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Original Article

Differential Modulation of IRF1, IRF3 and IRF7 Gene Expression by an n-Hexane Fraction of a Polyherbal Formulation in Human Peripheral Blood Mononuclear Cells: An In-Vitro Study

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Abstract

Background: Interferon regulatory factors (IRFs) coordinate antiviral gene programmes, but the effects of non-polar polyherbal fractions on individual IRF pathways in primary human immune cells remain poorly defined. This exploratory study evaluated the effect of an n-hexane fraction of a polyherbal formulation on IRF1, IRF3 and IRF7 expression in peripheral blood mononuclear cells (PBMCs) from HIV-negative donors.

Methods: PHA-stimulated PBMCs were cultured without treatment or with 50 µg/mL of the n-hexane fraction. Total RNA was extracted, reverse-transcribed and analysed by quantitative reverse-transcription PCR, using GAPDH as the reference gene and the 2^-ΔΔCt method for relative expression. The available dataset comprised four observations per group. Independent-samples t-tests were used, and Welch's result was prioritised when variance homogeneity was violated.

Results: IRF1 expression decreased from 1.06 ± 0.05-fold in untreated cells to 0.50 ± 0.09-fold after treatment (52.7% reduction; $p < 0.001$). IRF3 increased from 1.06 ± 0.05-fold to 2.05 ± 0.63-fold (93.5% increase). The pooled-variance test yielded $p = 0.021$, but the variance-robust Welch test yielded $p = 0.052$. IRF7 changed from 1.06 ± 0.05-fold to 0.96 ± 0.62-fold and was not significant (Welch $p = 0.763$).

Conclusion: The n-hexane fraction produced a gene-specific transcriptional pattern characterised by marked IRF1 suppression, a large but statistically uncertain increase in IRF3 and no clear change in IRF7. These findings identify candidate immunomodulatory activity but do not establish antiviral efficacy. Larger donor-matched, dose-response and time-course studies with MIQE-compliant qPCR reporting, protein-level validation, cytotoxicity assessment and functional viral challenge are required.

Keywords: interferon regulatory factor; IRF1; IRF3; IRF7; innate antiviral immunity; PBMCs; polyherbal formulation; n-hexane fraction; RT-qPCR

Introduction

1. Introduction

Innate antiviral immunity provides the first coordinated cellular response to viral invasion. Recognition of viral RNA or DNA by pattern-recognition receptors, including RIG-I-like receptors, Toll-like receptors and cGAS-STING, activates adaptor proteins and kinases that converge on nuclear factor-κB and interferon regulatory factors (Al Hamrashdi & Brady, 2022). The resulting programme induces type I interferons and interferon-stimulated genes that restrict multiple stages of viral replication and shape subsequent adaptive immunity. (Lazear *et al.,* 2019). IRF1, IRF3 and IRF7 occupy distinct but overlapping positions within this network. IRF3 is constitutively expressed in many cell types and is activated rapidly by TBK1- or IKKε-mediated phosphorylation, dimerisation and nuclear translocation (Wang & Fish, 2019). IRF7 is generally expressed at lower basal levels but amplifies type I interferon production through positive-feedback signalling (Schwanke *et al.,* 2020). IRF1 contributes to constitutive and inducible antimicrobial defence, antigen presentation, apoptosis and inflammatory transcription (Schwanke *et al.,* 2020). Consequently, a compound that increases one IRF while suppressing another should be interpreted as a selective pathway modulator rather than a uniformly stimulatory or suppressive agent (Feng *et al.,* 2021; Ikushima *et al.,* 2013).

Peripheral blood mononuclear cells are useful for exploratory immunomodulation studies because they contain lymphocytes, monocytes and smaller dendritic-cell populations. Their cellular diversity improves physiological relevance compared with a single transformed cell line, but it also introduces donor variability and the possibility that bulk-RNA changes reflect altered cell survival, activation or subset composition. Experimental interpretation therefore depends on careful control of culture conditions, viability, biological replication and reference-gene stability (Ikushima *et al.,* 2013; Mogensen, 2019). Polyherbal formulations contain chemically diverse constituents that may act on multiple host and microbial targets. n-Hexane preferentially extracts lipophilic compounds, including some terpenoids, steroids, fatty acids and other non-polar metabolites (Al Hamrashdi & Brady, 2022). Plant-derived immunomodulators may alter oxidative stress, receptor-proximal kinases, transcriptional activity or cytokine production; however, their composition and activity can vary with species, plant part, geography, processing and extraction conditions. Botanical authentication and chemical standardisation are therefore essential for reproducibility (Balasubramaniam *et al.,* 2024; Song *et al.,* 2025).

