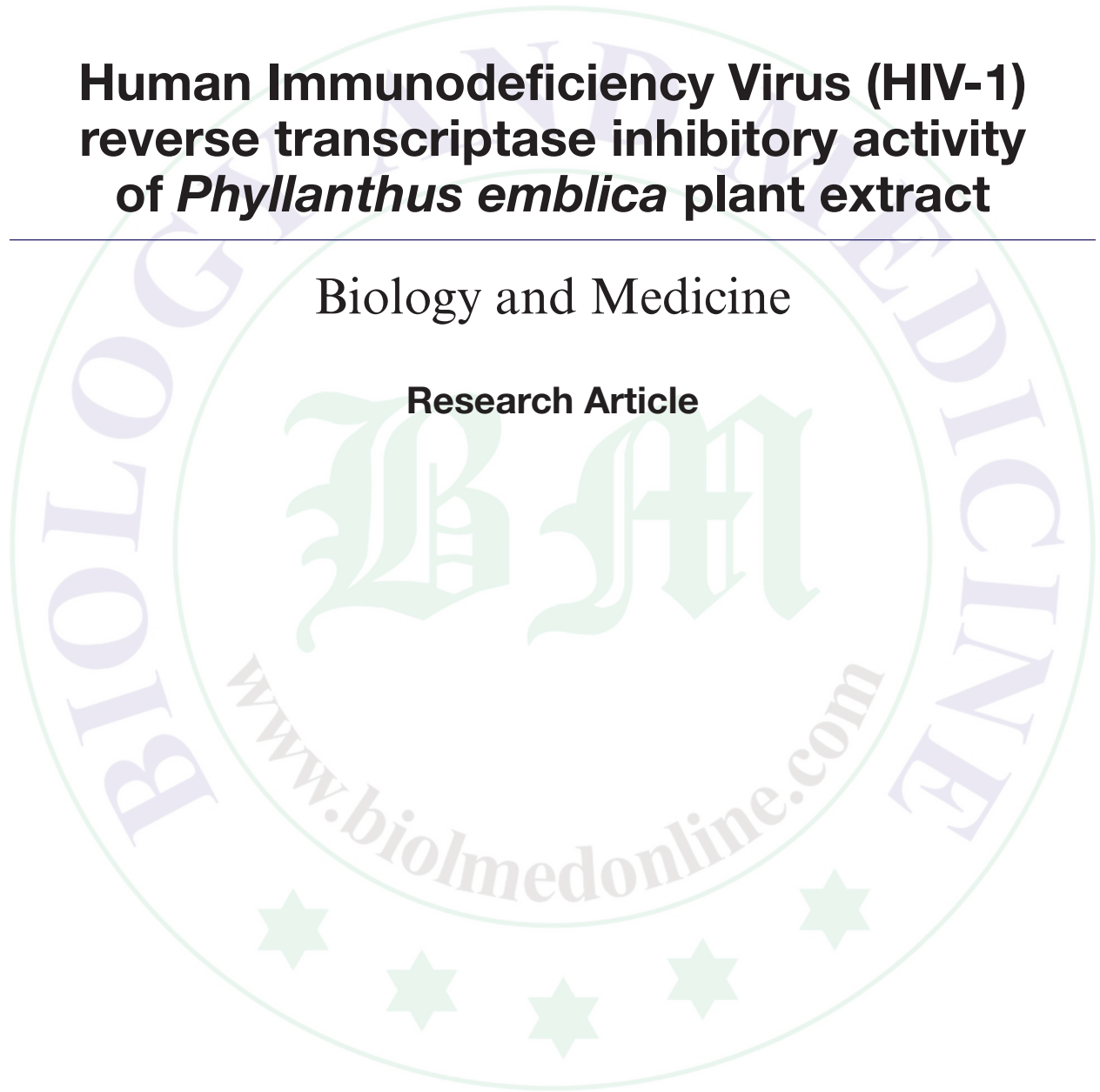


eISSN: 09748369

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Biology and Medicine

Research Article



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Human Immunodeficiency Virus (HIV-1) reverse transcriptase inhibitory activity of *Phyllanthus emblica* plant extract

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Accepted: 10th Nov 2012, Published: 23rd Nov 2012

