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Title:

Eradication of HIV in association with treatment of phyto Antiviral regimen

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ABSTRACT:

One of the current challenges in the suppression or eradication of HIV-1 proviral DNA in latently infected CD4 lymphocytes and other reservoirs, which can replenish and revive viral infection upon activation. The use of Highly Active Anti Retroviral Therapy (HAART) in Human Immunodeficiency Virus (HIV) infected patients can often significantly reduce the viral RNA to undetectable levels in plasma.. It has been reported that HIV-1 Proviral DNA has been eliminated from PBMC and lymphnode with the plant based therapy. The phytochemicals which are present in plants have been reported to have anti-HIV, immunostimulant properties. The ideal antiviral drug regimen would be one that induces strong and persistent suppression of viral replication, gives prolonged immunologic and clinical benefits without toxicity. A 30 year old female a symptomatic HIV-1 positive patient was given with the polyherbal regimen containing anti-HIV properties, anti-viral properties and immunostimulant properties for about more than three years. The qualitative proviral DNA, quantitative RT-PCR, quantitative p24 antigen assay, qualitative antibody assays, CD4 cells enumeration, CD8 cells enumeration, CD4/CD8 ratio, IFN-γ levels and IL-2 levels were assayed before the treatment, in-between the treatment, after the treatment with both positive and negative controls. After the treatment the viral load came to undetectable level, the proviral DNA vanished. The CD4 and CD8 cells increased. From the interpretations of the data, the treatment has the significant effect towards elimination of HIV-1 from the infected patient.

KEYWORDS: HIV elimination, phytochemicals, Herbal treatment

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INTRODUCTION:

AIDS results from infection with the human immunodeficiency virus (HIV), and nearly 40 million adults and children worldwide live with HIV/AIDS(1). The opportunistic infections characteristic of AIDS results from the progressive depletion of $CD4^+$ T lymphocytes in infected individuals (2). During the acute phase, virus levels increase rapidly with a concomitant loss of $CD4^+$ T cells. As cellular and humoral antiviral immune responses take effect, virus levels decreases and $CD4^+$ T-cell numbers temporarily recover. During the subsequent phase of clinical latency, $CD4^+$ T-cell numbers slowly, but inexorably, decline. Finally, as immune system is exhausted by HIV, clinical immunodeficiency and lethal infections occur (3).

The tropism of HIV-1 is governed by the distribution of cellular proteins that the virus requires to gain access to the cell's interior. Hot on the heels of the discovery of HIV-1(4-6) came studies demonstrating that CD4 is a receptor for the virus (7, 8). Over a decade later, the chemokine receptors CCR5 and CXCR4 were identified as co receptors for HIV-1(9-13). The distribution of these receptors permits infection of not only $CD4^+$ T cells, but also cells that are central to antigen presentation, including macrophages and dendritic cells (DCs). HIV-1 seems to have adapted to the unique biological characteristics of these types of cells. As a consequence, HIV-1 may exist in various replication states and tissue compartments, which would allow it to persist even in the face of host immune responses and highly suppressive antiretroviral regimens. Understanding these adaptations is important if we are to design strategies to restrict viral replication and pathogenicity.

In the course of effective immune response first the virion is internalized by phagocytosis or endocytosis by the Antigen Presenting cells (APCs) like macrophages, dentritic cells etc and presents its antigen protein along with cell membrane protein associated molecules called HLA or MHC molecules. The CD4 cells recognize this MHC associated antigen and produces the cytokines. This cytokines increases the B cell and T cell activities, thus increases the humoral and cell mediated immune response.

Infection by HIV is characterized by several effects on the host immune system. B cells decline in number and function (14), and, because of the toxicity of HIV antigens, cytokine regulation is distorted causing a decrease in CD4+ T-cells (15). There is a distinct interplay between HIV and the immune defenses. Typical non-progressors (those who have been infected with HIV but do not show symptoms) display several responses that are different than those of progressors. Non-progressors show more TH1-type cytokines like IL-2 and IFN-γ and an elevated response by CD4+ T-cells and cytotoxic CD8+ T-cells towards HIV is observed.

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Additionally, there is an increased synthesis of chemokines also. The HIV virus encounters these defenses by varying antigenic sites (preventing an effective immune response and overwhelming the immune system) and by reducing MHC on the surface of cells, and reducing the number of CD8+ T-cells.