The present experiment investigated baseline host-cell modulation in PBMCs obtained from HIV-negative donors. It did not expose cells to HIV-1 and therefore cannot demonstrate anti-HIV activity. This distinction is important because HIV-1 can evade innate sensing, and type I interferon responses may be protective early in infection yet contribute to chronic immune activation during established disease. A gene-expression change in uninfected PBMCs should therefore be regarded as a mechanistic signal that requires confirmation in functional infection models (Chen *et al.,* 2021). This study aimed to determine whether exposure to 50 µg/mL of the n-hexane fraction alters IRF1, IRF3 and IRF7 gene expression in HIV-negative human PBMCs. We hypothesised that the fraction would differentially modulate at least one of the selected IRF genes.

2. Materials and Methods

2.1 Study design

An exploratory in vitro comparative study was conducted using HIV-negative human PBMCs cultured under two conditions: untreated control and treatment with 50 µg/mL of the n-hexane fraction of a polyherbal formulation. The available statistical output contained four observations per condition. The conference abstract treated these observations as independent groups. The biological relationship between conditions must be confirmed because aliquots from the same donors would constitute a paired design.

2.2 Ethical Approval

Ethical approval was obtained from the Human and Biomedical Research Ethics Committee (HBREC) of Usmanu Danfodiyo University, Sokoto (Approval No: HBREC/25/025). The study complied with the Declaration of Helsinki on ethical principles for medical research involving human subjects.

Written informed consent was obtained from all participants, including permission for genetic testing and blood sample use. Participants were assured of confidentiality, and data were anonymized.

All HIV-related work was performed in a certified Biosafety Level 2 (BSL-2) facility with Class II biosafety cabinets. Staff underwent training in handling infectious materials.

2.3 Informed Consent

Written informed consent was be obtained from the all the study participants which include permission for genetic testing relevant to the research. They were made aware that all the information obtained will be treated with confidentiality and would be used for research purposes only and the research will be published.

2.4 Source of n-hexane fraction

The n-hexane fraction was sourced from the Department of Immunology, Usmanu Danfodiyo University, Sokoto, where it had been previously extracted and stored at 5°C in an airtight container. However, the PHF consists of five medicinal plants: *Acacia polyacantha* Willd. (bark), *Bauhinia rufescens* Lam. (bark), *Acacia senegal* (bark), *Adansonia digitata* (leaves), and Allium sativum (bulb).

A stock solution of the n-hexane fraction was prepared in dimethyl sulfoxide (DMSO) to concentrate. The stock was diluted in RPMI-1640 medium supplemented with 10% foetal bovine serum (FBS) and 20 U/mL interleukin-2 (IL-2) to achieve working concentrations of 25 µg/mL and 50 µg/mL, based on preliminary cytotoxicity assays and literature (Dispinseri *et al.,* 2014).

2.5 Blood sample collection

Six millilitres (6 mL) of whole blood were collected from the volunteers; a HIV-negative healthy volunteer, aseptically using EDTA Vacutainer. All collected samples were promptly transported on ice to the Centre for Advanced Medical Research and Training (CAMRET) at Usmanu Danfodiyo University, Sokoto, for PBMCs and HIV isolation.

2.6 PBMCs Isolation Method

The PBMCs were isolated using the Ficoll density gradient method; the procedure was used to isolate PBMCs from HIV negative volunteers. It was carried out according to the manufacturer's instructions as well as described previously by Hamid *et al.* (2021). Three millilitres (3 mL) of Histopaque-1077 (Sigma-Aldrich® Co. UK) were added to a 15 mL Falcon tube and brought to room temperature. Three millilitres (3 mL) of the 6 mL blood were carefully layered onto the Histopaque-1077. Afterwards, 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 a Pasteur pipette into a clean conical centrifuge tube. The cells were washed by adding 10 mL of isotonic phosphate-buffered saline (PBS) solution using a Pasteur pipette and mixed by gently drawing in and out of a Pasteur pipette. The cells were washed by centrifugation at 250 × g for 10 min, and the supernatant was discarded. The cell pellets were suspended in 5 mL of isotonic PBS solution with a Pasteur pipette and were mixed by gently drawing in and out of the pipette. The cells were then centrifuged at 250 × g for 10 minutes. The step was repeated two times; the cell pellets were resuspended in 2 mL of RPMI medium and treated with 10% heat-inactivated foetal bovine serum (FBS) and 10% penicillin streptomycin antibiotics.

