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Effects of alkaloidal extract of *Phyllanthus niruri* on HIV Replication

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ABSTRACT

Phyllanthus niruri has been found to exhibit marked inhibitory effect on hepatitis B virus evident by its exhaustive utility in cases of chronic jaundice. However, till date, research has not been focused on identification and validation of active pharmacophores of *Phyllanthus niruri* responsible for the reported inhibitory effect of its aqueous extract on anti-human immunodeficiency virus. The present investigation examines the anti-HIV effects of the alkaloidal extract of *Phyllanthus niruri* in human cell lines. The inhibitory effect on HIV replication was monitored in terms of inhibition of virus induced cytopathogenicity in MT-4 cells. The alkaloidal extract of *Phyllanthus niruri* showed suppressing activity on strains of HIV-1 cells cultured on MT-4 cell lines. The CC_{50} for the extract was found to be $279.85\mu\text{g mL}^{-1}$ whereas the EC_{50} was found to be $20.98\mu\text{g mL}^{-1}$. Interestingly the Selectivity Index (SI) was found to be 13.34, which showed a clear selective toxicity of the extract for the viral cells. The alkaloidal extract of *Phyllanthus niruri* was thus found to exhibit sensitive inhibitory response on cytopathic effects induced by both the strains of human immunodeficiency virus on human MT-4 cells in the tested concentrations.

Key Words: *Phyllanthus niruri*, Alkaloid, HIV, MTT assay.

INTRODUCTION

Phyllanthus niruri Linn has been used in Ayurvedic medicine for over 2,000 years and has been used in wide number of traditional ailments such as jaundice, gonorrhea, frequent menstruation, and diabetes and topically as a poultice for skin ulcers, sores, swelling, and itchiness.¹ The plant has a role in liver disorders due to its febrifuge, antiseptic, astringent, stomachic, deobstruent and diuretic actions.² It corrects GIT troubles like dyspepsia, colic, diarrhea and dysentery and tones the GIT tract back to function.³ The young shoots of the plant are administered in the form of an infusion for the treatment of

chronic dysentery.⁴ *Phyllanthus niruri* primarily contains lignans (e.g., phyllanthine and hypophyllanthine), alkaloids, and bioflavonoids (e.g., quercetin).¹ *Phyllanthus* blocks DNA polymerase, the enzyme needed for the hepatitis B virus (HBV) to reproduce.⁵ It has been found to inhibit DNA polymerase of HBV and binds to HbsAg, in vitro.⁶ The fact that both HBV DNA polymerase inhibitory activity and HbsAg binding activity are present in an isolable component of *Phyllanthus niruri* served as a harbinger of this research work. While it remains unknown as to which of these ingredients has an antiviral effect, research shows that this herb acts primarily on the liver. This action in the liver confirms its historical use as a remedy for jaundice. A comprehensive research focused on identification of the specific pharmacophore of *Phyllanthus niruri* remains the need of the hour. The prime objective of this study was thus focused on identification and evaluation of anti-HIV activity of *Phyllanthus niruri* with a viewpoint to broad base the pharmacological profile of the plant species.

MATERIAL AND METHODS

Plant Material

The whole plant of *Phyllanthus niruri* (Bhuiamlaki) was obtained from Amrutlal brothers Ltd. Mumbai. M/s. Anushka Herbals, Mumbai, India, performed the identification

and authentication of the plant and a voucher specimen of *Phyllanthus niruri* has been deposited with them. The plant was then ground into a mixer to obtain the coarse powder.

Plant Extraction: (Alkaloidal Fraction)

The dried, coarsely powdered whole plant of *Phyllanthus niruri* (200g) was moistened with 25% ammonium hydroxide, allowed overnight standing and then Soxhlet extracted with 95% ethanol. After concentration under vacuum, the syrupy residue (30g) was treated with concentrated hydrochloric acid. The acidic filtrate was washed with benzene, made basic (pH 10) with 25% ammonium hydroxide and extracted with chloroform to afford the alkaloidal fraction (2g).

