viral infections resistant to available antiviral drugs are alarming threats and serious health concerns [13].
Cytokines can be classified as pro and anti-inflammatory and are considered important initiators and mediators of inflammation [14]. Infection with HIV results in the production of cytokines by infected cells and cells of the immune system. Such cytokines regulate the immune function and affect viral replication [15]. Changes in cytokine levels in HIV-infected individuals can affect the function of the immune system and have the potential to directly impact the course of HIV disease by enhancing or suppressing HIV replication [16]. Immune deregulation during the development of AIDS is a result of cytokines imbalance. Cytokines that suppress HIV include interferon-alpha (IFN-α), interferon-beta (IFN-β), and interleukin 10 (IL-10) [15].
A lot of herbal medical practitioners in Nigeria, claim to have herbal formulations with immunomodulatory activities. The polyherbal formulation (PHF) used in this study contains five plant materials, which include the following: Black cutch- Bark (*Acacia polyacentha* wild), orchid bush-Bark (*Bauhinia rufescens* lam), Gum Arabic tree- Bark (*Acacia Senegal*), Baobab-leaves (*Adensonia digitata*), and Garlic-Yellow-bulb (*Allium sativum*). It is used by some people locally as a source of remedy for different diseases including HIV without prior knowledge of its adverse effects or which specific fraction of the formulation is responsible for the therapeutic effect. Previous studies on this PHF have reported improved neutrophil adhesion and T-dependent antibody response [17], immunostimulatory activity on murine Th1/Th2 cytokines secretion and cell-mediated immune response [18], and immunomodulatory activity on macrophage function and humoral immune response against sheep red blood cells in mice [19]. Indeed, a recent in vivo study shows it has protective potential in cyclophosphamide-induced immunosuppression [20]. This provides insight into its potential use in the management of immunocompromised conditions. However, the mechanistic bases of the in vivo effects are not well understood and the bioactive fractions of the PHF have not yet been studied. This study aims to identify the PHF fractions responsible for their therapeutic effect and to study their proliferative effect on the human PBMCs and cytokine secretions that are important in the suppression of viral replications. Therefore, it may pave the way for the production of a compound from a natural source for better treatment and/or management of immunocompromised conditions or viral infections. The study evaluates the in-vitro effects of fractions of PHF on the proliferation of PBMCs and the level of type-I interferons.

METHODS

Study design and setting

This is an *in vitro* study investigating the cytotoxic and or proliferative effect of polyherbal formulations fraction on human PBMCs of newly diagnosed HIV patient and secretion of type I interferons.

Polyherbal formulation

The polyherbal formulation consists of five plant materials namely: Black Cutch-Bark (*Acacia polyacentha* wild), Orchid Bush-Bark (*Bauhinia rufescens* lam), Gum Arabic tree-Bark (*Acacia Senegal*), Baobab-leaves (*Adensonia digitata*) and Garlic-Yellow-bulb (*Allium sativum*). The aqueous extract of the PHF was collected from the Department of Immunology, School of Medical Laboratory Sciences, Usmanu Danfodiyo University, Sokoto, which was initially stored and preserved at 5°C in airtight bottles and was labelled as PHF aqueous extracts. In stock preparation, 1 g of the PHF was dissolved in 10 ml of the distilled for 1 h at room temperature with gentle rocking at intervals to ensure complete and total dissolution of the PHF particles.

Thin layer chromatography

Thin Layer Chromatography (TLC) was carried out at the Central Laboratory of Usmanu Danfodiyo University, Sokoto. The fractions in the PHF were identified by using TLC, the technique used for the separation of non-volatile mixtures. The developing chamber was prepared by obtaining a jar thatfits the entire TLC plate. Two 200 ml of the mixture ethanol/propanol/water/acetonitrile (1:1:1:1 V/V) was measured and 1 ml of the dissolved PHF in distilled water was added into the jar. The TLC plate was held next to the chamber to make sure that the solvent level is below the level of the spots. The TLC plate was inserted evenly into the chamber. The line and the spots are at the bottom of the plate and are above the level of the solvent. The chamber was covered with a lid. Once the solvent was close to the top of the plate, the plate was removed from the chamber, and the solvent line was marked with a pencil. The plate was allowed to dry and was then visualized under ultraviolet light.