2.7 Pre-Treatment Cell Counts

The procedure was conducted strictly according to the manufacturer's instructions and as described

by Hamid *et al* (2021). Ten microliters (10 µL) of 0.4% Trypan blue (Sigma-Aldrich® Co. UK) solution (w/v) were combined with 10 µL of the PBMCs suspension in a cryovial tube (dilution factor = 2) and mixed. It was allowed to stand for 5 minutes in the dark. While the coverslip is in place, 10 µL of the Trypan blue-PBMCS suspension mixture was transferred to both chambers of the improved Neubauer counting chamber using a pipette. The viable and non-viable cells were counted under a light microscope. Non-viable cells appear blue, while viable cells remain colourless. The percentage of viable cells was then calculated using the formula below.

Calculations of percentage yield of viable cells

Cells/ml = the average count per square x dilution factor x 10^4 (Viable)

Total Cells = cells per ml x the original volume of fluid from which the cell sample was removed (Viable) (Appendix VII).

$$\text{Viable cells\%} = \frac{\text{Total number of viable cells}}{\text{Total number of cells (viable and non – viable)}} \times 100$$

2.8 Treatment of PBMCs with n-Hexane Fraction of the PHF

2.8.1 Cell Seeding and Treatment

HIV-infected PBMCs containing 1 × 10^5 cells were suspended in RPMI-1640 medium supplemented with 10% foetal bovine serum (FBS), and 190 µL of the cell suspension was dispensed into each well of a sterile 48-well microtiter plate (Appendix IX). Thereafter, 10 µL of phytohemagglutinin (PHA; 10 µg/mL) was added to each well to serve as a mitogen for PBMC activation. The cultures were incubated at 37°C in a humidified incubator containing 5% CO_2 for 24 hours to activate the PBMCs. After activation, treatments were added in 10 µL volumes according to the experimental grouping to achieve the required final concentrations. The experimental groups were arranged as follows:

Group A: Uninfected PBMCs without treatment (normal control).

Group B: Uninfected PBMCs treated with 10 µL of 50 µg/mL n-hexane fraction.

All experimental groups were set up in quadruplicate to ensure reproducibility and statistical reliability. The plates were subsequently incubated for an additional 24 hours at 37°C in a humidified atmosphere containing 5% CO_2. At the end of the incubation period, the treated PBMCs and controls were harvested aseptically for downstream molecular and protein analyses.

2.9 Determination of Gene Expression

2.9.1 Primer design

Primers for the target genes were designed using Primer-BLAST, with in silico validation for specificity using BLAST. The RNA sequences for the IRF1, IRF3, IRF7 and APOBEC3G genes was retrieved from the National Centre for Biotechnology Information (NCBI) database. Primer efficiency was assessed using a standard curve, and acceptable efficiencies fall within 90–110% as described by Rossi *et al.* (2019).

2.9.2 Extraction of mRNA

The mRNA extraction was carried out according to the procedure described by the manufacturer (Hunan Run Mei Gene Technology Co., Ltd, China). Two hundred microlitre (200 µL) of PBMC suspension was taken and placed in a clean Eppendorf tube. Then 200 µL of Lysis Solution was added to the tube, vortexed and shaken for 10 seconds. Afterwards, the tube was left to stand at room temperature for 10 minutes. Then, 200 µL of absolute ethanol was added to the tube, followed by vortexing and shaking for another 10 seconds. Afterwards, the Adsorption Column was inserted into a collection Tube. All the liquid from the centrifuge tube was transferred to the Adsorption Column, which was centrifuged at 8000 rpm for 1