Chemicals and Reagents

The chemicals/reagents used included RPMI 1640 medium (Gibco, grand Island, NY, USA), Foetal Calf Serum (FCS) (Whittakar Bioproduct, MD, USA), 3-(4,5 Dimethylthiazole-2-yl) 2,5-Diphenyltetrazolium bromide (MTT) (Wako Pure Chemical, Osaka, Japan), Fluorescein isothiocyanate-Conjugated F(ab')₂ fragments of rabbit anti-human immunoglobulin antibody (Cappel, Organon Taknika Corp., West Chester, PA, USA).

Culture of T-Cells

The HTLV-1 infected MT-4 Cell line was established by co-cultivating leukocytes from an adult T-cell leukemia (ATL) patient with cord blood Leukocytes (CBL) (Miyoshi et al 1981).⁷

All cell lines were grown and maintained under the same conditions as the H9/HTLV-III_B cell line.

Virus Growth and Partial Purification

HIV was obtained from the supernatant of a persistently HIV-infected H9 cell line (H9/HTLV III_B).

Clone H9 is an OKT4⁺ T-cell line that is permissive for HIV replication. H9/HTLV_{IIIB} cells were grown in 75-cm² loosely capped canted-neck culture flask (Nunc) in RPMI 1640 medium supplemented with 10% v/v heat inactivated foetal calf serum (FCS), 2.5µg/ml fungizone and 20µg/ml gentamycin. The cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. Every 3-4 days, cells were spun down and seeded at 500 000 cells/ml in new cell culture flasks. The cell-free culture fluids were pooled for virus purification and kept frozen at -70°C. Virus production by the H9/HTLV_{IIIB} cell was monitored by measuring the percentage of infected cells by indirect immunofluorescence assay. For virus purification, the cell supernatants were filtered through a 0.22µm Millipore filter (Millipore Corporation, Bedford, Massachusetts). Virus was subsequently pelleted in a Beckman ultracentrifuge (model L3- 50) at 4°C (SW 27 rotor at 26000 rpm for 2h). The viral pellets were suspended in RPMI medium (1/30 of the original volume) and stored at -70°C in cryotubes (Nunc). Infectivity titres of the virus stocks, when kept under these conditions, remained stable for at least 3 months. The virus stock used in this experiment had a titer of 10^{3.5} CCID₅₀/ml (50% cell culture infective dose). Titration was based on the viral cytopathic assay described below a 50% endpoint method.

Human anti-HIV serum

A serum with high titre of antibodies against HIV was obtained from a patient. The serum was found positive by ELISA-HTLV-III (ratio> 13.5), Western blot analysis and indirect immunofluorescence in lymphadenopathy associated Molt cells (titre of 1:1000). A dilution in PBS of the positive serum (stored in aliquots at -20°C) was used in all experiments.

Biosafety Laboratory Precautions followed during Experimentation

The culturing of HIV virions on human MT-4 cells was carried out adhering to 'Containment procedures for HIV' laid down by WHO and CDC, Atlanta USA. The most important element of containment was strict adherence to standard tissue culture and microbiological practices and techniques required for safe handling of viral strains and culturing of hitherto infected T-cells. Cultivation of HIV was carried out in the laboratories that met International standards prescribed for this purpose. The laboratory set up was in tune with the recommended P3 containment and utmost care was adopted with respect to handling of blood, body fluids and tissues. The laboratory had developed an operational manual for identification of hazards that may be encountered along with specification of procedures for minimization or elimination of risk factors involved. A scientist trained and knowledgeable in the laboratory techniques, safety procedures and hazards associated with handling infectious agents directed the laboratory activities pertaining to HIV experiments.