Column chromatography

Column chromatography was carried out at the Central Laboratory of Usmanu Danfodiyo University, Sokoto. Column chromatography was prepared and filled using cotton wool and fine sand (1.0 cm) while silica gel was used to pack the column for the stationary phase using a wet packing funnel. The mobile phase was poured into the column using a funnel. The column was gently tapped to remove air bubbles. Five millilitres (5 ml) of the dissolved PHF were loaded into the column, and four (4) different solvents were used to elude the PHF solution starting with the least polar i.e. propanol, methanol, distilled water, and acetonitrile. Fractions were collected from each solvent and were labelled fraction A, Fraction B, Fraction C, and Fraction D according to the solvent used to elute them mentioned above respectively. The collected fractions were again subjected to TLC to ascertain purity. Collected fractions were then transferred into a clean beaker and allowed to stand in a clean area to evaporate the excess solvent. After the evaporation fraction was then transferred into clean sterile cryovials and labelled Fractions A, B, C, and D. The cryovials were then stored at 5°C in a refrigerator.

Source of PBMCs

Three millilitres (3 ml) of whole blood was collected using the Monovette Vacutainer system containing lithium heparin as an anticoagulant from a 35-year-old volunteer who is diagnosed with HIV and has not commenced antiretroviral treatment. The patient gave his informed written consent to participate in the study which is to be published, having understood fully the research at hand. The sample was immediately transported to the Centre for Advanced Medical Research and Training (CAMRET), Usmanu Danfodiyo University, Sokoto for analysis. The study was conducted in accordance with the Helsinki declaration [21].

Isolation of PBMCs

The procedure for the isolation of the human PBMCs was carried out according to the manufacturer's instructions and also described previously [22]. Briefly, 3 ml of Histopaque-1077 (Sigma-Aldrich® Co. UK) was added to a 15 ml falcon tube and was brought to room temperature. Three milliliters (3 ml) of whole blood was layered onto the Histopaque-1077 carefully. Afterward, it was centrifuged at 400 × *g* for 30 min at room temperature, the middle layer which contains the PBMCs was aspirated carefully with a Pasteur pipette to within 0.5 cm of the opaque interface containing mononuclear cells and was carefully transferred with aPasteur pipette into a clean conical centrifuge tube. The cells were washed by adding 10 ml of isotonic phosphate-buffered saline (PBS) solution using a Pasture pipette and were mixed by gently drawing in and out of a Pasteur pipette. The cells were washed by centrifugation at 250 × *g* for10 min and the supernatant was discarded. The cells pellets were suspended with 5 ml of isotonic PBS solution with a Pasture pipette and were mixed by gently drawing in and out of the pipette. The cells were then centrifuged at 250 × *g* for 10 min. The step was repeated two times, the cells pellets were resuspended in 2 ml of RPMI-1640 (Beijing Solar Bio Science and Technology Co. Ltd, China) and treated with 10% heat-inactivated fetal bovine serum (FBS).

Trypan blue assay

The procedure was carried out according to the manufacturer's instructions and described in [22]. Ten microliters (10 µl) of 0.4% Trypan blue (Sigma-Aldrich® Co. UK) solution (w/v) and 10 µl of the PBMCs suspension were transferred into a cryovials tube (dilution factor = 2) and mixed, it was then allowed to stand for a maximum of 5 min. While the coverslip was in place, the pipette was used to transfer 10 µl of Trypan blue-PBMCs suspension mixture to both chambers of the haemocytometer. Both the viable and non-viable cells were counted using a light microscope. Non-viable cells are stained blue, whereas viable ones remain colourless. The percentage of viable cells was calculated.