minute. The filtrate was discarded. Afterwards, 600 µL of Washing Buffer I (with absolute ethanol added) was added to the Adsorption Column. It was then centrifuged at 8000 rpm for 1 minute, and the filtrate was discarded. Afterwards, 600 µL of Washing Buffer II (with absolute ethanol added) was added to the Adsorption Column. It was centrifuged at 8000 rpm for 1 minute, and the filtrate was discarded. Afterwards, the Adsorption Column was returned to the Collection Tube and centrifuged at 8000 rpm for 2 minutes. Afterwards, the filtrate and the Collection Tube were discarded. Afterwards, the adsorption Column was placed into an RNase-free 1.5 mL centrifuge tube, and 50 µL of Elution Solution was added to the centre of the membrane and left at room temperature for 1 minute. The column was centrifuged at 8000 rpm for 1 minute, then discarded. The nucleic acids were collected in the 1.5 mL centrifuge tube and stored at -20 °C as described by Rossi *et al.* (2019).

2.9.3 Determination of RNA Concentration and Purity

The concentration and purity of the extracted mRNA were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Briefly, the instrument was initialized and blanked using 2 µL of RNase-free water. Thereafter, 1 µL of the extracted RNA sample was carefully loaded onto the measurement pedestal, and the absorbance was measured at wavelengths of 280 nm. The RNA concentration was automatically calculated by the instrument and expressed in nanograms per microlitre (ng/µL) based on the absorbance at 280 nm. The purity of the extracted RNA was assessed using the A260/A280 absorbance ratio. Samples with A260/A280 ratios between 1.8 and 2.1 were of acceptable purity for downstream molecular applications. Samples exhibiting acceptable purity ratios were selected for subsequent complementary DNA (cDNA) synthesis and gene expression analysis. The quantified RNA samples were stored at -80°C until further use as described by Rossi *et al.* (2019).

2.9.4 Synthesis of cDNA and Relative Quantitative RT-PCR

The cDNA synthesis was performed using TranScript® Green One-step qRT-PCR Super Mix (SYBR Green) (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. Quantitative RT-PCR was conducted using gene-specific primers for IRF1, IRF3, IRF7, and GAPDH. Fold change was calculated using the $2^{-\Delta\Delta Ct}$ method s described by Rossi *et al.* (2019).

2.9.5 The cDNA synthesis and quantitative RT-PCR analysis.

In an Eppendorf tube, qPCR reaction components were mixed to make the recommended 20 µL reaction system cocktail. The components of the reaction mixture included mRNA template from each fraction (1 pg), forward primer (0.4 µL), reverse primer (0.4 µL), TransScript® Green One Step-RT/RI Enzyme Mix (0.4 µL), 2× PerfectStart™ Green One-step qPCR SuperMix (10 µL), and RNase-free water (8 µL). The mixture was transferred into 0.2 mL PCR tubes. The tube was placed in a PCR machine to run (Rossi *et al.,* 2019).

2.10 Outcome measures and statistical analysis

The primary outcomes were relative fold-expression values for IRF1, IRF3 and IRF7. Data were summarised as mean ± standard deviation (SD). The submitted analysis used independent-samples t-tests and Levene's test for equality of variances, with two-sided $\alpha = 0.05$. When Levene's test was significant, the Welch unequal-variance result was treated as primary. Percentage change and Hedges' g were calculated to describe magnitude. No multiplicity adjustment was applied because this was an exploratory analysis. Raw Ct values and donor identifiers were unavailable; therefore, normality, outliers, technical replicate variability, reference-gene stability and donor pairing could not be reassessed.

Table 1: List of primer sequences used

Gene product mRNA		Primer sequence	Sequence position		GC (%)	Tm	Amplicon size (bp)
			Start	Stop			
IRF 1	F	5' CAAGGCCAAGAGGAAGTCAT 3'	633	653	50	62	112
	R	5' CATGTAGCCTGGAACTGTGTAG 3'	723	745	50	62	
IRF 3	F	5' TATGTTCCCGGGAGGGATAAG 3'	425	446	52.4	63	89
	R	5' GCTAAACGCAACCCTTCTTTG 3'	493	514	47.6	62	
IRF 7	F	5' ACACACACATGCTGGACTC 3'	883	902	52.6	62	148
	R	5' CTTGGTTGGGACTGGATCTG 3'	1011	1031	55	62	
GAPDH	F	5'CGGATTTGG TCGTATTGG 3'	343	361	50	58	97
	R	5'GTTGAGGTCAATGAAGGG 3'	422	440	50	57	

3. Results

3.1 Overall expression pattern

Untreated cultures had a reported mean relative expression of 1.0575-fold for each gene (SD 0.0506). After treatment, IRF1 decreased to 0.5006 ± 0.0854-fold, IRF3 increased to 2.0462 ± 0.6325-fold and IRF7 was 0.9551 ± 0.6188-fold. The pattern therefore comprised strong IRF1 downregulation, a large upward shift in IRF3 and little overall change in IRF7 (Table 2 and Figure 1).