Effect of alkaloidal extract of *Phyllanthus niruri* on cytopathic effects induced by HIV-1 /HIV-2 virions human MT-4 cell lines

The anti-HIV activity of the alkaloidal extract of *Phyllanthus niruri* on HIV-1 replication was monitored in terms of its inhibition of virus-induced cytopathic effect in MT-4 cells by MTT assay.⁸ Clinically active Azidothymidine (AZT) was also tested as control reference compound. Flat bottom, 96-well plastic microtitre (Falcon, Becton Dickinson Labware, Lincoln Park, NJ, USA) trays were filled with 160µl of complete medium using a Titretrek multidrop dispenser (Labystems, Finland). Subsequently, stock solutions (10 x final test concentration) of the extracts were added in 40µl volumes to two series of triplicate wells so as to allow simultaneous evaluation of their effects on HIV- and mock-infected cells. Two fold serial dilutions were made directly in the microtitre trays using an eight-channel Titertrek pipette. Untreated control, HIV and mock-infected cell samples were included for each concentration of

the extract. MT-4 cells were infected with HIV-1_{IIIB} at a multiplicity of infection (MOI)⁹ of 0.01. HIV- or mock-infected MT-4 cells (2×10^5 cells per ml, 200 μ l) were placed into 96-welled micro titre plates and incubated in the presence of various concentrations of the extract. After 5 days of culture at 37°C in a carbon-dioxide incubator, cell viability⁹ was quantified by MTT assay. MTT assay is based on the reduction of the yellow colored 3-(4,5-dimethylthiazol-20yl)-2,5-diphenyltetrazolium bromide mitochondrial dehydrogenases of metabolically active cells to a blue colored formazan product, which can be measured spectrophotometrically. Solubilization of the formazan crystals was achieved by 10 per cent (v/v) Triton X-100 in acidified isopropanol. Finally, the absorbances were read in an eight-channel computer controlled photometer at two wave lengths (540nm and 690nm). The absorbance measured at 690nm was automatically subtracted from the absorbance at 540nm, so as to eliminate the effects of non-specific absorption. Blanking was carried out directly on the micro titre trays with the first column wells that contained all reagents except for the MT-4 cells. All data represents the average values for a minimum of three wells. The 50% cytotoxic concentration (CC₅₀) was defined as the concentration of extract that reduced the absorbance (OD₅₄₀) of the mock-infected control sample by 50 per cent. The percent protection achieved by the extract in HIV-infected cells was calculated by the following formula:

$$\frac{(\text{OD}_T)_{\text{HIV}} - (\text{OD}_C)_{\text{HIV}}}{(\text{OD}_C)_{\text{mock}} - (\text{OD}_C)_{\text{HIV}}}$$

$$(\text{OD}_C)_{\text{mock}} - (\text{OD}_C)_{\text{HIV}}$$

Where (OD_T)_{HIV} is the optical density measured with a given concentration of the test extract in the HIV-infected cells; (OD_C)_{HIV} is the optical density measured for the control untreated HIV-infected cells; (OD_C)_{mock} is the optical density measure for the control untreated mock-infected cells; all OD values were determined at 540nm. The concentration achieving 50 per cent protection according to the above formula was defined as the 50 per cent effective concentration (EC₅₀). The ratio between CC₅₀ and EC₅₀ was calculated as Selectivity Index (SI).

