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Evident stabilization of the clinical profile in HIV/AIDS as evaluated in an open label clinical trial using a polyherbal formulation

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***Background & objectives*: The complementary and alternative medicines (CAM) have not been systematically evaluated for the management of HIV/AIDS patients. In a prospective, single-site, open-label, non-randomized, controlled, pilot trial, we evaluated a polyherbal formulation (PHF) for its safety and efficacy in treating subjects with HIV-AIDS.**

***Methods*: A total of 32 and 31 subjects were enrolled under the PHF and highly active antiretroviral treatment (HAART) arms, respectively, and followed up for a period of 24 months. Plasma viral RNA, CD4 cell count and blood chemistry were monitored at 3-month intervals. Following mid-term safety evaluation, 12 subjects from the PHF arm were shifted to HAART and were followed separately as PHF-to-HAART arm, for the rest of the period.**

***Results*: The HAART arm was characterized by significant improvements in CD4 cell count (154.4 cells/µl/year, $P<0.001$) and reduction in plasma viral load within 3 to 6 months (-0.431 ± 0.004 $\log_{10}$ IU/month, $P<0.001$). In contrast, the PHF arm showed a profile of CD4 cell loss at remarkably slower kinetics (14.3 cells/µl/year, $P=0.021$) and insignificant reduction in the viral load. The PHF and HAART arms did not differ significantly in the occurrence of AIDS-related illnesses over the study period of 24 months. In the PHF-to-HAART arm, the rates of CD4 count and reduction in viral load were significant and comparable to that of the HAART group. In the PHF arm, at 1 month, a significant increase in CD4 cell count and a concomitant decrease in viral load were seen.**

***Interpretation & conclusions*: The PHF appears to have provided protection by delaying the kinetics of CD4 cell reduction. Given the several study limitations, drawing assertive inferences from the data is challenging. Future studies with a stringent study design are warranted to confirm these findings.**

Key words Chronic immune activation - complementary and alternative medicine - disease progression - HIV-AIDS - human clinical trial - immunomodulation - polyherbal formulation

Despite success in improving clinical prognosis, the application of highly active anti-retroviral therapy (HAART) for HIV/AIDS suffers from several limitations including drug resistance, toxicity, adherence, treatment duration, high costs, limited immune reconstitution and lack of viral eradication. Further, in the absence of an effective vaccine, complementary and alternative medicines (CAM) have become an alternative option to vast majorities of people in industrialized as well as resource-constrained countries regardless of the economic and cultural differences[1]. Only a few clinical trials have been conducted to evaluate the immunomodulatory properties of CAM and herbal medicines for HIV/AIDS. Most of these clinical trials were limited in terms of study design, allocation concealment, high rates of loss to follow up, lack of pre-defined end points, inadequate drug description, small study size and short duration, and absence of rigorous statistical evaluation, *etc*[2,3].

India is native for several systems of CAM including *Ayurveda, Siddha*, *Unani* and *Yoga* in addition to the popular use of Homeopathy and Naturopathy and many others[4,5]. Several thousands of medicines presently in use and listed in modern pharmacopoeia are of herbal origin with many of these having anti-microbial and/or immuno-modulatory properties[6]. The pathogenesis of HIV-1 infection is significantly associated with the loss and/or dysfunctioning of the CD4 T-cell which is believed to be the consequence of chronic activation of the immune system[7]. Any therapeutic strategy that could alleviate the immune activation, therefore, must confer significant clinical benefit in HIV/AIDS.

We report here the evaluation of an Indian polyherbal formulation (PHF), a unique herbal extract and mineral combination devoid of heavy metals, in a pilot clinical trial, for its safety and efficacy for treating patients with HIV-AIDS. We also report findings on stabilization of CD4 count and viral load and clinical progression in study participants during a follow up of 24 months.

Material & Methods

Study participants: All the study participants were recruited, counseled, and monitored at the Seva Free Clinic which provides free clinical services in the city of Bangalore, Karnataka, India, under the auspices of *Samraksha,* a non-governmental organization (NGO). The potential volunteers were subjected to four or five rounds of counselling by trained counsellors at the Seva Free Clinic and admitted to the study after obtaining an informed consent form. The study participants were balanced at baseline for several characteristics including gender, age group, location, income, occupation, education and marital status (Table I). A total of 475 potential volunteers were screened for CD4 count and 63 of them were qualified by the selection criteria. The study participants, all hailing from the communities within and around the city of Bangalore, were regular attendees receiving medical attention at the *Seva Free Clinic*. The study participants have been recruited in several batches over a period of 9 months (Fig.1).

*Trial characteristics***:** In a prospective, single-site, open-label, non-randomized, controlled, investigator-blinded trial, polyherbal formulation (PHF) was compared with highly active anti-retroviral therapy (HAART) for safety and efficacy in treating HIV/AIDS. Participants in both arms of the study, 31 and 32 under HAART and PHF, respectively, were balanced at baseline for several parameters (Table I). The major inclusion criteria were a CD4 count between 200-250 cells/µl and the subjects being drug naïve and free of presenting AIDS-related clinical symptoms. The major exclusion criteria were the presence of opportunistic infections and complications including dementia, signs of acute systemic illness, prior antiretroviral treatment, pregnancy and age below 18 years. During the first year of the study, only two arms of intervention were examined. After midterm safety evaluation, the PHF arm was further divided into two subgroups (Fig. 1) and

Table I. Baseline characteristics of the study participants

Characteristic	HAART (n=31) No. (Range or %)	PHF (n=32) No. (Range or %)
Age, yr	36 (27-50)	34 (26-46)
Female gender	15 (48)	12 (38)
Education, high school and above	15 (48)	15 (47)
Employed	31 (100)	30 (94)
Income, > ₹3,000	11 (35)	16 (50)
Marital status, married	22 (71)	18 (56)
Location, urban dwellers	23 (74)	24 (75)
CD4 count, cells/µl	226 (140-329)	222 (159-313)
Plasma viral load (log_{10} IU/ml)	4.6 (2.3-5.9)	4.8 (3.7-6.6)

HAART, highly active antiretroviral therapy; PHF, polyherbal formulation

all the three arms were followed up for the second year of the study. At mid-term safety evaluation, performed approximately 18 months after study initiation and 6 months after recruitment of the last volunteer, it was noted that in some of the participants under the PHF arm, the CD4 counts dropped below 200 cells/µl although there were no clinical manifestations in any of the study participants. Following the institutional ethics committee (IEC) directive, the study participants were informed of this development, counselled to shift to HAART and the decision was left to each participant whether to continue in the PHF arm or to be shifted to HAART. Twelve of the 28 participants of the PHF arm decided to be shifted to HAART and one of them died shortly. Clinical profile of these 12 PHF subjects who have been shifted to HAART has been summarized (Table II). Of the remaining 16 participants who continued in the PHF arm, six had CD4 cells below 200 cells (S006, S103, S170, S177, S237 and S410). Eventually, three of these six participants (S103, S237 and S410) dropped out of the study voluntarily while the other three continued. At mid-term evaluation, three of the 13 participants (S006, S170 and S177) had a CD4 count of approximately 180 cells/µl. After the interim safety analysis, co-trimoxazole prophylaxis was provided to several study participants as per the standard clinical practice.

