



IN VITRO CYTOTOXIC AND ANTI-HIV ACTIVITY OF *PHYLLANTHUS NIRURI* WHOLE PLANT EXTRACTS

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ABSTRACT

HIV is the etiological agent of AIDS that has created major health care problem throughout the world. The aim of the present study is to evaluate the cytotoxic effects and anti-HIV activity of *Phyllanthus niruri* whole plant extracts. Hexane, chloroform, ethyl acetate, acetone and methanol solvents were used to prepare the extracts by sequential maceration method. RetroSys HIV-1 RT (Innovagen, Sweden) kit was used to determine the anti-HIV activity of all solvents extracts. Cytotoxicity study was performed on all extracts by MTT assay using PBMC's. All extracts exhibited the most notable activity. The chloroform and hexane extracts of *Phyllanthus niruri* showing highest (88.7% and 87.8%) HIV-RT inhibition at 2mg/ml concentration. While control drug (AZT) showing 91.7% at 2mg/ml concentration. IC₅₀ value of all extracts determined below 40mg/ml. This result suggests that *Phyllanthus niruri* whole plant extracts contains medicinally important bioactive compounds towards anti-HIV.

KEYWORDS: HIV, AIDS, PBMC's, Cytotoxicity, *Phyllanthus niruri*



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INTRODUCTION

HIV (Human Immunodeficiency Virus) virus that causes AIDS (Acquired Immunodeficiency Syndrome) is one of the hottest areas of medical research today. It is estimated that 35.3 million people throughout the world were infected with HIV by the end of 2012¹. Several treatments (ART and HAART) that have been developed dramatically improved the outlook for many HIV patients. However, these treatments have many severe side effects, and in most cases are too costly to be administered widely in many parts of the world. According to WHO² traditional medicine safer, more accessible and sustainable. Thus, more research is needed to improve the available treatments, making them more tolerable to patients. Medicinal plants have always been the main constituents of the traditional medicine³. Medicinal plants are a good source for the discovery of novel antimicrobial chemotherapeutic agents⁴. The ancient civilizations of the Chinese, Indians and North Africans provide written evidence for the use of natural sources for curing various diseases⁵. Current therapies that directly target to virus are often ineffective due to the emergence of drug resistance and viral variants⁶. Plants have a great potential for producing new drugs for human benefit⁷. Therefore, the discovery of novel drugs with new mechanisms of action, low toxicity, high activity and well tolerability is still a challenging issue in HIV treatment⁸. Medicinal plants play an important role in supporting the healthcare system in the World⁹. Natural products have been the basis of treating and preventing human diseases¹⁰. Several studies have described the inhibitory properties of medicinal plants on different targets in the life cycle of HIV^{11, 12}. Various studies have shown anti-HIV properties of the extracts prepared from variety of plants^{13, 14, 15}. Therefore, screening of potential anti-HIV agents from medicinal plants may be a rapid and effective way for drug discovery. Based on previous research work¹⁶ the *Phyllanthus niruri* whole plant selected to test anti-HIV activity and cytotoxic

effects. It was therefore decided to analyze the anti-HIV activity of *Phyllanthus niruri* whole plant extracts and also evaluate its cytotoxicity in PBMC cells.

MATERIALS AND METHODS

Plant collection

Plant was selected for this study is based on its traditional medicinal use. Whole plant of *Phyllanthus niruri* was collected from the Chintur mandal, Khammam district of Telangana, India, in the month of September 2012. The plant voucher specimen identification was done with the help of Prof. Vastsavaya.S.Raju Department of Botany Kakatiya University, Warangal and the same was deposited at Infectious Diseases & Metabolic Disorders Research Lab, Department of Zoology, Kakatiya University, Warangal.

Preparation of plant extract

After collection of selected medicinal plant material sample was dried at room temperature until they were free from moisture. The selected part of plant subjected to size reduction to get coarse powder was then stored in a clean dry air tight container. The air dried powder was subjected to the sequential maceration method used by different solvents (hexane, chloroform, ethyl acetate, acetone, and methanol etc ;) for seven days. The extract was filtered mass was obtained and it was finally dried at low room temperature under pressure in a rotary vacuum evaporator (Thermotech, buchi type model th-012).