Determination of HIV antigen expression by indirect Immunofluorescence

MT-4 cells in microtray wells were infected with HIV and exposed to varying concentrations of the alkaloidal extract of *p.niruri* as described for the cytopathic effects. After 4 days of incubation, the MT-4 cells were transferred to Falcon-2054 tubes and washed three times with cold PBS by centrifugation at 10,000 rpm for 5 min. The same PBS was also used for preparing the serum dilutions, monoclonal antibodies and the fluorescein isothiocyanate (FITC) conjugated IgG. The cell pellet was resuspended in PBS at concentration of 3×10^6 cells per ml of which 10ml was put into each well of the PTFE (Polytetrafluoroethylene) coated multispot microscope slides. Mock-infected MT-4 cells were treated in a similar fashion and included in each slide. The slides were air-dried and fixed in cold acetone at -20°C during 10min. The fixed MT-4 cells were wrapped in absorbent paper, packed in aluminium foil, and stored at -70°C until further analysis. Then, the slides were unwrapped, thawed to room temperature, and 25 μ l of HIV-positive serum (1/1000 dilution) was spotted onto each well. The slides were subsequently incubated in the dark for 45min at 37°C in a humid chamber. The serum was shaken off with one quick motion, and the slides were washed twice with PBS for 10min while under magnetic stirring. The cells were then dried on absorbent paper, and stained by incubation at 37°C for 45 min with 20 μ l of a mixture of goat antihuman IgG conjugated with fluorescein isothiocyanate (FITC) (diluted 1:40 in PBS) and 0.0125% evans blue. The mixture was removed and the slides were washed with PBS for 10 min under

magnetic stirring and dried with absorbent paper. Finally, the cells were immersed in glycerol 70% in PBS, and stored in this form for further analysis. The slides were then examined for immunofluorescence¹⁰ with the fluorescence microscope. Uninfected MT-4 cells were consistently negative under these assay conditions.

Virus (HIV) Adsorption Assay by Cytofluorographic analysis

The inhibitory effect of the alkaloidal extract of *Phyllanthus niruri* on virus adsorption to the cells was measured by an indirect immunofluorescence – Laser Flow Cytofluorographic method.¹⁰ MT-4 cells were exposed to high concentrated HIV-1_{IIIB} virions in the presence or absence of *Phyllanthus niruri*. The extract was added one minute before virus was added. The cells were incubated for one hour at 37°C and washed twice in phosphate buffered saline (PBS, pH 7.4) to remove unabsorbed virus. Then a high titre polyclonal antibody derived from a patient with AIDS related complex (diluted 1/500 in PBS) was added. After one-hour incubation at 37°C, the cells were washed twice with PBS. The cells were then incubated with Fluorescein is thiocyanateconjugated F (ab'2) fragments of rabbit anti-human immunoglobulin antibody (diluted 1/30 in PBS), resuspended in one ml of 0.5 per cent paraformaldehyde in PBS and analysed by Laser Flow Cytofluorography.

RESULTS

Efforts to gain information on the anti-HIV activity of alkaloidal extract of *Phyllanthus niruri* showed promising activity for *Phyllanthus niruri* when tested on strains of HIV-1 cells cultured on MT-4 cell lines as shown in Table 1. The CC_{50} for the extract was found to be $279.85 (\pm 15.24) \mu\text{g mL}^{-1}$ whereas the EC_{50} was found to be $20.98 (\pm 4.12) \mu\text{g mL}^{-1}$. Interestingly the Selectivity Index (SI) was found to be 13.34, which shows a clear selective toxicity of the alkaloidal extract for the viral cells. The SI is an important parameter as it includes both antiviral activity and eventual toxicity of the test compound (*Phyllanthus niruri*). Higher the value of SI, lower is the toxicity to the host cells and higher is the effect against virus. The SI of reference compound Azidothymidine (AZT) was found to be greater at 2046.11. The alkaloidal extract of *Phyllanthus niruri* when tested on HIV-2 cells also showed significant inhibition evident by an EC_{50} of $11.53 (\pm 2.91) \mu\text{g mL}^{-1}$ with a Selectivity Index of 25.83 as shown in Table 2 which was higher than that observed for HIV-1 cell lines.

The growth characteristics of HIV-infected and mock-infected MT-4 cells indicate that from the 3rd day post infection, the viability of HIV-infected MT4 cells rapidly declined and by the 5th day, all cells were dead. When the alkaloidal extract of *Phyllanthus niruri* and Azidothymidine were evaluated for their inhibitory effects on the cytopathogenicity of HIV in MT-4 cells, they proved quite effective in protecting cell destruction by the virus.