While the study participants, clinicians and the counselors were in the knowledge of the group allocation, the research laboratory at Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR), Bangalore, responsible for measuring CD4 cell count and viral load was blinded until unblinding was performed. The protocols, donor information brochures, any amendments and patient consent forms were approved by the Institutional Ethics Committee of the NGO *Samraksha* which monitored the progress of the clinical trial (CTRI No. CTRI/2008/091/000021). The IEC of JNCASR also approved the study. Given that there was no other similar study earlier and that clinical benefits accrued by this formulation were not known, the number of study participants and effect size

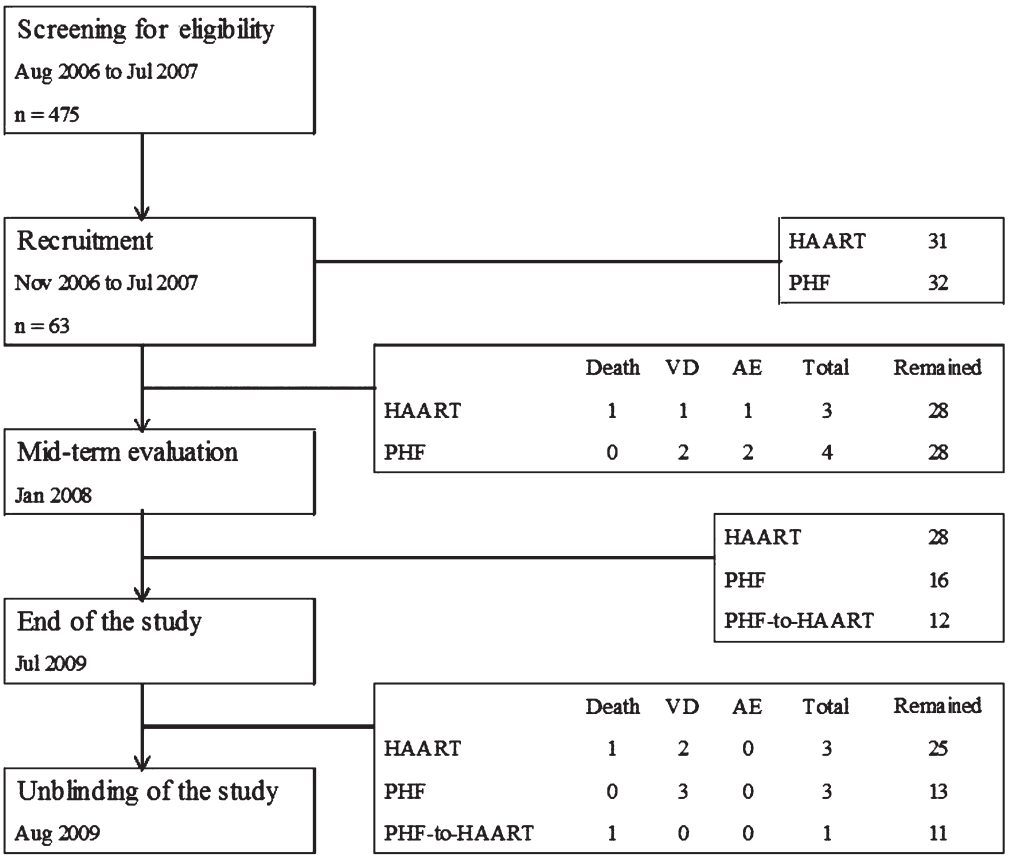


Fig. 1. Trial profile. PHF, polyherbal formulation; VD, voluntary dropout; AE, adverse event; HAART, highly active antiretroviral treatment.

Table II. Clinical, immunological and virological profiles of the 12 PHF-to-HAART participants

Subject Id	Time of shifting (months)	CD4 cell count (cells/µl)		pVL (log_{10}) copies/ml	
		Baseline	At shifting	Baseline	At shifting
S010	15	162.4	113.8	4.97	4.34
S017	21	195.9	190.1	5.00	4.74
S042	18	199.3	185.0	4.83	4.34
S064	12	159.0	174.1	4.98	5.28
S071	15	159.0	54.4	4.73	4.34
S135	18	214.6	179.1	4.08	3.56
S179	15	251.3	207.5	4.18	3.69
S189*	18	251.4	165.4	5.78	5.64
S220	12	171.5	133.7	4.88	5.08
S225	15	255.8	158.5	4.15	4.26
S235	9	247.0	209.7	5.08	4.68
S375	9	182.8	138.3	5.04	5.26

*died soon after switching to HAART; pVL, plasma viral load

required for power calculations could not be calculated. It was decided to include 30 participants in each arm of the study hoping that withdrawal rate would be minimal given the involvement of the NGO. The identity of the participants was protected during the study and after it ended. After the clinical trial was formally concluded, all the study participants of the antiretroviral arms were transferred to the National ART programme sponsored by the Government of India.

Medicines and treatment administration: The HAART arm primarily received the administration of 30 mg of stavudine, 150 mg of lamivudine and 200 mg of nevirapine, twice a day as per the 2004 guidelines of The National AIDS Control Organization (NACO), India[8]. Alternative regimens, with nevirapine substituted with efavirenz/indinavir or stavudine replaced by zidovudine were used as required.

The PHF procured from Vedic Drugs Pvt. Ltd., India, consisted of ingredients from 58 different plant species (Table III) and formulated into 6 primary formulations (F_{BP}, F_{TP}, F_{KC}, F_{MC}, F_{CK} and F_{PB},) and five supplementary formulations (F_{AJ}, F_{AD}, F_{AZ}, F_{OT} and F_{SWT}). The drug administration schedule of the PHF has been depicted (Table IV). The polyherbal formulations were administered orally only for 4 months following recruitment. Study participants were counselled on the use of the PHF and provided quantities of medicines sufficient between visits to the clinic. To circumvent the generic problem of the highly variable nature of the herbal formulations, a single lot of PHF was used for the entire clinical trial, drug toxicity testing, quality analyses and *in vitro* virus inhibition assays.

Adherence and clinical management: Adherence to antiretroviral treatment was monitored by pill count method by checking the number of pills brought back by the participants. More than 95 per cent adherence was found among the study participants. Administration of the antiretroviral medicine was monitored by three trained Allopathy physicians throughout the study period. Side effects in the HAART arm were managed according to guidelines of NACO (National Guidelines on Second-line ART for adults and adolescents 2008) [8] by one of the investigators (KSS). At least one of a team of seven physicians affiliated with the Shanmugha Arts, Science, Technology and Research Academy (SASTRA) University, Tanjavur, Tamil Nadu, India was available at the Clinic to monitor the clinical trial throughout the duration of the study. Minor complications in the PHF arm were managed by the attending CAM clinicians and serious manifestations were referred to KSS. The costs of treatment under both of the arms and other incidental costs were borne by the research project.

Unblinding of the study: The study was unblinded on August 8, 2009 in the presence of an Unblinding Committee consisting of three members not related to study. Hard copies of the data sheets containing raw CD4 counts and viral load values from the JNCASR laboratory and the allocation code from The Seva Free Clinic were presented to the Committee members. Each of the original data sheets and the clinical code were attested by all the Committee members and placed in the possession of the Committee Chairman.

Drug toxicity testing: Drug toxicity testing for the PHF was performed by Sri Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvanathapuram, Kerala, India. Three different tests - acute oral toxicity, sub-acute oral toxicity and sub and chronic oral toxicity tests - performed in rats confirmed the non-toxic nature of the polyherbal formulation (data not shown).

Study end points: The primary end points of the study were CD4 count and the plasma RNA viral load. Secondary end points were manifestation of AIDS-related clinical symptoms including opportunistic

Table III. Plant species that constituted six primary and five supplementary polyherbal formulations

S.No.	Name of the plant	Common name	Family	Part	Dosage (mg)										
					Primary formulations						Supplimentry formulations				
					BP	TP	KC	MC	CK	PB	AJ	AD	AZ	OT	SWT
1	*Abies webbiana*	East Himalayan silver fur	*Pinaceae*	Whole plant			275								
2	*Aloe barbadensis*	Kui Aloe Vera	*Xanthorrhoeaceae*	Leaf pulp				**150**		**3000**					
3	*Anacyclus pyrethrum*	Pellitory	*Asteraceae*	Roots/ leaves, flowers								+		+	
4	*Argemone Mexicana*	Brahm dandi	*Papaveraceae*	Seeds						400					
5	*Azadirachta indica*	Neem	*Meliaceae*	Fruit, Bark, Leaf, Twigs						**400**					
6	*Bambusa arundinaceae*	Bambusa salt manna	*Poaceae*	Inner portion of stem			250				100				
7	*Borago officinalis*	Ox tongue	*Boraginaceae*	Leaves / flowers							300				
8	*Carum carvi / Cuminum cyminum*	Cumin seeds	*Apiaceae*	Seed					190						
9	*Caryophyllus aromaticus*	Clove	*Myrtaceae*	Flower							200				
10	*Cassia angustifollia*	Swarnmukhi patra	*Caesalpinaceae*	Leaf (Dry)	600	700	350			400					3000
11	*Cinnamomum zeylanicum*	Cinnamon Bark	*Lauraceae*	Bark			300				200				
12	*Citrus vulgaris Schard*	Kalinda	*Cucurbitaceae*	Seed	400										
13	*Colchicum luteum*	Suranjana	*Colchicaceae*	Corm/ tuber		450	100								
14	*Coriandrum sativum*	Choriander seed	*Apiaceae*	Seeds					150	**400**					
15	*Crocus sativus*	Saffron	*Iridaceae*	Stamen							200				
16	*Cucumis melo*	Sweet mask melon	*Cucurbitaceae*	Seed	400										
17	*Cucumis sativas*	Tikta karkatika	*Cucurbitaceae*	Seed	400				475						
18	*Curcuma longa*	Turmeric	*Zingiberaceae*	Rhizome					550	**400**					
19	*Curcuma zedoaria*	Round Zedoary	*Zingiberaceae*	Rhizome					175		450				
20	*Cyperus rotundus/ C. scariosus*	Nut grass tubers	*Cyperaceae*	Rhizome					375						
21	*Dalbergia nigra /D. sisso*	Rosewood	*Fabaceae*	Heart wood						**400**					
22	*Diospyros ebenum*	Ebony	*Ebenaceae*	Heart wood						**400**					
23	*Eclipta alba*	Garga – Bringaraj	*Asteraceae*	Whole plant					250						
24	*Elettaria cardamomum*	Cardamoms	*Zingiberaceae*	Fruit (Dry)	300		65		125		200				
25	*Embelia ribes*	Baebadang	*Myrsinaceae*	Fruit						400					

Contd...