Cytotoxicity assay

Cytotoxicity of the PHF fractions on PBMCs was determined using WST-8 cell proliferationassay kit (Beijing Solar bio Science and Technology Co. Ltd, China) and the procedure wascarried out according to manufacturers' instructions. The cells (100 µl/well) were seeded in 48-well microplate with RPMI-1640 medium at a concentration of 1 x 10^5 cells/well andincubated at 37^{o}C and a 5% CO_2 humidified atmosphere incubator for 24 h. The cells in the48-well microplate were then treated with different concentrations (25 µg/ml, 50 µg/ml, 100µg/ml, and 200 µg/ml) of the PHF Fractions (A, B, C, D) at 10 µl/well. The treated cells werethen incubated for 4 h at 37^{o}C and a 5% CO_2 humidified atmosphere incubator. After theincubation period, 10 µl of WST-8 reagent was added to the treated cells and also the control.The cells were incubated for 1 h at 37^{o}C and 5% CO_2 humidified atmosphere incubator. Theabsorbance was read using an enzyme-linked immunosorbent assay (ELISA) microplate reader (Rayto-RT-2100C) at 450 nm and percentage cytotoxicity was calculated. All steps for the cytotoxicity test were done in duplicate as outlined by the manufacturer.

Proliferation assay

The proliferation of the PBMCs was determined according to the manufacturer's instructions. PBMCs suspension (100 μl) was dispensed in 48-well microtiter plates at a density of 1 x 10^5 cells/well in RPMI-1640 at 37^{o}C in a 5% CO_2 humidified incubator for 24h.

Phytohaemagglutinin (Sigma-Aldrich® Co. UK) (PHA: 10 µg/ml) was added to each of the PBMCs cultures then 10 μl /well of the PHF fractions (A, B, C, D) at 25 µg/ml concentration (i.e. IC_{50}) was added. The cells were incubated for 4 h at 37°C and 5% CO_2 in a humidified atmosphere incubator. Then 10 µl /well of WST-8 cell proliferation assay reagent (Beijing Solar bio Science and Technology Co. Ltd, China) was added and then incubated for 1 h at 37°C and 5% CO_2 in a humidified atmosphere incubator. PBMCs not treated with the extract, RPMI, FBS, and PBS were considered as controls. All treatments were performed in duplicate. The absorbancewas read using an ELISA microplate reader (Rayto-RT-2100C) at 450 nm. The corrected absorbance is proportional to cell number (proliferation).

Levels of type I interferons

Cell culture supernatants were collected from the microtiter plate wells in clean cryovial tubes. It was centrifuged at 1000 x *g* for 10 min to remove debris and was assayed immediately for the cytokines level. The concentration of the interferons (IFN-α, IFN-β) was measured by quantitative sandwich ELISA technique (Beijing Solarbio Science & Technology Co., Ltd, China). The procedure was carried out with strict adherence to the manufacturer's instructional manual. The assay range for IFN-α and IFN-β was 10.90 pg/ml- 2500 pg/ml and 7.81 pg/ml–500 pg/ml respectively. The assay sensitivity for IFN-α and IFN-β was 20.0 pg/ml and 4.0 pg/ml respectively.

Statistical analysis

The results obtained were entered into SPSS version 25 (IBM, USA) for analysis. Continuous variables were expressed as mean and standard deviation (SD), whereas categorical variables were expressed in percentages. One-way between-groups analysis of variance (ANOVA) with a post hoc test (Bonferroni) was carried out to compare between groups. Independent sample t-test was carried out to compare the mean absorbance of control and treatment on the level of type-I interferons. The $P \leq 0.05$ was used to determine the level of statistical significance.

RESULTS

PHF fractions

The PHF when subjected to Thin Layer Chromatography and Column Chromatography yielded four different fractions according to the type of solvent used in the separation, the fractions extracted were named Fractions A, B, C, and D according to the sequence of collection, fraction A was collected from propanol, fraction B was collected from methanol, fraction C was collected from water and fraction D was collected from acetonitrile.

PBMCs count and percentage viability

The total viable cell count of the isolated PBMCs was 1944 x 10^4 cells/ml while the non-viable total cell count was 80 x 10^4 cells/ml. The percentage of PBMC viability was found to be 96.05%.