The recent studies reveals that the levels of cytokines present in body fluids are useful for understanding pathogenesis and as diagnostic and prognostic indicators in many diseases, including those induced by human immunodeficiency virus (HIV) infection(16). The IFN-γ has antiviral property towards HIV also, if with greater yield by the secreting cells it can completely eradicate the virus from body including the proviral DNA possessing cells by apoptosis mechanism. Besides antiviral molecules, some phytochemical molecules present in some herbs has induced greater yield of IFN-γ to the level of eradication of virus, it is dose dependant (17). We could eradicate HIV, including proviral DNA possessing cells by combining both the properties of antiviral and IFN-γ induction with phytochemical molecules which are present in some herbs. I have reported in my previous study that the HIV-1 proviral DNA has been eliminated from PBMC and lymph node (18), to study in deep about my previous work, this work was undertaken.

MATERIALS AND METHODS:

STUDY DESIGN

A Randomized Controlled Trial (RCT) study was performed with one negative control (Regular Normal Healthy blood donor), five positive controls (Asymptomatic HIV-1 positive treatment naïve individuals) and one 30 year old asymptomatic female HIV-1 positive patient was selected for treatment.

I selected nine medicinal plants, having antiviral and immunostimulant property were selected which are traditionally used in Indian System of Medicine.

1.*Curcuma longa* have antiviral property[19]

2.*Coriandrum sativum* has antiviral property[20]

3.*Glycyrrhiza rhiza* has antiviral property[21]

4.*Ocimum sanctum* has IFN-γ inducing property[22]

5.*Piper longum* has IFN-γ inducing property[22]

6.*Phylanthus emblica* has IFN-γ inducing property[22]

7.*Phylanthus niruri* has IFN-γ inducing property[22]

8.*Withania Somnifera* has IFN-γ inducing property[22]

9.*Eclipta alba* has IFN-γ inducing property[22]

The antiviral regimen capsule preparations were carried out as per the previous works and the LD50 and toxicological studies had been carried out previously (18, 29).

Informed consent from the subject and Institutional Ethics Committee approval obtained for this study. Before starting the treatment the proviral DNA, Viral load, HIV-1 antibodies, P24 antigen assay, CD4, CD8 cell counts Interferon- γ (IFN-γ) and Iinterleukin-2 (IL-2) levels were recorded and all the parameters were recorded every six months once during the treatment. The patient's serum and blood cells (PBMC) were demonstrated in cell culture for the presence of virus before starting the treatment, during the treatment and after the treatment. The negative control normal blood donor is same age, same sex without clinical complaints or signs of disease and negative for anti-HIV-1/2 antibodies, the positive control HIV-1 infected individuals also same age, same sex without clinical complaints or signs of disease and positive for anti-HIV-1 antibodies, were included in this study. Both control and patient were monitored for routine laboratory values such as hemoglobin, liver or renal function tests, the patient also monitored for any adverse effects during treatment. Both were monitored for other illness and co-infections for entire study period. Both the controls were given placebo.

Detection of HIV-1 Proviral DNA by PCR

DNA extraction and purification of PBMCs, Lymphnode biopsy and other types of cells for HIV-1 DNA detection were performed as per previous work (18)

The samples were also send to Reliance life science laboratory, Mumbai and assay was performed in The National HIV Repository Center, Bangkok for counter check up.

HIV-1 (RT-PCR) Quantitative assay, Real time RT-PCR assay, Estimation of P24 Antigen assay, CD4 and CD8 Counts and Estimation of IFN-γ were performed by following previous work (18,30,31).

Estimation of Interleukin-2 (IL-2)

The serum Interleukin-2 was estimated using Roche kit by ELISA method. **HIV-1 co-culture**

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This assay was performed in Naval Institute of Molecular Research in Namakkal, Tamilnadu as per the previous work (18) as well as in "The National HIV Repository Center", Bangkok.

Detection of HIV-1 antibody

The HIV-1 antibodies were detected using ELISA method and Western-Blot method using J.Mitra & Co. Ltd. Kits as per manufacturer's recommandations. This assay was performed in Naval AIDS Research Center in Namakkal, Tamilnadu.

Statistical Analysis: The Test of Proportion study that is Fisher's Z test was performed for proviral DNA data analysis with SPSS Software Version 17.For other virological and immunological markers between controls and treatment, the one way ANOVA was performed using the Graph pad prism 5.0 Software.

RESULTS:

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TABLE 1: VARIOUS PARAMETERS BEFORE, DURING AND END OF THE TREATMENT. The data are the mean of three different assays.