S.No.	Name of the plant	Common name	Family	Part	Dosage (mg)										
					BP	TP	KC	MC	CK	PB	AJ	AD	AZ	OT	SWT
27	*Foeniculum vulgare*	Mishreya	*Apiaceae*	Fruit (Dry)	1200	900	450			400	500				1000
28	*Fumaria officinalis*	Common fumitory	*Fumariaceae*	Areal parts								+	+		
29	*Fumaria parviflora*	Shatera	*Fumariaceae*	Root			**650**			400					
30	*Glycyrrhiza glabra*	Liqourice	*Fabaceae*	Roots	**500**	**500**			**300**		700				4000
31	*Hyoscyamus niger linn*	Khurasani ajwayan	*Solanaceae*	Seeds				100							
32	*Juglans regia linn*	Wallnut	*Juglandaceae*	Fruit (Dry)	300										
33	*Lawsonia inermis*	Henna	*Lythraceae*	Leaf						**400**					
34	*Mentha piperita*	Pudina	*Lamiaceae*	Leaves								+	+		
35	*Myristica fragrans*	Nutmeg	*Myristicaceae*	Fruit/ seeds							350				
36	*Nardostachya jatamansi*	Musk root	*Valerianaceae*	Rhizome					275						
37	*Pimpinella anise/P. anisum*	Sweet fennel aniseed	*Apiaceae*	Fruits							400				
38	*Piper longum*	Pippali	*Piperaceae*	Dried Inflo-resence / Fruit			200		225						
39	*Piper nigrum*	Black pepper	*Piperaceae*	Fruit			300	200							
40	*Prunus amygdalus satch*	Badam	*Rosaceae*	Seed	300	350	135								
41	*Psoralea corylifolia*	Bavanchi	*Fabaceae*	Seeds						400					
42	*Rosa centifolia / Damoascena shathapatri*	Sata rose petals	*Rosaceae*	Petals		350	200			**400**					2000
43	*Ruta chalepensis Linn / R. graveolens*	Garden common rue	*Rutaceae*	Leaves									+	+	
44	*Saccharum officinarum*	Sugar	*Poaceae*	Kanda Saara	600	1000	1500								
45	*Santalum album*	Sandalwood	*Santalaceae*	Heart Wood					450	400	200				
46	*Saussurea lappa*	Khost /Kuth	*Asteraceae*	Rhizome		350	75								
47	*Sphaeranthus indicus / S. hirtus*	East Indian globe thist	*Asteraceae*	Whole plant						**400**					
48	*Swertia chirata*	Chirayata	*Gentianaceae*	Herb						**400**					
49	*Tephrosia purpurea*	Sharapunkha	*Fabaceae*	Whole plant						**400**					
50	*Terminalia arjuna/ Galega purppure*	Arjuna	*Combretaceae*	Bark						**400**					
51	*Terminalia belerica*	Beleric	*Combretaceae*	Fruit						**400**					
52	*Terminalia chebula*	Baal halda	*Combretaceae*	Fruit pulp (Dry)		400	75		250	**400**	300				2000

Contd...

S.No.	Name of the plant	Common name	Family	Part	Dosage (mg)										
					BP	TP	KC	MC	CK	PB	AJ	AD	AZ	OT	SWT
54	*Tricholepis glaberrima*	Brahmadandi	*Asteraceae*	Whole plant and seeds									+		
55	*Trigonella foenum-graecum*	Fenugreek	*Fabaceae*	Seeds						400					
56	*Vitis vinifera*	Raisin	*Vitaceae*	Fruit					300		500				
57	*Zingiber offcinale*	Fresh ginger	*Zingiberaceae*	Rhizome			75		**400**		200				
58	*Zizyphus vulgaris*	Unnab	*Rhamnaceae*	Fruit						400					

PHF consists of 6 primary herbal formulations (F_{BP}, F_{TP}, F_{KC}, F_{MC}, F_{CK} and F_{PB}) and 5 supplementary formulations (F_{AJ}, F_{AD}, A_{AZ}, F_{OT} and F_{SWT}). The plus sign represents presence of the pant ingredient in the formulation. BP contains added sugar. MC contains added sodium borate (50 mg/dose). CK contains added Peter salt (150 mg/dose). While the items shown in bold were aqueous extractions, the other ingredients were used in the powdered form.

infections and/or death, as defined by Centers for Disease Control and Prevention (CDC)[9].

Blood collection and processing: A peripheral blood sample of 50 ml was collected from each participant at each of the following time points, day 1, day 14, months 1, 3 and at 3 month intervals thereafter up to month 24. The blood samples were collected between 10.00 to 12.00 h and sent to the JNCASR laboratory within 4 h after collection. Although this was an open-label study, the samples were sent under a code to blind the investigators at JNCASR. A part of the blood was used directly for CD4 cell count determination while from the remaining blood, mononuclear cells (PBMC) were isolated using density gradient centrifugation using Ficol-Hypaque. PBMCs were stored in multiple aliquots in liquid nitrogen. At every sample collection, blood was analyzed for the following parameters – differential blood count, haemoglobin, erythrocyte sedimentation rate (ESR), liver enzymes (ALT, AST), random blood sugar, urea, and creatinine. Levels of sodium, potassium, and chloride ions were checked at baseline and at the end of every year. Further, thymus scans were performed at baseline and at the end of the study. Helical scan was performed starting from apices of lungs down to mid chest level by employing 5 mm sections.

CD4 cell count determination: CD4 cell counts were determined 12-24 h from the time of sample collection using MultiTest antibodies, counting beads (340499 and 349480, Becton and Dickinson, USA) and the Lyse-No-Wash protocol as described by the manufacturer. CD4 cell count of each sample was performed in duplicate. An Immunotrol control sample (660798, Beckman and Coulter) was included in each run to validate the analysis. Samples were acquired on a BD FACSCalibur and instrument calibration was performed using Calibrite beads (340486 and 340487, Becton and Dickinson, USA).

Plasma viral load quantitation: Plasma was separated from whole blood within 6 h of collection and stored frozen in a deep freezer as aliquots of 0.5 ml. Viral loads were assayed using NucliSens EasyQ v1.1.1 (bioMerieux, France). Viral loads below the detection limit of <25 IU/ml, were assigned a value of 25 IU/ml. A plasma sample of known viral load and a different sample negative for the virus were included in each run and the assay was validated only if the internal controls gave expected results. Samples were thawed only once and used immediately for estimating viral load. The CD4 cell count and viral load were determined at every time point of sample collection from every study participant.

HLA typing of the subjects using genomic DNA: HLA typing of all the study participants was performed at 2 digit resolution for MHC Class I loci A and B and the Class II loci DRB1, 3, 4 and 5. Genomic DNA extracted from frozen PBMCs using an in-house protocol was subjected to PCR using sequence-specific primers essentially as described previously[10]. Allele frequencies of this cohort was compared to the frequencies observed in the general southern Indian population (obtained from the dbMHC database, *http://www.ncbi.nlm.nih.gov/projects/gv/mhc/main.fcgi?cmd=init*). Alleles were classified as either protective or suspectible to HIV infection and disease progression[11-13]. Statistical testing was performed using chi-squared test.