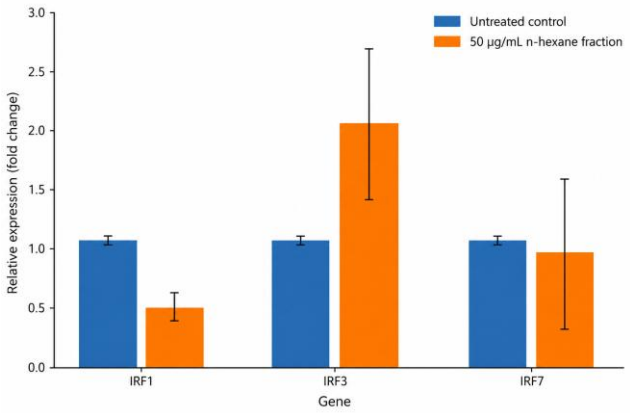

Figure 1: Relative expression of IRF1, IRF3 and IRF7 in untreated PBMCs and PBMCs treated with 50 µg/mL n-hexane fraction. Bars show mean ± SD.

3.2 Gene-specific comparisons

IRF1 expression was 0.5569-fold lower in treated than untreated cultures. Variance homogeneity was not rejected (Levene p = 0.425), and the pooled t-test showed a statistically significant difference (t = 11.22 for control minus treatment, df = 6, p < 0.001). The pooled 95% confidence interval for treated minus control was approximately −0.678 to −0.435. The estimated Hedges' g was −6.90, although this unusually large, standardised effect is inflated by the very low control-group variance.

IRF3 expression was 0.9887-fold higher after treatment. Variability was substantially greater in treated cultures, and Levene's test was significant (p = 0.011). The pooled test gave p = 0.021, whereas Welch's test gave t = −3.12, df = 3.04 and p = 0.052. The Welch 95% confidence interval for treated minus control ranged from −0.014 to 1.991. Hedges' g was 1.92. The result therefore represents a large, biologically notable but statistically uncertain upward signal. IRF7 expression was 0.1024-fold lower after treatment. Levene's test indicated unequal variances (p = 0.045), and Welch's test showed no evidence of a difference (t = 0.33, df = 3.04, p = 0.763).

Table 2: Relative IRF gene expression in untreated and treated HIV-negative PBMCs

Gene	Untreated control, mean ± SD	50 µg/mL n-hexane fraction, mean ± SD	Percentage change	Levene's *p* value	Primary *p* value
IRF1	1.0575 ± 0.0506	0.5006 ± 0.0854	−52.7%	0.425	<0.001[a]
IRF3	1.0575 ± 0.0506	2.0462 ± 0.6325	+93.5%	0.011	0.052[b]
IRF7	1.0575 ± 0.0506	0.9551 ± 0.6188	−9.7%	0.045	0.763[b]

Note: n = 4 observations per group. Welch's test is primary for IRF3 and IRF7 because Levene's test indicated unequal variances. The conference abstract reported IRF3 p = 0.021 from the pooled-variance row.

Table 3: Effect estimates for IRF gene expression in n-hexane fraction-treated versus untreated HIV-negative PBMCs

Gene	Mean Difference Treated − Control	Statistical test	*t* (df)	95% CI for mean difference	Hedges' *g*
IRF1	−0.5569	Student's *t*-test[a]	−11.22 (6.00)	−0.678 to −0.435	−6.90
IRF3	+0.9887	Welch's *t*-test[b]	+3.12 (3.04)	−0.014 to 1.991	+1.92
IRF7	−0.1024	Welch's *t*-test[b]	−0.33 (3.04)	−1.083 to 0.878	−0.20

Note: CI, confidence interval; df, degrees of freedom. All differences and effect-size signs are expressed as treated minus untreated control. Positive values indicate higher expression following treatment, negative values indicate lower expression. [a] Equal variances assumed because Levene's test was not significant. [b] Equal variances were not assumed because Levene's test indicated heterogeneity of variance. Hedges' *g* estimates should be interpreted cautiously because each group contained only 4 observations and the untreated-control variance was unusually low.