		Viral load(C)	Viral load(R)	P24 Antigen	CD4 Count/µl	CD8 Count/µl	CD4/CD8	IFN-Gamma	IL-2
BT	PC	LDL	LDL	LDL	900	1020	.88	125	1800
	NC	LDL	LDL	LDL	980	980	1	122	1750
	TMT	54000	54100	2.7	360	830	.43	9	90
6 M	PC	7000	6900	LDL	700	1000	.7	101	1500
		LDL	LDL	LDL	960	970	.98	125	1780
	NC	45000	45000	2.2	450	880	.51	19	200
	TMT								
12 M	PC	36000	35900	2	650	950	.68	90	1400
		LDL	LDL	LDL	990	1010	.98	123	1800
	NC	27000	27000	1.5	540	910	.59	27	350
	TMT								
18 M	PC	90000	90000	4.5	450	890	.5	60	1200
		LDL	LDL	LDL	980	980	1	125	1750
	NC	18000	18200	1	630	1000	.63	45	700
	TMT								
24 M	PC	120000	121000	6	400	860	.46	40	900
		LDL	LDL	LDL	970	1000	.99	122	1780
	NC	9000	9500	LDL	720	1030	.69	54	900
	TMT								
30 M	PC	150000	149000	7.5	350	850	.41	30	700
		LDL	LDL	LDL	990	990	1	125	1750
	NC	LDL	LDL	LDL	810	1080	.75	90	1400
	TMT								
36 M	PC	210000	211000	10	320	850	.37	21	500
		LDL	LDL	LDL	990	1000	.99	124	1800
	NC	LDL	LDL	LDL	900	1090	.82	108	1650
	TMT								
39 M	PC	300000	300000	15	300	860	.34	15	300
		LDL	LDL	LDL	980	980	1	125	1770
	NC	LDL	LDL	LDL	900	1090	.82	115	1700
	TMT								
42 M	PC	460000	459500	27	250	850	.29	9	120
	NC	LDL	LDL	LDL	1000	1000	1	125	1750
	TMT	LDL	LDL	LDL	990	1160	.85	118	1800

*PC-Positive Control,NC-Negative Control, THT-Treatment, **M-Months, *** Viral load(C)-Analysed by Roche Cobas amplicor, Viral load(R)- Analysed by Real Time PCR Assay-Copies/ml, P24-Picogram/ml, IFN-γ- Picogram/ml, IL-2- Picogram/ml

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TABLE 2: PRESENCE OF HIV-1 PROVIRAL DNA BEFORE, DURING AND END OF THE TREATMENT

	POSITIVE CONTROL	NEGATIVE CONTROL	TREATMENT
BEFORE TREATMENT	1	0	1
6TH MONTH OF TREATMENT	1	0	1
12TH MONTH OF TREATMENT	1	0	1
18TH MONTH OF TREATMENT	1	0	1
24TH MONTH OF TREATMENT	1	0	1
30TH MONTH OF TREATMENT	1	0	1
36TH MONTH OF TREATMENT	1	0	0
39TH MONTH OF TREATMENT	1	0	0
42TH MONTH OF TREATMENT	1	0	0

*1-Represents proviral DNA present. **0-Represents proviral DNA absent.

The treatment with herbal molecules was carried out for about 42 months. The viral load has come down from 54000 copies/ ml of serum to an undetectable level in 30 months (Table 1) and there is no significant difference in viral load level between two laboratories and two test methods' result. Likewise there is no significant difference in assay results carried out in different laboratories. The proviral DNA disappeared in 30 months both in PBMC (Fig 1) and Lymphnode (Electropherogram not given) and other type of HIV infectable cells (Electropherogram not given). The CD4 cells increased from 360 cells/µl of blood to 990 cells/µl of blood in the duration of treatment about 42 months. The CD8 cells also increased from 830 cells/µl to 1160 cells /µl (Table 1). The control showed the normal CD4 and CD8 range all the times where as the positive controls (mean value of all the five individuals) showed the decrease of CD4, CD8, CD4/CD8 ratio, IFN-γ , IL-2 and viral load, p24 antigen vice versa (Table1) The P24 antigen in the blood has decreased from 2.7 pg/ml to undetectable level in treatment.

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However, the haemogram, kidney function tests, liver function tests reveals almost normal in all the times both in controls and patient (Data not shown) and no other illness and co-infections recorded for the entire study period.

In the statistical analysis, the positive control verses negative control gives the Z value 4.24, P value 0.0006 is statistically highly significant, the positive control verses treatment gives the Z value 2.63, P value 0.0182 is statistically significant and the negative control verses treatment gives the Z value 2.27, P value 0.0375 is statistically significant. In the analysis of other virological and immunological data reveals that there is a significant difference between means ($P<0.0001$, F value is 6.020) and variance ($P<0.0001$) among treatment and controls. This is statistically highly significant.