Table IV. Drug administration schedule of the polyherbal formulation

Day of administration	Time of administration	Formulation code	Dosage/day
1-15	0700, 2200	BP	5 g powder
16-30	0700, 1400	KC	5 g powder
	1500	MC	2 capsules
	Alternate day (Flexible time)	AJ	5 g jam
31-60	0700, 1700	CK + M	5 g powder
	2200	M + BP	5 g powder
61 to 90	0700, 1700	CK + BP + H	5 g powder
	2200	BP + TP + PB	5 g powder
	1800	FeC	1 capsule
91 to 120	2200	CK + BP + H	5 g powder
	2200	BP + TP + PB	5 g powder
	1800	FeC	1 capsule
Maintenance 1	0800	PB	
	1800	FeC	
	Alternate days	TP + BP	
	(1900)		
Maintenance 2	0800	AZ + OT	
	2100	AD	
	Bimonthly	SWT	

In addition to the primary and supplementary formulations, mineral combinations (FeC, H, M, Borax) were used as required at the discretion of the attending physician as preventive or therapeutic measure for treating seasonal or opportunistic infections. The day and time of administration, the formulation code and dosage have been shown. The names of the plant species that constitute each of the formulations have been presented in Table III.

AJ, Amrakha Jam available commercially; AD, formulation used for treating acidity in patients; AZ, formulation used for alleviating liver dysfunction; OT, decoction of the herbs used for the alleviation of the bronchial infections; SWT, a decoction of herbs that contains a purgative action for cleansing the system; FeC, Commercial Iron tablets (500 mg) used for iron supplement; H, Mineral combination of $CaCO_3$ and Mg used in discreet quantities (5 mg) as indicated; M, $CaCO_3$ used in discreet quantities (5 mg) in combination with other formulations; Borax, naturally occurring sodium borate, Preservative - sodium benzoate (0.5%); Honey, *Apis mellifica*; Filler, Talc used as required.

In vitro virus-inhibition assay: The PHF formulation was dried and the aqueous phase was extracted in a Soxhlet extractor at 100 °C for 36 h. The crude extract was cooled and dried under reduced pressure and stored at -20 °C until use. The remaining material was dried at room temperature and extracted with ethyl acetate at 80 °C for 24 h. The extract was cooled, dried and stored at -20 °C until use. To test in vitro activity, CEM CCR5 T-cells were treated with varying amounts of the extract (aqueous extract re-dissolved in water and ethyl acetate extract in DMSO) for 72 h. Viability was tested by staining the cells with 7 amino actinomycin (7-AAD) and per cent dead cells was assessed by flow cytometry (FACSCalibur, Becton and Dickinson, USA). Camptothecin treatment was used as positive control for cell death. Cell proliferation was assessed by determining live cell counts using Trypan blue. Antiviral activity was estimated using CEM CCR5 cells infected with NL4-3 strain[14]. Supernatants were collected on day 5 and the levels of p24 were assayed by capture ELISA using a commercial kit (Microlisa, J. Mitra and Co, Bangalore, India).

Immunophenotying of the T-cells in the clinical samples using flow cytometry: Frozen PBMC were thawed and rested overnight in RPMI medium supplemented with 10 per cent fetal calf serum (FCS) and antibiotics. On the following day, $1x10^6$ cells were stained with

100 µl of viability dye (L23101, Invitrogen, USA) for 30 min. Cells were washed once in 2% FCS and stained with anti- CCR7-Cy7-PE (557648, BD, USA) in a final volume of 50 µl for 20 min at 37°C in azide-free buffer. CD4 T-cells were classified as recent thymic emigrants (CD45RA+ CD31+), naïve (CD45RA+ CCR7+), central memory (CD45RA- CCR7+), effector/effector memory (CD45RA- CCR7-) and Ki67+. Cells were washed and stained with a 100 µl cocktail of antibodies to other surface antigens - CD31 PE (555446, BD, USA), CD4 PerCP (347314, BD, USA), CD45RA APC (550855, BD, USA) and CD3 APC-H7 (641397, BD, USA)- for 30 min on ice, fixed in 4 per cent PFA, permeabilized with Perm/Wash buffer (BD, USA) and fixed again in 0.5 per cent PFA. In an alternate panel, the CD31 PE antibody was replaced with intracellular Ki67 PE (556027, BD, USA) staining. Only samples with >70 per cent viable cells were included in the analysis. A minimum of 9,000 live CD3+ CD4+ events was acquired on a BD FACSCanto and analyzed using FlowJo 7.6.1 for Windows (TreeStar, USA). Compensation was performed using single-stained controls and gates were set using appropriate FMO controls. Data was analyzed using t-tests with Bonferroni correction.

Statistical analyses: Longitudinal analyses were carried out individually for each dependant variable using generalized estimating equations (GEE) with time as a repeated factor under a first-order auto-regressive covariance structure. The values of the measured clinical parameters are presented as mean $\pm$ 1 SD with 95% confidence intervals (CI). Duplicate T-cell counts at each time point were averaged and viral load was $\log_{10}$-transformed. The analysis utilized a total of 500, 475 and 535 observations for CD4 count, CD8 count and plasma viral load, respectively. The missing values in CD4 count and plasma viral load (<10% of total observations) were not imputed. Within-group longitudinal analysis was performed as a comparison of each time point with the baseline and *P* values are presented with and without Bonferroni correction. Inter-group means at each time point were compared by an unpaired two-tailed t-test. Slopes of the dependant variables were obtained by linear regression. Incidence of opportunistic infections was analyzed using Kaplan-Meier survival curves and significance using logrank test. Voluntary dropouts were censored at the time of leaving the study. Statistical testing was performed with PASW18.0, Hog Kong and GraphPad Prism 5.0, USA and *P* values below 0.05 were considered to represent statistical significance. The data have been presented as mean, rather than median values as the number of observations has fallen to small figures in the PHF and PHF-to-HAART groups especially in year-2. The statistical analysis included all data points available at any given point even after some volunteers left the study, died or lost for follow up and this was applicable to all the three arms of the study. The missing values were ignored and not imputed for statistical evaluation. Statistical evaluations based on median values, however, provided similar inferences. Values from the month 1 time point were excluded from the linear regression as it was significantly different from the other time points and tended to alter slope values.

Results

Baseline characteristics: Between August 2006 and July 2007, a total of 475 volunteers were screened and 63 subjects were identified who were qualified for the study (Fig. 1). Following several rounds of counselling, 31 and 32 subjects voluntarily opted to join the HAART and PHF arms of the study, respectively. The study was terminated in July 2009 and the unblinding protocol was performed in August 2009. At baseline, the mean CD4 count and viral loads were comparable between the two groups (Table I). By the time the study was terminated at 24 months, 14 of the 63 participants (Fig. 1) discontinued for one of the following reasons - voluntary decision (8 subjects), adverse events (3 subjects) and death (3 subjects). The clinical profiles of the 14 subjects who dropped out of the study are summarized in Table V. Some of the participants dropped out of the study at an early point and sufficient number of data points could not be collected from them, and data from such participants were not included in the data analysis.

Primary end points: In the HAART arm, a progressive enhancement in the mean CD4 count was observed from the time anti-retroviral therapy was initiated. The mean CD4 count increased from 225.7 ± 53.5 cells/µl (95% CI, 205.9-245.5) at baseline to 632.8 ± 225.2 cells/µl (544.5-721.0) at 24 months (Fig. 2, top panel) translating to a recovery of 154.4 ± 13.5 cells/µl per year (R^2 = 0.3419, P<0.001). Concomitantly, a steep suppression in the viral RNA load was achieved in 26 of the 28 participants within 3-6 months (Fig. 2, middle panel). The mean baseline value of 4.64 ± 0.73 $\log_{10}$ IU/ml (4.37-4.91) was reduced to 1.78 ± 0.62 $\log_{10}$ IU/ml (1.55-2.01) within 3 months (P<0.001), an approximate reduction of three orders of magnitude, and remained

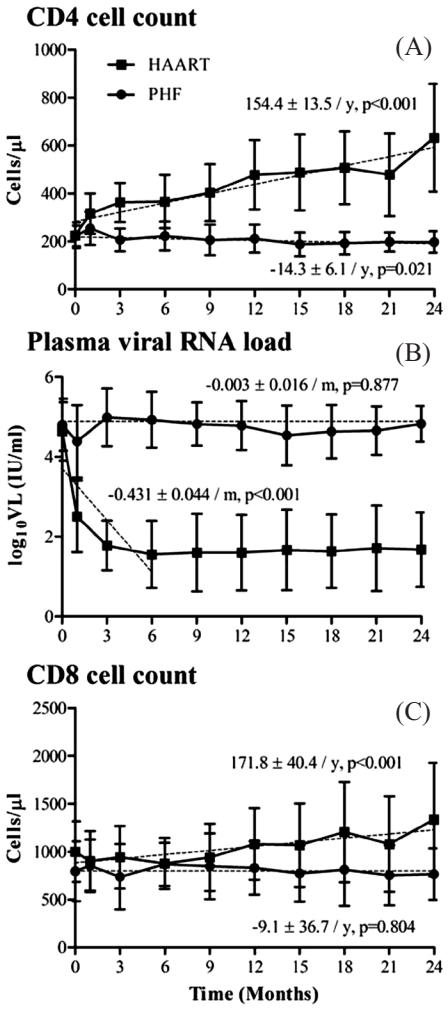
Table V. Clinical, immunological and virological profile of the 14 participants who dropped out of the study