Determination of percentage cytotoxicity of the PHF fractions on PBMCs

The minimum inhibitory concentration (IC_{50}) of each of the four fractions was determined using different concentrations of the fractions A, B, C, and D (25 µg/ml, 50 µg/ml, 100 µg/ml, and 200 µg/ml). The concentrations of each of the fractions at 25 µg/ml had the lowest mean percentage cytotoxicity: Fraction A (0.39%), Fraction B (0.43%), Fraction C (0.28%), and Fraction D(2.43%), whereas the concentration of each fraction at 200 µg/ml had the highest mean percentage cytotoxicity: Fraction A (10.51%), Fraction B (13.59%), Fraction C (6.59%), and Fraction D (16.17%). Therefore, the minimum concentration with the lowest cytotoxicity was 25 µg/ml concentration of each fraction (Figure 1).

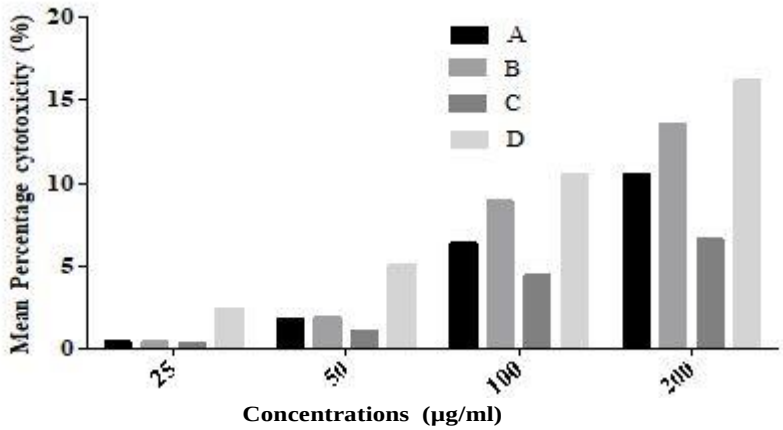

Figure 1: Mean percentage Cytotoxicity of each of the fraction on the PBMCs. The cytotoxicity increased with an increase in concentrations. Fraction C at 25 µg/ml shows the least cytotoxicity on the PBMCs. Whereas Fraction D displayed the highest cytotoxicity at 25 µg/ml.

Effect of different concentrations of PHF fractions on the PBMCs

A one-way between-groups analysis of variance was conducted to explore the effect of an aqueous extract of PHF fractions on PBMCs. As depicted in figure 2, there was a difference in PBMC mean absorbance scores across the different concentrations of the PHF fractions (P<0.0001).