The concentration of *Phyllanthus niruri* obtained in MTT assay for HIV-1 cells was tested in Virus adsorption assay at almost twice the higher concentration of EC_{50} . A complete inhibition of virus adsorption to the cells was found at this higher concentration of *Phyllanthus niruri* as shown in Table 3.

DISCUSSION

Phyllanthus niruri is the most effective of a group of closely related species that grow in India, China, and tropical locations ranging from the Philippines to Cuba and has been used in folk medicine to treat a variety of maladies particularly its role in viral hepatitis.² Scientists have not identified the ingredient responsible for its medicinal effect, but the herb has been shown to block an enzyme that plays a crucial

role in reproduction of the hepatitis B virus (HBV). Insofar as is known, however, only the aqueous extract of *Phyllanthus niruri* has been proposed to inhibit HIV strains but then the active constituents responsible for its anti-viral activity have not yet been identified. In the present pharmacological investigation, the alkaloidal extract of *Phyllanthus niruri* specifically exerted growth inhibition on both the HIV-1 and HIV-2 strains cultured on human MT-4 cells. Small amounts of *Phyllanthus niruri* were found to reduce viral replication while higher concentrations totally inhibited its reproduction. More importantly, with a higher selectivity index, the alkaloidal extract was non-toxic to the human cells at concentrations stopping viral replication. Based on these research findings, it would be optimistic to analyse the utilization of the alkaloidal extract of *Phyllanthus niruri* in alleviating immune-linked life-threatening diseases like AIDS on account of its promising anti-HIV activity. The data obtained holds prominent significance in the light of the fact that studies on *Phyllanthus niruri* till date have never focused on the identification of constituent's responsible for inhibitory effect on cytopathogenicity induced by HIV on human MT-4 cell lines. The results obtained herewith calls for unlocking the hidden potentials of this indigenous herb focusing on other pertinent biological effects associated with it. More studies in different clinical settings and different populations with a broader view point to characterize the individual functional components of *Phyllanthus niruri* which could serve as potential leads for further optimization continues to be future focus of research.

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Table 1: Inhibitory activity of alkaloidal extract on cytopathic effects induced by HIV-1 virions on human MT-4 cells

<i>Sr. No.</i>	<i>Drug</i>	<i>CC₅₀ (µg/ml)</i> (50% Cytotoxic concentration)	<i>EC₅₀ (µg/ml)</i> (50% Effective concentration)	<i>SI</i> (Selectivity Index)
1	Phyllanthus niruri	297.85* (±15.24)	20.98* (±4.72)	13
2	Azidothymidine(µm)	12.89* (±1.29)	0.0063** (±0.02)	2046.11

* P<0.1, ** P<0.005, Values in µm: micromolar

(Data ± mean S.D of triplicate experiment)

Table 2: Inhibitory activity of alkaloidal extract on cytopathic effects induced by HIV-2 virions on human MT-4 cells

<i>Sr. No.</i>	<i>Drug</i>	<i>CC₅₀ (µg/ml)</i> (50% Cytotoxic concentration)	<i>EC₅₀ (µg/ml)</i> (50% Effective concentration)	<i>SI</i> (Selectivity Index)
1.	Phyllanthus niruri	297.85* (± 15.24)	11.53* (± 2.91)	25.83
2.	Azidothymidine (µm)	12.89* (± 1.29)	0.0097** (± 0.02)	1328.56

* P<0.1, ** P<0.005, Values in µm: micromolar

(Data ± mean S.D of triplicate experiment)

Table 3:HIV - Adsorption assay

<i>Sr. No.</i>	<i>Drug</i>	<i>Concentration (µg/ml)</i>	<i>Inhibition %</i>
1	Phyllanthus niruri	500	93.86