Period	Group	Volunteer Id	Reason for Dropping Out	CD4 cell count (per µl)									Plasma viral load (log_{10})									CD8 cell count (per µl)								
				0	1	3	6	9	12	15	18	21	0	1	3	6	9	12	15	18	21	0	1	3	6	9	12	15	18	21
Year 1	HAART	S020	Death	209.5	227.6	177.8	419.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	673.2	471.5	352.6	856.8	-	-	-	-	-
		S142	Skin rash	223.5	511.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1127.8	817.6	-	-	-	-	-	-	-
		S392	Voluntary	157.8	287.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	544.4	938.9	-	-	-	-	-	-	-
	PHF	S038	Voluntary	200.7	190.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1493.9	1309.3	-	-	-	-	-	-	-
		S111	PCP	250.9	233	177.4	127.1	88.8	-	-	-	-	-	-	-	-	-	-	-	-	-	1075.9	1074.3	1099.8	438.1	502.6	-	-	-	-
		S324	Carcinoma	243	203.4	299.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		345.7	568.2	-	-	-	-	-	-
		S345	Voluntary	313.3	204.3	339	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1836.5	1017.7	1542.9	-	-	-	-	-	-
Year 2	HAART	S167	Pregnancy	190.4	245	-	293.5	239.3	243.9	226.7	157.4	112.7	5.11	4.90	4.49	4.11	5.36	5.15				1238.8	1096.8	-	973.2	991.1	1306.4	937.9	829.3	734.5
		S398	Death	197.5	-	257.8	331.2	361.2	391.2	347.1	352.8	461.3	5.00	2.08	1.40	1.40	1.40	1.40				1066.7	-	985.6	1180.9	1232.6	1135.6	1258.4	1190.4	1494.6
		S459	Voluntary	287.3	343.7	-	552.5	609.5	543.1	525			4.80	2.08	1.40	1.40	1.40	1.40		-	-	698.2	528.2	-	721.6	825.1	740.8	796.7		
	PHF	S103	Voluntary	234.6	250.2	184.6	170.9	131.7	-	-	-	-	5.11	4.98	5.32	5.32	4.93	-	-	-	-	971.8	906.6	1037.9	1130.7	561.1	-	-	-	-
		S237	Voluntary	187.8	127.3	168.7	102.2	138.1	275.2	-	-	-	5.59	5.04	5.46	5.46	5.18	-	-	-	-	1837.7	1048.1	2019.6	1384.2	1711.1	1221	-	-	-
		S410	Voluntary	199	183.6	120.5	120.9	118.7	140.7	-	-	-	4.58	2.04	4.69	4.69	4.18	4.40	-	-	-	1083.5	1089.2	775.9	955.9	1029.5	1405.1	-	-	-
	PHF-to-HAART	S189	Death	251.4	362.6	215.6	261.8	279.3	245.1	182.2	165.4	-	5.78	4.99	5.52	5.52	5.20	5.20	4.80	5.60	-	1135.1	1125.1	908.8	1001.6	1158.1	1222.2	965.8	620.3	-

The causes of participant drop-out, group allocation and identity code have been indicated. Cell count and plasma viral load values at different time points (in months) have been presented.

at the low level for the rest of the study period with a slope of -0.431 ± 0.044 log_{10} copies per month (R^2 = 0.472, *P*<0.001). The CD8 count remained largely stable over this period with 1000.0 ± 315.8 (868.1-1132.0) and 1334.3 ± 593.4 cells/µl (1101.6-1566.9) at the baseline and 24 months, respectively (*P*= 0.007) (Fig. 2, bottom panel).

In the PHF arm, the mean CD4 count of 222.2 ± 43.0 cells/µl (206.3-238.1) at the baseline appeared to have remained stable over the study period of 24 months. This was in spite of a perceptible loss in the mean CD4 count, to 211.0 ± 58.5 (187.6-234.4) and 197.4 ± 45.3 cells/µl (172.7-222.0) at 12 and 24 months, respectively (Fig. 2A). Using a post-hoc analysis, the observed power of the study for the difference at 24 months was 56 per cent. The slow kinetics of the CD4 cell reduction in the PHF arm, 14.3 ± 6.1 cells/µl lost per year (R^2 = 0.029, *P*=0.021), contrasted that of the HAART and the difference between the two arms was statistically significant at all the time points (*P*<0.001) subsequent to the baseline. The CD4 count of the PHF participants was determined on more than one occasion, at and around baseline, at screening (typically 4 to 6 wk prior to recruitment), at recruitment (day 0) and at day 15 and found to be stable. The viral load, on the other hand, remained stable over the study period with 4.80 ± 0.65 log_{10} IU/ml (4.56-5.04) at the baseline and 4.78 ± 0.61 (4.54-5.03) and 4.83 ± 0.45 log_{10} IU/ml (4.59-5.07) at 12 and 24 months, respectively, (Fig.



Time	HAART					PHF					IGC
	N	Mean	SD	p	p'	N	Mean	SD	p	p'	p
0	28	225.7	53.5	-	-	28	222.2	43.0	-	-	0.7883
1	23	316.4	84.2	<0.001	<0.001	26	254.5	68.9	0.001	0.013	0.0068
3	18	362.5	81.1	<0.001	<0.001	25	206.1	47.3	0.095	0.855	<0.001
6	25	366.6	111.2	<0.001	<0.001	19	223.0	60.7	0.950	1.000	<0.001
9	28	403.7	118.5	<0.001	<0.001	27	205.8	63.9	0.130	1.000	<0.001
12	26	478.1	144.8	<0.001	<0.001	24	211.0	58.5	0.279	1.000	<0.001
15	28	487.8	158.5	<0.001	<0.001	21	187.7	50.0	0.001	0.008	<0.001
18	27	507.4	152.1	<0.001	<0.001	17	192.2	47.1	0.001	0.008	<0.001
21	27	478.5	173.0	<0.001	<0.001	14	197.8	39.7	0.035	0.314	<0.001
24	25	632.8	225.2	<0.001	<0.001	13	197.4	45.3	0.041	0.368	<0.001

Time	HAART					PHF					IGC
	N	Mean	SD	p	p'	N	Mean	SD	p	p'	p
0	28	4.64	0.73	-	-	28	4.80	0.65	-	-	0.390
1	27	2.51	0.90	<0.001	<0.001	26	4.39	0.91	0.001	0.013	<0.001
3	28	1.78	0.62	<0.001	<0.001	28	4.99	0.72	0.028	0.254	<0.001
6	28	1.56	0.84	<0.001	<0.001	28	4.93	0.70	0.199	1.000	<0.001
9	28	1.60	0.97	<0.001	<0.001	28	4.82	0.54	0.860	1.000	<0.001
12	28	1.60	0.95	<0.001	<0.001	24	4.78	0.61	0.890	1.000	<0.001
15	28	1.67	1.01	<0.001	<0.001	21	4.54	0.75	0.064	0.576	<0.001
18	27	1.64	0.92	<0.001	<0.001	17	4.63	0.67	0.210	1.000	<0.001
21	27	1.71	1.07	<0.001	<0.001	14	4.66	0.61	0.233	1.000	<0.001
24	25	1.68	0.93	<0.001	<0.001	13	4.83	0.45	0.762	1.000	<0.001

Time	HAART					PHF					IGC
	N	Mean	SD	p	p'	N	Mean	SD	p	p'	p
0	22	1000.0	315.8	-	-	27	794.7	312.9	-	-	0.0275
1	22	902.1	310.4	0.037	0.332	22	852.4	274.0	0.310	1.000	0.5764
3	18	942.1	324.9	0.485	1.000	24	736.8	340.7	0.207	1.000	0.0557
6	25	876.0	267.4	0.034	0.308	19	868.4	224.9	0.136	1.000	0.9209
9	28	939.6	350.8	0.404	1.000	20	846.9	344.0	0.408	1.000	0.3677
12	26	1080.5	374.5	0.366	1.000	17	829.8	279.8	0.527	1.000	0.0232
15	28	1067.5	434.4	0.453	1.000	21	771.9	293.0	0.695	1.000	0.010
18	27	1204.2	523.6	0.039	0.352	17	812.4	377.7	0.842	1.000	0.0106
21	27	1079.1	499.5	0.453	1.000	14	752.9	312.1	0.595	1.000	0.0322
24	25	1334.3	593.4	0.007	0.060	13	765.6	269.2	0.716	1.000	0.002

Fig. 2 (A-C). T-cell count and plasma viral RNA load profiles in HAART and PHF arms of the study. All values calculated using generalized estimating equations model are presented as mean ± 1 SD. *P* values are presented without (p) or with (p') Bonferroni correction. IGC represents *P* value of inter-group comparison by an unpaired t-test. The kinetics of T-cell reconstitution or viral load reduction is estimated using linear regression analysis (dotted lines). Under HAART, the regression slope for viral load analysis was based on observations only up to 6 months. The slope kinetics and *P* values are shown. Months 12 and 24 are highlighted only for clarification.