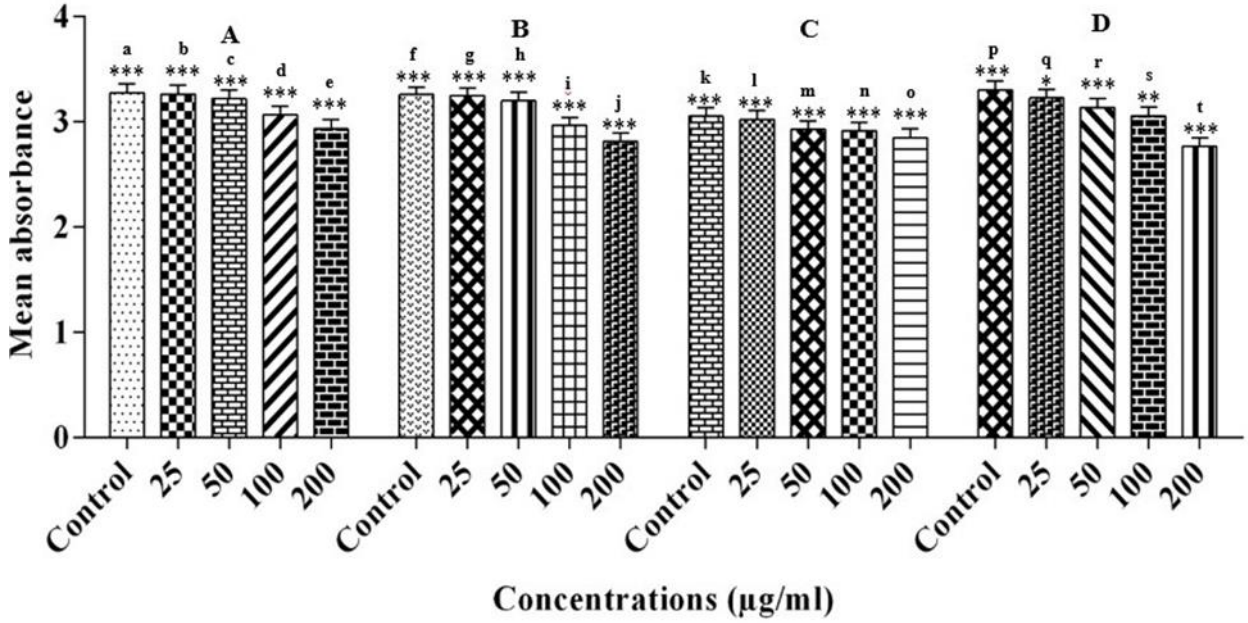

Figure 2: Effect of different concentrations of PHF fractions on the PBMCs. The mean absorbance decreased with an increase in fraction concentrations. Post-hoc comparison shows that all the fractions at 100 and 200 concentrations significantly differ from control. Absorbance is directly proportional to the number of viable cells. a; control vs 100 µg/ml, b; 25 µg/ml vs 100 µg/ml, c; 50 µg/ml vs 200 µg/ml, d; control vs 100 µg/ml, e; 25 µg/ml vs 200 µg/ml, f; control vs 200 µg/ml, g; 25 µg/ml vs 100 µg/ml, h; 50 µg/ml vs 200 µg/ml, i; control vs 100 µg/ml, j; 25 µg/ml vs 200 µg/ml, k; control vs 200 µg/ml, l; 25 µg/ml vs 100 µg/ml, m; 50 µg/ml vs 200 µg/ml, n; control vs 100 µg/ml, o; 25 µg/ml vs 200 µg/ml, p; control vs 200 µg/ml, q; 25 µg/ml vs 100 µg/ml, r; 50 µg/ml vs 200 µg/ml,s; control vs 100 µg/ml, t; 25 µg/ml vs 200 µg/ml. *P= 0.05, **P=0.008, ***P<0.0001. A= fraction A, B= fraction B,C= fraction C, D= fraction D

Effect of different PHF fractions on the proliferation of PBMCs

A one-way between-groups analysis of variance was conducted to explore the effect of aqueous extract of the PHF fractions at 25 µg/ml on the proliferation of PBMCs. As depicted in figure 3, there was significant difference in PBMCs mean absorbance scores across the different fractions of the PHF and control (P<0.0001).