Fig 1.a

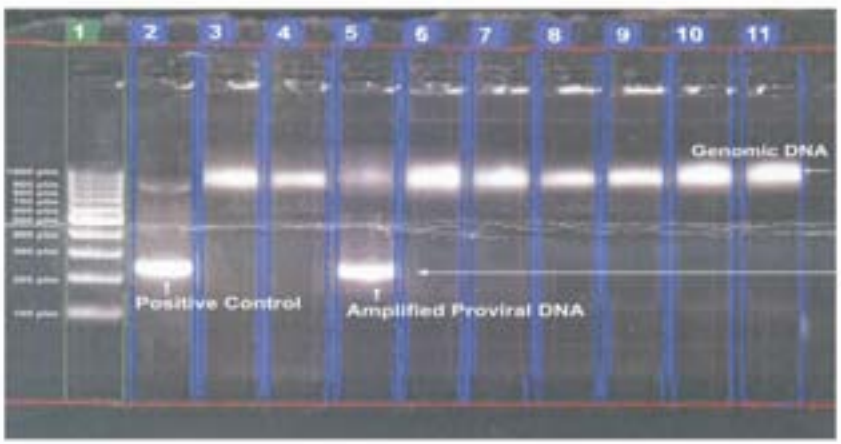

Fig 1.a.Electrophoresis: This is the picture shows the presence of Proviral DNA beforer treatment in PBMC. Lane No.1: 100bp Molecular weight marker. 2:Positive Control. Lane: 5 Our Study Sample. Lanes 3, 4,6,7,8,9,10 and 11:: Negative Controls. :

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Fig1.b

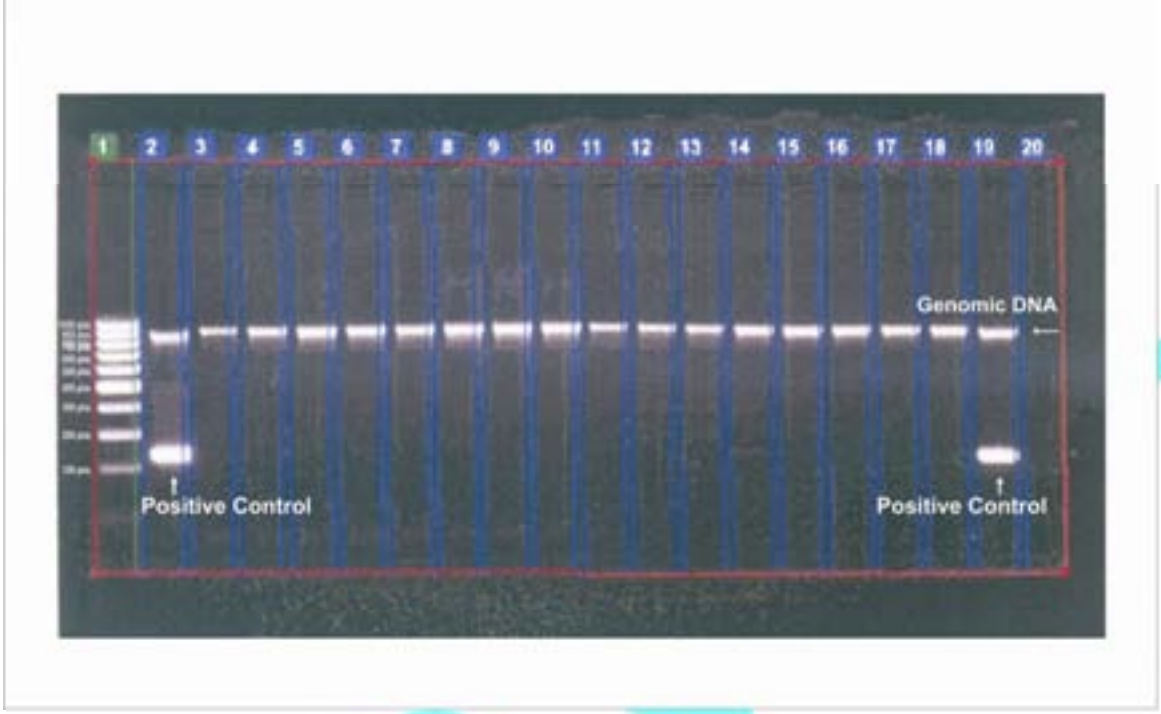

Fig 1.b.Agarose Gel Electrophoresis: This is the picture shows the absence of Proviral DNA after treatment in PBMC. Lane No.1: 100bp Molecular weight marker. Lane 2 and 19: Positive Control. .Lane 5: Our Study Sample. Lanes 3,4,6,7,8,9,10,11,12,13,14, 15, 16, 17 and 18: Negative Controls.