2B) these changes being non-significant. Likewise, the CD8 count of 794.7 ± 312.9 cells/µl (676.7-912.7) at baseline in the PHF arm, did not change significantly at 12 or 24 months with 829.8 ± 279.8 (696.8-962.8) and 765.6 ± 269.2 cells/µl (619.3-911.9), respectively (Fig. 2C).

The secondary end points and adverse events: The occurrence of AIDS-defining illnesses was analyzed in all the three arms (Fig. 3). At the termination of the study, there was no significant difference among the three arms in either AIDS-defining or other illnesses. All the parameters of the blood chemistry determined at every visit largely remained within normal reference ranges in all the study participants (data not shown). Appearance of several adverse events has been recorded in all the three arms of the study. In the HAART arm, chronic diarrhoea, one event of lymphoma followed by death, skin rash, herpes zoster, pulmonary tuberculosis, extrapulmonary tuberculosis, hodgkins lymphoma and nevirapine-related allergy were observed. In the PHF arm, appearance of Herpes zoster, *Pneumocystis carinii* pneumonia, oral ulceration, persistent lymphadenopathy, skin lesion, oesophageal carcinoma, and an event of extrapulmonary tuberculosis were seen. In the PHF-to-HAART arm, thrombocytopenia, skin lesion, pulmonary tuberculosis, herpes zoster, cryptococcal meningitis and oral candidiasis were observed.

Immunological and virological responses in the PHF versus PHF-to-HAART arms: After the PHF arm was divided, a retrospective comparison performed between the 16 remaining PHF subjects and the 12 PHF-to-HAART participants led to several interesting observations (Fig. 4). First, at baseline, these two groups differed significantly in the mean CD4 count (P=0.05). Second, the CD4 count remained relatively stable in the PHF group over 24 months. In contrast, in the PHF-to-HAART group, the reduction in the CD4 count became significant at 15 months (P=0.043) and thereafter, although fewer data points were available for this analysis. Lastly, the rate of CD4 cell loss in PHF-to-HAART group (27.8 ± 9.9 cells/year) was nearly twice that of the PHF group (14.4 ± 7·4 cells/year).

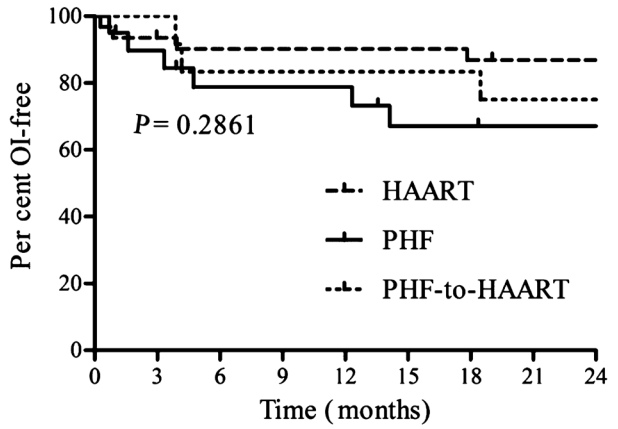
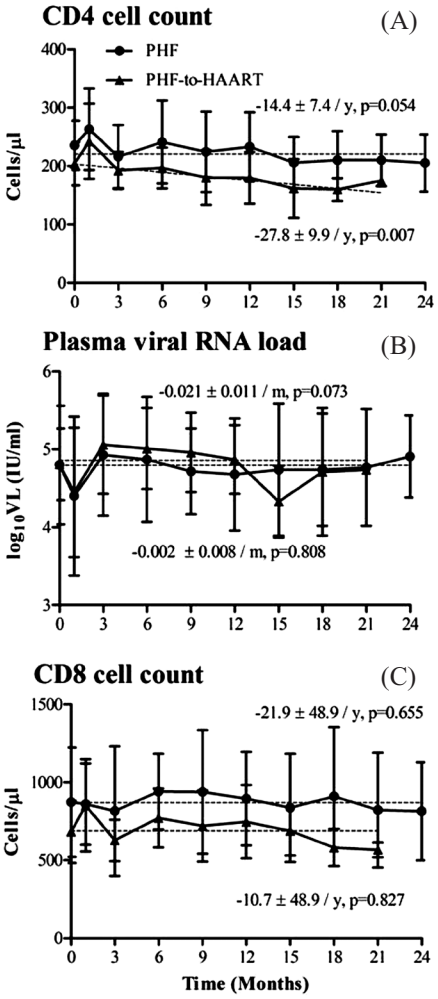


Fig. 3. AIDS-related clinical manifestations: Kaplan-Meier plots depicting the incidence of AIDS-related illnesses including death, infectious diseases and malignancies under the HAART, PHF and PHF-to-HAART arms of the study. Opportunistic infections (OI) were classified according to the CDC system. Statistical significance was tested using logrank test.

Immunological and virological responses in the HAART versus PHF-to-HAART arms: Through a similar comparative analysis between the HAART and PHF-to-HAART subgroups, a statistically significant improvement was observed in the CD4 count in both the groups at a rate comparable between HAART and PHF-to-HAART arms with 159.5 ± 15.2 and 169.3 ± 34.0 cells reconstituted per year, respectively (Fig. 5A), suggesting efficient immune reconstitution even in the PHF-to-HAART group regardless of low CD4 cell count at the inception. While the profiles of viral load reduction were indistinguishable between these arms (Fig. 5, middle panel), the CD8 count remained stable in both the arms (Fig. 5C).

Presence of specific HLA alleles in the arms of the study: Specific HLA alleles are known to be strongly associated with protection or susceptibility in HIV-1 infection. To understand if the HLA allele distribution between the HAART and PHF arms of the study differed from each other, we determined the HLA profile of each of the study participants. Additionally, allele frequencies of the two arms were compared to the frequencies observed in the general population of southern India (retrieved from the dbMHC database, *http://www.ncbi.nlm.nih.gov/projects/gv/mhc/main.fcgi?cmd=init*). This analysis failed to identify a significant association between the study arms with any of the alleles known to promote disease progression or resistance in the viral infection (Fig. 6). This observation ruled out the possibility that the HLA profile differences between the study arms could have influenced the clinical outcome.