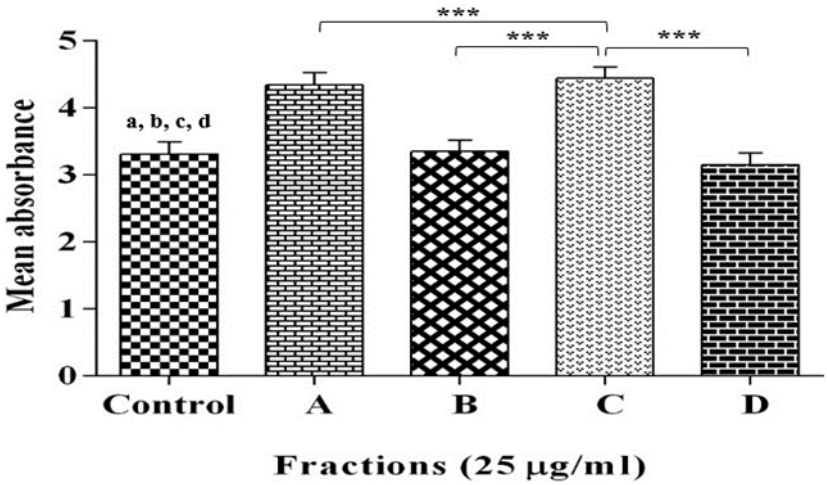

Figure 3: Effect of Different PHF Fractions on Proliferation of PBMCs. Post-hoc comparisons using the Bonferroni test indicated that there was significant difference in the mean absorbance scores for the proliferation of control PBMCs when compared with the different fractions; A, B, C, and D. So also when fraction C was compared with other fractions, there was significant difference. Absorbance is directly proportional to cellular proliferation. a; control vs fractions A (P<0.0001), b; control vs fraction B (P<0.0001), c; control vs fraction C (P<0.0001), d; control vs fraction D (P<0.0001). ***P<0.0001

Effect of PHF fraction C on the level of type -I interferon

An independent samples t-test was conducted to compare the mean concentration of supernatant IFN-α and IFN-β secreted by PBMCs for treatment and control after treatment with 25 µg/ml (IC50) concentration of fraction C. There was significant difference between the treatment mean concentration of the cytokines (IFN-α, IFN-β) and control (P<0.0001) (figure 4).

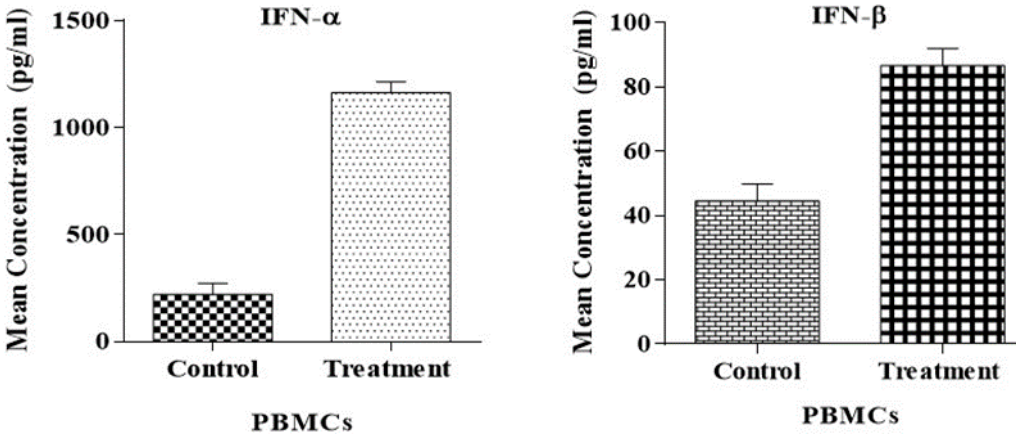

Figure 4: Effect of Fraction C on the Level of Type-1 Interferons. The secretion of the cytokines was significantly increased compared with that of control after treatment of PBMCs with 25 µg/ml of fraction C.

DISCUSSION

Medicinal plants' acceptability for the treatment of pathological conditions is increasing worldwide [23]. So much research is needed to identify the untapped therapeutic potential of the plant's material [24]. For safety purpose, thorough investigations are required on any plant compound aimed for therapeutic use [25]. The percentage cytotoxicity on PBMCs in this study significantly increases with an increase in the concentration of the studied PHF. Cytotoxic

compounds are toxic to cells as they induce undesirable necrosis or apoptosis [26]. We reported 0.28% as the least percentage of cytotoxicity from fraction C at 25 µg/ml concentration. This activity may be attributed to differences in the bioactive component between fraction C and other fractions. However, the lowest concentration (0.0625 mg/ml) of *Commiphora kerstingii* leaves extract exhibit cellular growth inhibition of 52.85% [27]. Some constituents of medicinal plants are potentially toxic [28], as such are not free from toxic effects [29].