HIV-1 Reverse Transcriptase Inhibition Assay

The HIV reverse transcriptase enzyme inhibition due to each extract was determined using HIV- RT inhibition assay by using of Retro Sys HIV-1 RT activity kit (Innovagen, Sweden). To determining RT activity on inhibiting substances that are to be analysed are serially diluted. The diluted

substances are then added to a plate with reaction mixture. After 30 minutes of pre-incubation at 33°C, the reaction is started by the addition of a standardised amount of RT. The RT will now incorporate BrdUMP depending on the level of inhibition. The product is quantified by the addition of the RT Product Tracer which binds to the incorporated BrdUMP. After removing excess tracer the amount of bound tracer is determined by an alkaline phosphatase / pNPP colour reaction¹⁷. After correction for background signal, the measured

residual RT activity for each substance dilution is calculated as a percentage of the measured RT activity in absence of inhibiting substances. Plot the percentage of residual RT activity against the concentrations of the substance dilutions for each of the tested substances. AZT (Azidothymidine) was used as control. The inhibitory effect of each substance is expressed by RT activity and is determined with the aid of the obtained graph. The percentage inhibition of HIV-1 RT was calculated as,

$$\text{Inhibition (\%)} = \left(\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100.$$

Where, A is Optical Density (OD).

Preparation of Peripheral Blood Mononuclear Cells (PBMCs)

Aseptically 2.5ml of HiSep media transferred in to a 15 ml heparin coated test tubes and overlay with 7.5ml diluted blood (blood sample from healthy volunteers were collected by venipuncture and blood sample were diluted at 1:1 ratio with PBS). Centrifuged at 1,000 x g for 30 minutes. During the centrifugation the PBMC's moved from the plasma and were suspended in the density gradient, isolating them from erythrocytes and granulocytes. The PBMC's layer was removed and then washed twice with PBS centrifuged at 400 x g. The supernatant was then removed and the PBMC's were resuspended in RPMI 1640 medium.

Cytotoxicity Screening by MTT assay

Cell viability was determined by the MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test method. MTT (5 mg/ml) was dissolved in PBS. PBMC cells were cultured in 96-well plates

containing 100 µl medium prior to treatment with different solvent extracts of *Phyllanthus niruri*. To that, 100 µl DMSO solution containing various concentrations (0.0625, 0.125, 0.25, 0.5, 1.0 and 2.0mg/ml) of extracts was added to each well, and incubated for 37°C for 24 h. Diluted extracted solutions were freshly prepared in DMSO prior to each experiment. The metabolic activity of each well was determined by the MTT assay and compared to those of untreated cells. After removal of 100 µl medium, MTT dye solution was added (15 µl / 100 µl medium) and the plates were incubated at 37°C for 4 h. After that, 100 µl of DMSO were added to each well, and mixed thoroughly. The absorbance was measured at 570 nm with a reference wavelength of 630 nm. High optical density readings corresponded to a high intensity of dye colour that is to a high number of viable cells able to metabolize MTT salts. The fractional absorbance was calculated by the following formula.

$$\% \text{ cell inhibition} = 100 - [(A_t - A_b) / (A_c - A_b)] \times 100$$

Where, A_t = Absorbance value of test compound,
 A_b = Absorbance value of blank
 A_c = Absorbance value of control

The effects of extracts were expressed by IC₅₀ values (the drug concentration, reducing the absorbance of treated cells by 50% with respect to untreated cells). Dose-response curves between percentage of cell inhibition and concentrations of *Phyllanthus niruri* extractions were constructed. The IC₅₀ value was determined from the plotted curve.

RESULTS

Percentage of yield extract

The yield of sequential extracts of *Phyllanthus niruri* whole plant (g) is shown in (Table 1). The amount obtained from hexane, chloroform, ethyl acetate, acetone and methanol extractions are 6.22 gm, 5.12 gm, 3.66 gm, 4.01 gm, and 4.98 gm respectively.