Transient enhancement in CD4 cell count and immunemodulation: Following one month of PHF administration, a significant but transient increase



Time	PHF					PHF-to-HAART					IGC
	N	Mean	SD	p	p'	N	Mean	SD	p	p'	p
0	16	235.7	42.2	-	-	12	204.2	37.1	-	-	0.05
1	14	263.3	70.0	0.032	0.291	12	242.4	64.3	0.015	0.121	0.4386
3	14	216.2	53.9	0.151	1.000	11	192.5	31.7	0.386	1.000	0.210
6	12	241.2	71.0	0.750	1.000	7	196.9	35.0	0.705	1.000	0.144
9	16	224.4	68.7	0.467	1.000	11	180.5	46.8	0.088	0.704	0.078
12	14	233.3	58.4	0.870	1.000	10	180.0	44.3	0.081	0.649	0.024
15	13	205.9	43.8	0.036	0.322	8	161.7	50.2	0.005	0.043	0.047
18	13	210.2	49.6	0.030	0.269	4	160.0	19.6	0.000	0.000	0.071
21	13	210.0	44.3	0.080	0.718	1	175.1	5.3	0.002	0.014	-
24	13	205.4	48.9	0.024	0.220	0	-	-	-	-	-

Time	PHF					PHF-to-HAART					IGC
	N	Mean	SD	p	p'	N	Mean	SD	p	p'	p
0	16	4.80	0.76	-	-	12	4.81	0.46	-	-	0.968
1	14	4.40	1.02	0.047	0.420	12	4.45	0.83	0.023	0.187	0.7807
3	16	4.93	0.78	0.186	1.000	12	5.06	0.63	0.072	0.574	0.641
6	16	4.87	0.80	0.638	1.000	12	5.01	0.52	0.058	0.466	0.603
9	16	4.72	0.55	0.518	1.000	12	4.96	0.51	0.262	1.000	0.249
12	15	4.68	0.72	0.565	1.000	9	4.87	0.44	0.474	1.000	0.367
15	13	4.74	0.85	0.708	1.000	8	4.33	0.46	0.000	<0.001	0.435
18	13	4.74	0.72	0.703	1.000	4	4.71	0.82	0.754	1.000	0.923
21	13	4.77	0.75	0.830	1.000	1	4.74	0.00	0.613	1.000	-
24	13	4.91	0.53	0.323	1.000	0	-	-	-	-	-

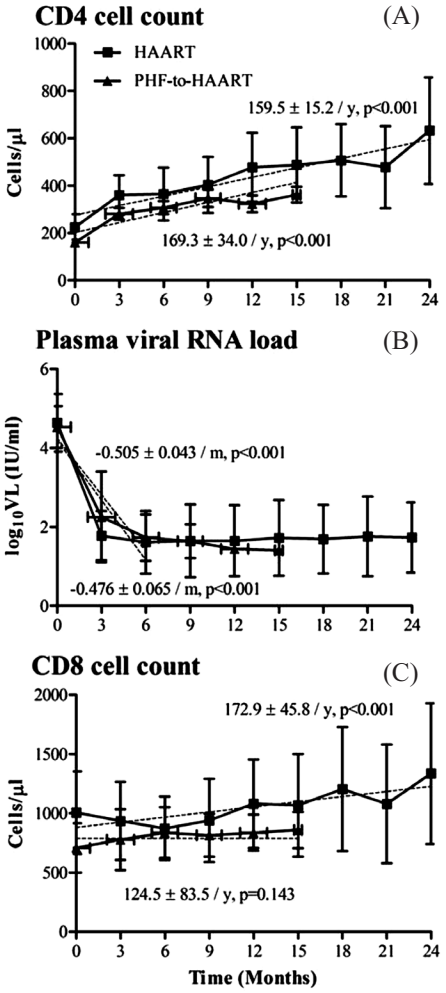
Time	PHF					PHF-to-HAART					IGC
	N	Mean	SD	p	p'	N	Mean	SD	p	p'	p
0	16	872.5	350.1	-	-	11	682.5	200.4	-	-	0.1177
1	13	860.2	261.0	0.856	1.000	9	852.2	295.3	0.043	0.348	0.9472
3	14	815.2	415.9	0.366	1.000	10	627.5	132.6	0.399	1.000	0.1843
6	12	941.5	243.0	0.271	1.000	7	771.6	188.3	0.235	1.000	0.1311
9	12	938.2	396.3	0.497	1.000	8	720.3	228.4	0.586	1.000	0.1785
12	11	895.6	300.2	0.764	1.000	6	747.1	235.6	0.377	1.000	0.3131
15	13	836.5	347.7	0.688	1.000	8	687.5	156.4	0.923	1.000	0.271
18	13	908.9	445.4	0.756	1.000	4	582.2	119.5	0.305	1.000	0.1756
21	13	822.5	368.1	0.597	1.000	1	566.5	47.6	0.189	1.000	-
24	13	814.6	314.6	0.550	1.000	0	-	-	-	-	-

Fig. 4. T-cell count and plasma viral RNA load profiles in the PHF and PHF-to-HAART arms of the study. Data presentation as described in Fig. 2. The original PHF arm was stratified into two subgroups after the mid-term safety evaluation. All data from the PHF-to-HAART arm have been collected prior to the initiation of the antiretroviral therapy.

was observed in mean CD4 count in 17 of the 26 PHF participants. The mean CD4 count of 222.2 ± 43.0 cells/µl at baseline increased to 254.5 ± 68.9 cells/µl from (228.0-281.0, *P*=0.001) with a corresponding reduction in the viral load from 4.80 ± 0.65 to 4.39 ± 0.91 $\log_{10}$ IU (4.04 to 4.74, *P*=0.001) (Fig. 2A,B). A polychromatic flow cytometry analysis of the CD4 cells was done, drawn from six representative subjects - at baseline and months 1 and 3. CD4 T cells were classified as naïve (CD45RA+ CCR7+), central memory (CD45RA- CCR7+), effector (CCR7-), recent thymic emigrant (CD45RA+ CD31+) and proliferating (Ki67+) cells. There was a significant increase in the number of total $CCR7^+$ cells (*P*=0.010) at month 1. When the $CCR7^+$ cells were further divided into central memory and naïve, the increase was observed in central memory (*P*<0.001) but not in naïve cells. There were no notable differences in the effectors and proliferating cells. Similar transient changes in CD8 cell count was also observed although not reaching statistical significance but correlating with changes in CD4 count (data not shown). A consistent enhancement in the CD4 cells in a majority of the study participants was suggestive of the immunomodulatory property of the PHF.

Discussion

The main finding of the present study was the likely stabilization of the clinical profile, especially the CD4 count, in the PHF arms. However, the significance of changes in the CD4 count in the PHF arm depended partly on the statistical model used for evaluation. The mean rate of CD4 cell decrease



Time	HAART					PHF-to-HAART					IGC
	N	Mean	SD	p	p'	N	Mean	SD	p	p'	p
0	28	225.7	53.5		-	12	160.5	42.6		-	<0.001
3	18	360.6	84.2	<0.001	<0.001	12	281.6	88.0	<0.001	<0.001	0.020
6	25	365.2	112.0	<0.001	<0.001	10	307.4	87.3	<0.001	<0.001	0.154
9	28	403.7	118.5	<0.001	<0.001	8	346.8	103.7	<0.001	<0.001	0.228
12	26	478.1	144.8	<0.001	<0.001	4	323.6	70.6	<0.001	<0.001	0.048
15	28	487.8	158.5	<0.001	<0.001	2	361.8	47.1	<0.001	<0.001	0.279
18	27	507.4	152.0	<0.001	<0.001	0	-	-	-	-	-
21	27	478.5	172.9	<0.001	<0.001	0	-	-	-	-	-
24	25	632.7	225.2	<0.001	<0.001	0	-	-	-	-	-

Time	HAART					PHF-to-HAART					IGC
	N	Mean	SD	p	p'	N	Mean	SD	p	p'	p
0	28	4.6	0.7		-	12	4.5	0.5		-	0.641
3	28	1.78	0.62	<0.001	<0.001	12	2.25	1.15	<0.001	<0.001	0.101
6	28	1.61	0.79	<0.001	<0.001	10	1.73	0.59	<0.001	<0.001	0.6646
9	28	1.65	0.92	<0.001	<0.001	8	1.64	0.43	<0.001	<0.001	0.977
12	28	1.65	0.90	<0.001	<0.001	4	1.44	0.10	<0.001	<0.001	0.649
15	28	1.72	0.96	<0.001	<0.001	2	1.41	0.02	<0.001	<0.001	0.657
18	27	1.69	0.87	<0.001	<0.001	0	-	-	-	-	-
21	27	1.76	1.01	<0.001	<0.001	0	-	-	-	-	-
24	25	1.73	0.89	<0.001	<0.001	0	-	-	-	-	-

Time	HAART					PHF-to-HAART					IGC
	N	Mean	SD	p	p'	N	Mean	SD	p	p'	p
0	22	1006.4	348.2		-	12	708.2	207.1		-	0.0109
3	18	935.79	329.45	0.42	1.000	12	777.12	256.81	0.44	1.000	0.170
6	25	872.83	267.23	0.035	0.282	10	836.51	213.49	0.042	0.211	0.703
9	28	939.58	350.83	0.386	1.000	8	813.89	181.68	0.260	1.000	0.338
12	26	1080.36	373.92	0.428	1.000	4	836.61	153.20	0.202	1.000	0.215
15	28	1067.47	434.39	0.507	1.000	2	858.43	154.45	0.163	0.817	0.511
18	27	1204.42	523.77	0.052	0.413	0	-	-	-	-	-
21	27	1079.43	499.82	0.497	1.000	0	-	-	-	-	-
24	25	1334.61	594.11	0.011	0.086	0	-	-	-	-	-

Fig. 5 (A-C). T-cell and plasma viral RNA profiles in the HAART and PHF-to-HAART arms of the study. Data presentation as described in Fig. 2. Month 1 observations from the HAART group have been omitted from the data analysis since the PHF-to-HAART arm did not contain a corresponding time point. The mean ± 1 SD values presented here under HAART therefore differ marginally from those of Fig. 2. The 'zero' time point under PHF-to-HAART corresponds to the first time evaluation after the subject has been started on antiretroviral therapy.

in the PHF arm was 14.3 ± 6.1 cells/µl per year when regression analysis was performed. A more rigorous analysis performed using the Generalized Estimating Equations showed a non-significant mean CD4 cell reduction from baseline 222.2 cells/µl to 211.0 cells/µl at 12 months. At 24 months, this number dropped further which was significant compared to the baseline value but not if Bonferroni correction was applied. There is no consensus among the statisticians under what circumstances this correction must be used[15]. Application of Linear Mixed Model to evaluate the significance of CD4 cell changes demonstrated results similar to the GEE model (data not shown).