DISCUSSIONS:

In HIV co-culture and patient's PBMC, before the treatment, virus and P24 antigen were present and 24,30, 36 and in 42 months' results reveals that the proviral DNA were absent. But in all the time the HIV-1 antibodies were present. This has to be studied.

With the Cell-mediated immune response the host defenses that are mediated by antigen-specific T cells and various non-specific cells of the immune system. It protects against intracellular bacteria and viruses. CD8 T-cells or cytotoxic T- lymphocytes are white blood cells that find and kill infected cells in the body. The CD8 T-cell responses are important in controlling the HIV levels (23, 24, and 25).

Interferons are several glycoproteins produced and secreted by certain cells that induce an antiviral state in other cells and also help to regulate the immune response. Interferon gamma (IFN-γ) is secreted by T_H1, T_C, NK cells. The IFN-γ inhibits viral replication, enhances

macrophages activity and increases the expression of class I and class II MHC molecules (25).Interferon- mediated antiviral activity may involve several mechanisms. Due to HIV-1 infection there is sustained impaired activity in most of the cells including low level expression of MHC. The IFN-γ restores the expression of both I MHC and II MHC molecules, there by CTL activity against HIV-1 infected cells occurs (29, 30, 31).

Finally, IFN-γ increases the activity of T_C cells, macrophages, and NK cells, all of which play a role in the immune response to viral infected cells. All nucleated cells express class I MHC molecules, if the cells are infected with HIV-1, if the cells possess foreign DNA like HIV-1 proviral DNA that is altered self-cell. Each and every altered self- cell expresses class I MHC and HIV antigenic peptide complex, the CTL very well recognizes that type of cells and destroys that. By keep on increasing CTLs and its activity by IFN-γ with the induction of phytochemicals all the proviral gene possessing cells, viral infected cells could be destroyed (27).

Some phytochemical molecules have anti- HIV activity (26), the plants which were employed for this study namely *Curcuma longa* ,*Coriandrum sativum* ,*Glycyrrhiza rhiza* possesses antiviral activity but further study has to be carried out for isolation, characterization, structural identification and mechanism of action, how it acts as anti- HIV molecules. The Hyper Active Anti Retroviral Therapy (HAART) very often gets resistant to the HIV-1 mutants (26), likewise CTL mediated virus infected cell lysis also often failure due to CTL escape mutants (28). The statistical significant result of this study reveals and supports that the therapy is effective to eliminate the HIV from the host.

Even though our body has 2500 million cells, it is very difficult to demonstrate each and every cell is free from virus practically with laboratory methods. But hypothetically we can assume that the cells are free from virus and proviral DNA based on viral load and proviral DNA tests at different intervals repeatedly. The HIV can infect the following cell types 1.Haemotopoitic/immune cells(T lymphocytes, B lymphocytes, Primary monocyte /macrophages, Kuppfer cells, bone marrow precursor cells, dendritic cells and langerhans cells) 2.Brain/ Glial cells 3.Other types of cells (Fibroblasts, Sperm etc) (25).

In our study at 6 months intervals for about 3 times, that is for about 18 months there is no proviral DNA in PBMC. If there is a possibility of hidden virus and proviral DNA in other than PBMC in the host's any of the system, tissue and cells within this 18 months of period, naturally it would have infected the PBMC and showed the proviral DNA positive as similar as in the course of disease progression after HIV infection, but in this individual there is no such kind of viral marker identification during the middle and last phase of treatment. Either the virus has to infect and undergo replication cycle even it is in latent period in any one or any type of cells during this long period of last phase of treatment or it has to be lysed by body mechanisms.

More over the half life of the cell and HIV is too short when compare to the last phase of the treatment period, all proteins and nucleic acids would have been disappeared by metabolic activity unless they involve in the replication cycle. So we can consider positively, the host is free from virus and the antibody is persistent antibody.

In the case of above conclusions further research should be done to understand about CTL escape mutants and drug resistance to mutants with the response to this phytochemical therapy.

CONCLUSSION:

From the above study the herbal molecules possesses antiviral compounds, interferon inducers and immunostimulating molecules. Further work has to be done what kind of molecules involved in this activity and also to be studied the molecular mechanisms to this activity.

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Conflict of Interest Statement: No competing interest declared.

Informed consent and IEC approval was obtained as per helsinky declaration. The work has been approved by the Institutional Ethics Committee, Naval Aids Research Center, Namakkal.

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