Table 1
Extractive values of different extracts of *Phyllanthus niruri* whole plant

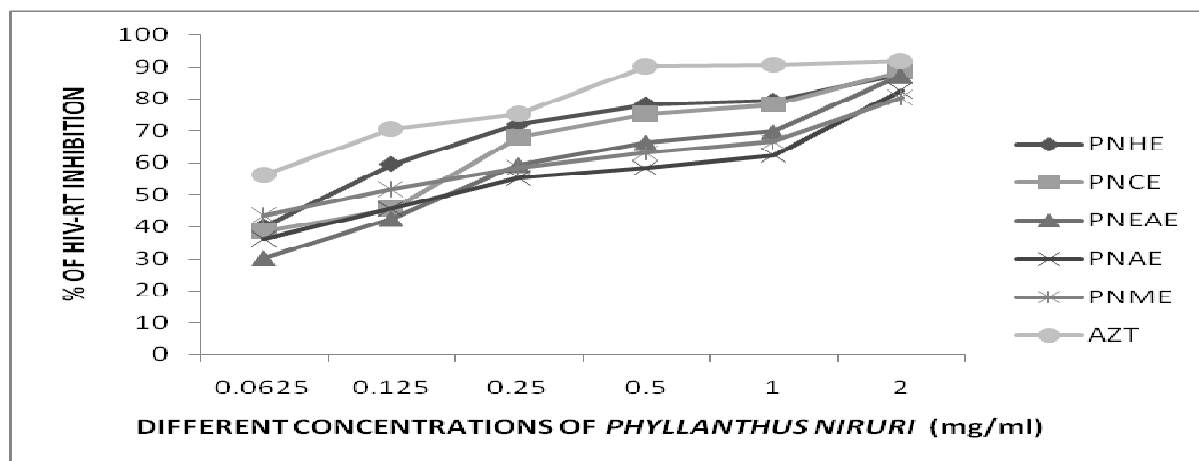
S.No	Solvent	Color of extract	Yield of the extract (in gm)	Percentage yield(%w/w)
1	Hexane	Dark green	6.22	2.07%
2	Chloroform	Dark green	5.12	1.70%
3	Ethyl acetate	Green	3.66	1.22%
4	Acetone	Green	4.01	1.33%
5	Methanol	Dark green	4.98	1.66%

Anti-HIV activity of *Phyllanthus niruri* whole plant extracts

Inhibition of HIV-RT by *Phyllanthus niruri* whole plant crude extractions were presented in Figure 1. Chloroform and hexane extracts shows highest inhibition of recombinant HIV-RT (88.7% and 87.8% respectively) at 2 mg/ml

concentration. Ethyl acetate, acetone and methanol extracts shows highest inhibition of HIV-RT at 2 mg/ml concentration (87.2%, 82.5% and 80.5% respectively). While control drug (AZT) showing 91.7% at 2mg/ml concentration.

Figure 1
In vitro HIV-RT inhibitory activity of *Phyllanthus niruri* whole plant crude extractions



Cytotoxicity of *Phyllanthus niruri* whole plant crude extraction on PBMC cells

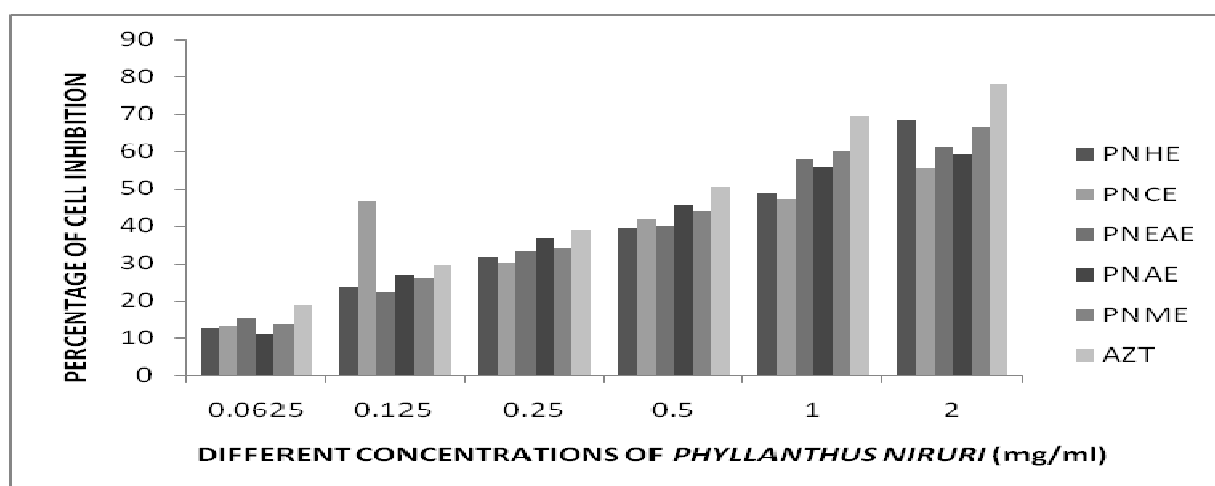
Cytotoxicity activity of *Phyllanthus niruri* whole plant crude extractions were carried out against PBMC's at different concentrations to