At the time the study was terminated, the three arms of study did not differ from one another significantly in AIDS-related clinical manifestations. Only two studies from India examined the survival rate in AIDS in subjects with <200 CD4 cells/µl. Kumarasamy *et al*[16] observed that the median duration of survival in 71 drug naive subjects was 33 months, and Hira *et al*[17] reported a median survival period of only 19.2 months after developing AIDS. We did not find AIDS-related mortality in the participants in the PHF arms with the exception of a single death. A few studies previously reported the existence of seropositive individuals whose CD4 counts dropped below 200 cells/µl but who remained symptom-free for extended periods[18,19].

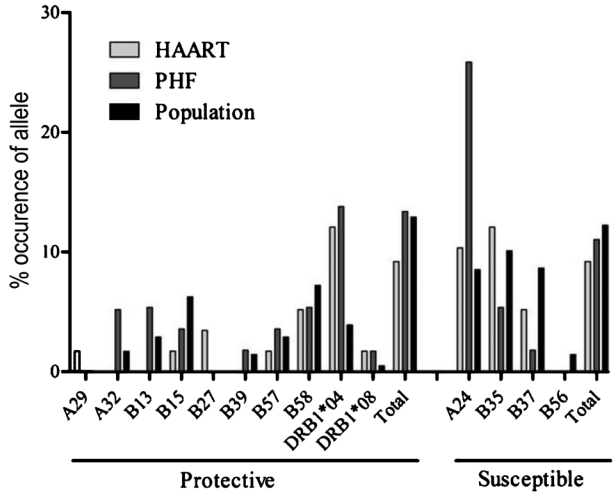


Fig. 6. HLA alleles in the HAART and PHF groups in comparison to the general populations of India. The PHF and PHF-to-HAART arms were pooled due to insufficient numbers. Allele frequencies were calculated using the HLA comparison software available at HIV Molecular Immunology Database (*http://www.hiv.lanl.gov/content/immunology/hla/hla_compare.html*). HLA allele frequencies of the southern India population were obtained from dbMHC database. Statistical significance was tested using chi-squared test.

The present study had several limitations. First, the study was an open-label trial. Given the nature of the medicines, the identity of the medicines could not be concealed from the participants, therefore, the placebo effect could not be eliminated from the study outcome. The investigators at the JNCASR laboratory were however, blinded to the identity of the group allocation until the time of unblinding. Second, participant recruitment could not be randomized given that the PHF formulation was not a conformed medicine. In an open-label study, non-randomization is permitted especially for ethical reason[19]. Third, lack of a placebo group to compare the study outcome imposed limitations on the study interpretation. As the issue of the placebo inclusion is highly contentious[21], the HAART arm was used for the purpose of comparison. Fourth, the quality control of the herbal medicines remains a universal problem with variations in the formulations, ingredients and administration. We prepared a single lot of the PHF that was used for the entire clinical trial, toxicity studies and laboratory assays thus partially addressing this question. Fifth, given the recruitment criteria, a 'selection bias' may have crept into the study design. Although the study subjects cannot be technically qualified as long-term non-progressors or elite controllers, some of them being intrinsically resistant to disease progression cannot be ruled out. Lastly, co-trimoxazole prophylaxis was offered to all the HAART and several PHF participants especially during year 2. Cotrimoxazole prophylaxis can have significant impact on the incidence of opportunistic infections and prognosis. However, the magnitude of benefits conferred by the prophylaxis is quite variable ranging from 0 to 50 per cent[22]. In spite of these limitations, inferences drawn from this prospective study were based on a large number of repeat observations from each participant. Monitoring the AIDS-related illnesses added strength to the present study.

The absence of apparent disease progression in the PHF participants could be partly explained if the CDC classification of AIDS-related clinical stages[8] is not applicable for India. The validity of the CDC classification system for non-caucasoid ethnic groups of many countries has been challenged[23-25]. A recent study commissioned by The National AIDS Control Organization (NACO) of India conducted simultaneously at eight different centres using the two-color immunophenotyping technique and uniform protocols and quality control measures and involving a total of 1,200 subjects representing diverse populations of India, identified that the mean CD4 count in these populations was 918.85 ± 311.39 cells/µl[26] similar to that of the Caucasoid populations. Thus, absence of AIDS-related clinical symptoms in the PHF groups at or below 200 CD4 cell/µl may not be attributed to ethnic differences in average CD4 count.

We asked if the kinetics of CD4 cell reduction, rather than the absolute CD4 count and/or cell percentage, could serve as a more reliable prognostic marker[27,28] to explain the slower CD4 cell loss observed in the PHF arm. Only two studies from India examined CD4 cell loss kinetics to date, and neither of them could be representative for technical reasons[29,30]. Several investigators mainly from the pre-HAART era, monitored the fate of the CD4 cell kinetics in drug-naïve subjects, however, only a few considered cell loss at or below 200 cells/µl. In a study of 111 haemophiliacs in London, followed up for more than 7 years[27], it was identified that the median slope for CD4 cell decline was 80 CD4 cells/year and this value was steeper for those who developed AIDS. This study proposed a uniform cell decline through all the stages of the viral infection including below 200 cells/µl. Another study involving 579 seropositive homosexual men in Baltimore, USA, with a follow up of 4 years

demonstrated a CD4 cell loss of 189 cells/year in those who progressed to AIDS in less than one year[18]. Urassa *et al*[31] in a prospective study with 171 seropositive subjects in Tanzania showed that the median CD4 cell loss was 52 cells/year in 35 subjects who progressed to AIDS and 22 cells in 156 participants that did not. In a prospective study among 187 homosexual men in Amsterdam and with a follow up of 5 years, the authors proposed a bimodal decline in the CD4 count among those participants who progressed to AIDS[32]. The CD4 cells declined at a slower rate of 40 cells/year until the level of 400 cells/µl reached and fell at a faster rate of 188 cells/year and the rate of cell reduction continued at the same pace even after 200 cells. The CD4 cell loss observed in the PHF and PHF-to-HAART arms in the present study, both in the absence of antiretroviral therapy was significantly smaller than that reported previously.

Given the evidently stabilized immune profile of the PHF arm, we tried to find if the immune function of the T-cell significantly improved in these subjects. It was reasoned that if the polyherbal formulation improved the quality of life of the study participants, such an improvement is likely reflected in the quality of the T-lymphocyte function itself. Extensive immunophenotyping of the CD4 T-cells, in a cross-sectional analysis, suggested reduced levels of immune activation of the T-cell with respect to four different biomarkers (Mangaiarkarasi *et al* personal communication). In this backdrop, the reduced serum levels of the liver enzymes in the PHF arm may be of significance. This is suggestive of hepatotoxicity which is manifested in several infections including in HIV-AIDS.

Considering the inherent limitations of the present study, it is not possible to ascertain the clinical benefits of the PHF. Another study, therefore, is urgently warranted with a stringent study design incorporating double-blinding, randomization and larger number of participants. To eliminate selection bias, participants with higher CD4 count need to be included, preferably with >350 cells/µl in light of the revised guidelines of the NACO[8]. Use of the placebo control will be the most critical component of the study design since it will permit meaningful interpretation of the data. It would also be critical to examine if the PHF formulations could complement the standard antiretroviral therapies.

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