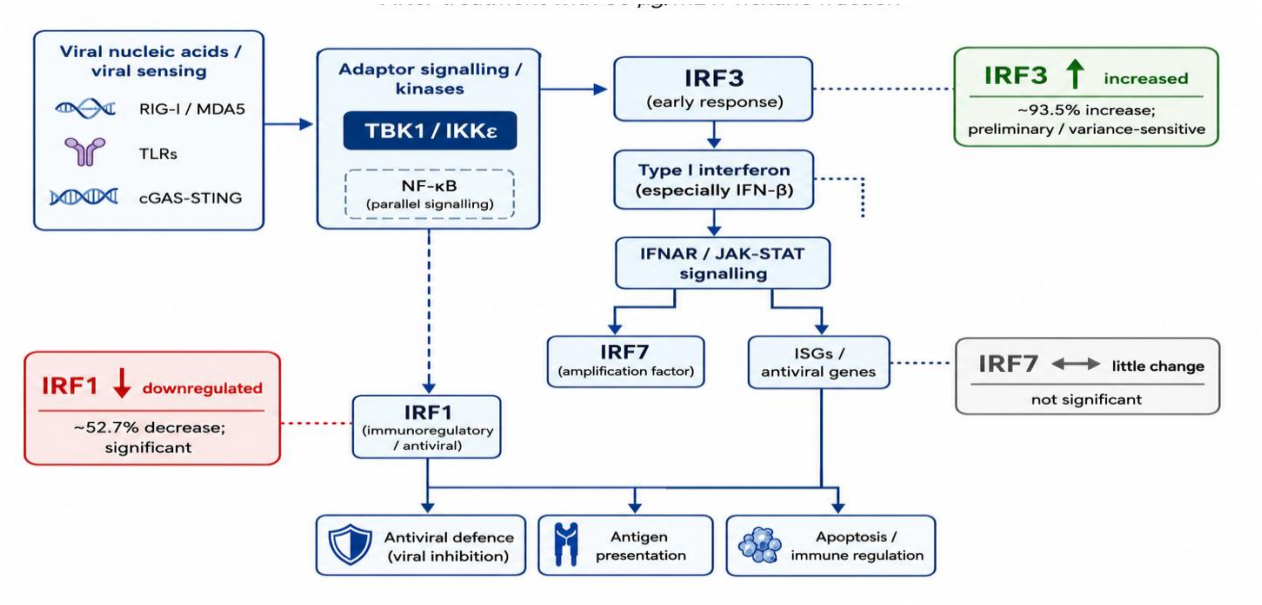

Figure 2: Simplified relationship of IRF1, IRF3 and IRF7 within antiviral signalling and the preliminary transcriptional pattern observed after treatment.

4. Discussion

This exploratory study found that the n-hexane fraction did not produce uniform activation of the three interferon regulatory factors examined. IRF1 was strongly suppressed, IRF3 showed a large but variance-sensitive increase and IRF7 remained broadly unchanged. The findings are therefore most consistent with selective transcriptional modulation rather than general stimulation of the type I interferon system.

The upward shift in IRF3 is potentially relevant because IRF3 is a sensor-proximal regulator of early antiviral signalling. Nevertheless, increased IRF3 mRNA does not demonstrate pathway activation. IRF3 activity depends on phosphorylation, dimerisation, nuclear translocation and interaction with transcriptional co-activators. Confirmation requires measurement of total and phosphorylated IRF3, nuclear localisation, IFN-β secretion and downstream interferon-stimulated genes such as MX1, OAS1, ISG15 and IFITM3 (Al Hamrashdi & Brady, 2022; Schwanke *et al.,* 2020).

The statistical interpretation of IRF3 is important. Although the pooled-variance row yielded $p = 0.021$, the treated-group SD was more than twelve times the control SD and Levene's test rejected variance homogeneity. Welch's $p = 0.052$ is therefore the more defensible primary result. This does not eliminate the observed biological signal; rather, it shows that the estimate is imprecise in a dataset of only four observations per condition. Reporting the effect estimate, confidence interval and individual biological replicates is more informative than classifying the result solely by a 0.05 threshold.