determine the IC₅₀ (50% growth inhibition) by MTT assay. Results of different concentrations of *Phyllanthus niruri* crude extractions including 0.0625, 0.125, 0.25, 0.5, 1.0 and 2.0mg/ml graphically represented in figure 2.

MTT assay of *Phyllanthus niruri* crude extractions shows significant effect on PBMC's in different concentration. The highest cytotoxicity of hexane extraction against PBMC's was found in 2.0 mg/ml concentration with 68.5% of cell growth inhibition while methanol, ethyl acetate, acetone and chloroform extractions showing 66.5%, 61.0%,

59.2% and 55.5% at 2mg/ml respectively and control drug (AZT) showing 78% of cell growth inhibition. It was found that the percentage of growth inhibition to be increasing with increasing concentration of test compounds. The standard drug showing IC₅₀ value at 0.25mg/ml and IC₅₀ value of chloroform assay was 0.40mg/ml.

Figure 2
Effect of *Phyllanthus niruri* whole plant crude extraction on PBMC cells



DISCUSSION

The herbal products today symbolize safety in contrast to the synthetics that are regarded as unsafe to humans¹⁸. Natural plant derived drugs continue to be excellent source of new drug candidates with anti HIV effect and the results and experiences with many of the anti HIV natural products will inspire and motivate even more researchers to look for new leads from plants and natural sources¹⁹. The need for novel antiviral for managing HIV/AIDS is incontestable. Our laboratory has been actively involved in the research of novel experimental moieties for their potential anti-HIV activity. Previous studies^{20, 21} are showed that the *Aerva lanata* roots, *Madhuca indica* inner bark and *Calotropis gigantea* flowers crude extracts were equally effective against isolates that were sensitive and potentially resistant to standard anti-retroviral drugs AZT. In the present study, the assay was optimized and standardized with respect to various experimental parameters and then applied to test the HIV-RT inhibitory

activity of the different extracts. At the concentration of 0.5 mg/ml to 2 mg/ml all extractions of *Phyllanthus niruri* whole plant shows significant inhibition of recombinant HIV-RT. The results obtained in the present investigation indicated that *Phyllanthus niruri* with chloroform extraction shows highest inhibition activity (88.7% at 2mg/ml) against HIV-RT when compared to other extractions, while control drug (AZT) shows 91.7% at 2mg/ml concentration. In the present study, the cytotoxic effect of *Phyllanthus niruri* extractions on PBMC's was evaluated by MTT assay. Different concentrations of extractions show more than 50% cell viability from 0.0625mg/ml to 0.5mg/ml.

CONCLUSION

Experimental results in the present investigation indicated *Phyllanthus niruri* as a rich source of secondary metabolites. The

whole plant of *Phyllanthus niruri* can provide lead molecules which could be useful substrate for the synthesis of new broad spectrum antibiotics for the treatment of infections caused by the organisms. Thus the present study does seem to justify the traditional use of plants for

the treatment of infectious diseases of viral origin. Therefore in order to assess the usefulness of this plant, it is necessary to isolate the active principle compound from crude extracts, identify them and study their mechanism of action.

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