This study demonstrated that the PHF fractions have significant immune-proliferative activity on the PBMCs at 25 µg/ml concentration. This suggests the fractions induce cell division amongthe PBMCs. Absorbance is directly proportional to the concentration of the PBMCs. The differencesin absorbance scores may be a result of the variation in bioactive compounds present in the respective fractions, with fraction C containing the bioactive compound responsible for the highproliferative activity. Indeed, the mean absorbance of fractions A, B, and C is reasonably higher than that of the control suggesting the presence of the stimulatory compounds responsible for the said activity. However, fraction D has a lower mean absorbance score compared with the control suggesting low immune-proliferative activity. Cellular proliferation refers to an increase in cell number due to cell division (cytokinesis), this occurs at the final step of the cell cycle. Healthy cells actively proliferate, whereas growth-arrested, senescent, and dead or dying cells do not [30]. *Rhaphidophora korthalsii* methanol extract was shown to stimulate PBMCs proliferation and maintain their viability [31]. Suberosin isolated from *Plumbago zeylanica* has a profound inhibitory effect on the activation and proliferation of human PBMCs stimulated with PHA [32]. Crude extract from the leaf of *Albizia gumifera* inhibits the proliferation of mononuclear cells in a dose-dependent manner [33].

Fraction C of the PHF studied here has the least cytotoxic effect coupled with the highest proliferative activity on the PBMCs, hence selected to be studied for cytokine secretion. Herein, we reported significant upregulation of IFN-α and IFN-β secretions compared with the control, which suggests that the fraction has compound(s) that can induce the secretion of type I cytokines. The same crude extract of the PHF induced the secretion of Th1/Th2 cytokines such as IL-2, IL-4, IL-6, and IFN-γ *in vivo* [18]. IFN-α and IFN-β are both secreted by immune and non-immune cells in response to a viral infection [34,35], with both anti-viral and immunomodulatory activities on target cells [36]. The application of these cytokines in the treatment of viral infection is remarkable [37]. For instance, in the treatment of hepatitis B and C viral infections [38].

Innate cytokine responses, particularly type I interferon, play an important role in coordinating the entire immune reaction to HIV and other pathogenic organisms, and have direct impacts on T cells, altering their functional properties. Understanding these multifaceted cytokine interactions may aid in the identification of valuable drug targets for therapeutics or vaccines [39]. IFN-α provides a therapeutic intervention target for viral infection, able to suppress HIV replication, but not expressed at higher levels in chronic HIV infection due to depletion of their rich source-plasmacytoid DCs (pDC). IFN-α potentiates cell mediate immunity that is deficient in HIV infection [39]. Infection of monocyte/macrophages by HIV induces secretion of IFN-β, which in turn restricts viral replication through blockade of p24 release [40]. Transduction of IFN-β into dendritic cells resulted in resistance to HIV infection and inhibition of HIV transmission [41].

CONCLUSION

Despite extensive research and the discovery of an array of pharmacological agents, human immunodeficiency virus (HIV) infections are becoming refractory to therapy. The use of herbal products for the treatment of HIV is growing in popularity worldwide. Type I interferons were shown to play an important defensive role in viral infections. This study demonstrated significant proliferative activity of Fraction A, B, and C on PBMCs. It revealed significant increase in IFN-α and IFN-β levels induced by selected Fraction C. These activities indicate the fractions possess compound(s) that potentiate the expression of type-I cytokines. Therefore, an in-depth research on the effect of the PHF fractions on individual cells of the PBMCs will provide useful data for therapeutics, also structural analysis to identify the structure of the compound responsible for the bioactivity.

Authors' contribution

Conceptualization and study design ABU, KMH, MHY, MUI, MA, and MUA. Experiments and data acquisition: ABU, KMH, MHY, UZ, RAA, HMA and NKA. Data analysis and interpretation: KMH, ABU, MA, UZ, RAA and MUI. Manuscript drafting and literature search: ABU, KMH, MUI, MUA, MHY, HMA, NKA and MA. All authors critically revised the manuscript and approved the final version for submission.

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Disclaimer
The article has not been previously presented or published.

Conflict of Interest
There are no financial, personal, or professional conflicts of interest to declare.

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