IRF1 decreased by approximately half and remained statistically significant in the supplied analysis. IRF1 supports antimicrobial defence, antigen processing and presentation, apoptosis and inflammatory transcription. Its suppression could weaken some protective responses, but it could also reflect regulatory or cytoprotective activity under a specific activation state. Without downstream gene, protein and functional measurements, the direction of biological benefit cannot be assigned (Feng *et al.,* 2021; Mogensen, 2019).

The absence of a clear IRF7 response may represent genuine selectivity, inadequate power or inappropriate sampling time. IRF7 often participates in a later positive-feedback phase of type I interferon induction; a single endpoint may therefore miss transient or delayed changes. Time-course sampling and multiple concentrations are needed to determine whether the fraction induces an early IRF3-centred response without subsequent IRF7 amplification (Ma *et al.,* 2023).

Changes in bulk PBMC RNA may also arise from altered cell viability, activation or subset composition. PHA promotes T-cell activation and proliferation, while an n-hexane fraction may affect lymphocyte and monocyte populations differently. Flow-cytometric immunophenotyping and formal cytotoxicity assays are required to distinguish direct transcriptional regulation from selective survival or expansion of PBMC subsets.

Chemical complexity is another major consideration. A non-polar fraction may contain multiple constituents with synergistic, additive or opposing effects. Bioassay-guided fractionation, chromatographic fingerprinting and testing of purified candidate compounds will be required to identify the active components and ensure batch consistency. Botanical authentication and extraction details are not optional reporting elements; they are necessary for scientific reproducibility and translational development (Balasubramaniam *et al.,* 2024; Song *et al.,* 2025).

The use of HIV-negative PBMCs establishes baseline host-cell effects but does not support claims of anti-HIV activity. Future experiments should expose donor-matched cultures to a controlled viral challenge or validated viral mimetic and measure viral replication alongside cellular viability. In an HIV model, relevant endpoints could include p24 antigen, viral RNA, proviral DNA and host restriction factors such as APOBEC3G, MX2, tetherin and SAMHD1. Appropriate antiretroviral and vehicle controls should be included.

RT-qPCR reporting must also be strengthened. GAPDH is frequently used as a reference gene, but activation and phytochemical exposure may change metabolic transcription. MIQE and MIQE 2.0 recommend validating reference-gene stability under the exact experimental conditions, reporting assay efficiency and specificity, distinguishing biological from technical replication and providing sufficient raw-data quality information for reproducibility (Bustin *et al.,* 2009, 2025; Livak & Schmittgen, 2001).

The principal limitation is the very small number of observations. Variance estimates are unstable, confidence intervals are wide and a single extreme value could alter the result substantially. The identical control means and SDs for all three genes should be checked against the raw dataset. In addition, uncertainty about donor pairing may affect the statistical model; if each donor supplied both untreated and treated aliquots, paired analysis is required. The single dose, single time point, absence of protein validation, lack of viability and PBMC-subset data, incomplete formulation characterisation and no adjustment for testing three genes further limit inference.

Despite these constraints, the use of primary human PBMCs and the gene-specific pattern provide a clear basis for follow-up. The most testable hypothesis is that one or more lipophilic constituents preferentially influence an IRF3-associated early signalling programme while suppressing IRF1-dependent transcription and leaving IRF7 amplification relatively unaffected. This hypothesis should be evaluated using a larger donor-matched design with chemical, cellular, molecular and functional endpoints.

5. Conclusion

Exposure of HIV-negative human PBMCs to 50 µg/mL of an n-hexane fraction of a polyherbal formulation produced differential IRF gene expression. IRF1 was strongly downregulated, IRF3 showed a large but statistically uncertain increase when unequal variance was considered, and IRF7 was not materially altered. The findings identify preliminary immunomodulatory activity but do not demonstrate antiviral efficacy. Confirmation requires larger donor-matched studies, multiple doses and time points, complete botanical and chemical standardisation, MIQE-compliant RT-qPCR, viability and immunophenotyping assays, protein-level pathway measurements and functional viral challenge experiments.

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