Abstract

Human Immunodeficiency Virus type-1 (HIV-1) is the cause of Acquired Immune Deficiency Syndrome (AIDS), a major human viral disease with about 33.2 million people infected worldwide. The high cost of the HAART regimen has impeded its delivery to over 90% of the HIV/AIDS population in the world. The aim of the present study was to evaluate the in vitro anti-HIV activity of *Phyllanthus emblica* plant extracts. Extracts were prepared from dried fruit in n-hexane, ethyl acetate, and n-butanol. Peripheral Blood Mononuclear Cells (PBMCs) isolated from healthy donors by ficoll-hypaque density gradient centrifugation method. A toxicity study was performed on all crude extracts by MTT assay using PBMCs isolated from whole blood. HIV-1 RT inhibition activity of the all solvent extracts of *P. emblica* was determined. AQF and HXF fractions show highest inhibition of recombinant HIV-RT (91% and 89% respectively) at 1 mg/ml concentration. CFF fraction shows highest inhibition of HIV-RT at 0.5 mg/ml and CTF fraction at 0.12 mg/ml concentration. Experimental results thus suggested that the *P. emblica* plant extracts which have been tested in the present study exert their anti-HIV activity via inhibition of HIV reverse transcriptase activity. Thus the present study seems to justify the traditional use of plant for the treatment of infectious disease of viral origin. However, in order to assess the usefulness of this herb, it is necessary to isolate the active principle(s) from the crude and fractions, identify them and study their mechanism of action.

Keywords: HIV; *Phyllanthus emblica*; PBMCs; HIV-1 RT; cytotoxicity; HIV-1.

Introduction

Since the discovery of the Human Immunodeficiency Virus as the causative agent of AIDS New chemical entities with such activity may be identified through a variety of approaches, one of them being the screening of natural products. Plant substances are especially explored due to their amazing structural diversity and their broad range of biological activities. Several plant extracts have been shown to possess activity against HIV by inhibiting various viral enzymes (Vermani and Garg, 2002). Various resource-poor settings, government-sponsored ART programmes discourage the use of traditional medicines, fearing that the efficacy of antiretroviral drugs may be inhibited by such natural products, or that their pharmacological interactions could lead to toxicity (Husain *et al.*, 1992). Medicinal plants like *Osimum sanctum* (Anuya *et al.*, 2010), *Phyllanthus myrtifolium*

(Chang *et al.*, 1995), *Linocera japonica* (Joshi, 2002; Harris *et al.*, 2001), *Rhus chinensis* (Wang *et al.*, 2008; Vermani and Garg, 2002) and *Jatropha curcas* (Chinsembu and Hedimbi, 2010) as potential sources of new active agents not only combine the advantage of being relatively non-toxic and hence more tolerable than rationally designed drugs, but also represent an affordable and valuable source of pharmacologically active substances that can be made sufficiently available through cultivation.

With the rapid explosion of new molecular targets available for drug discovery and advances in high throughput screening technology, there has been a dramatic increase in interest from the pharmaceutical and biotechnology industries in the huge molecular diversity present in plant sources. In this study the medicinal plant extracts used in tribal areas of Warangal districts exhibits significant potency against various bacterial and fungal pathogens, as well as potent

antioxidant activity. It was therefore decided to analyse the anti-HIV activity of these potential medicinal plant and also evaluate its cytotoxicity in PBMC cell cultures.

Materials and Methods

(i) Preparation of plant extracts

P. emblica fruits are collected from Parvatagiri Village of Torur Mandal, Warangal district, Andhra Pradesh. Voucher specimens were prepared and identified at the Department of Botany, Kakatiya University, Warangal. The *P. emblica* fruits were collected and left at room temperature for two weeks to dry, then ground into powder and extracted with Soxhlet techniques with methanol. Obtaining methanolic crude extracts of *P. emblica* were then fractionated successively using solvents of increasing polarity, such as, n-hexane (HX), carbon tetrachloride (CT), chloroform (CF), and aqueous fractions (AQ). All the four fractions (HXF, CTF, CFF, and AQF) were evaporated to dryness by using rotary evaporator at low temperature (39°C).

(ii) Isolation of PBMCs

Peripheral Blood Mononuclear Cells (PBMCs) were collected from the blood of healthy volunteers, by ficoll-hypaque density gradient centrifugation method (Indiveri *et al.*, 1980) by venipuncture and transferred into 15ml heparin coated test tubes. The samples were diluted at 1:1 ratio with PBS, layered onto HISEP media (Himedia, Mumbai) at a volume ratio of 3:1 and centrifuged at $1000 \times g$ for 30 min. During the centrifugation the PBMCs moved from the plasma and were suspended in the density gradient, the PBMCs layer was removed and then washed twice with PBS. The supernatant was then removed and the cells were resuspended in RPMI 1640 medium supplemented with 1mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin, 10% inactivated FBS, and adjusted to pH 7.2 by the addition of 15 mM HEPES. The PBMC cell density used in the cytotoxicity study was 1×10^5 cells/well of the 96-well tissue culture plate. Dose-response curves between percentage of cell viability and concentrations of the extracts were constructed. The IC_{50} value was determined from the plotted curve.

(iii) Cell viability by MTT assay

Cell viability was determined by the MTT 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test method (Mosmann, 1983). Briefly, MTT (5 mg/ml) was dissolved in PBS. PBMC Cells were cultured in 96-well plates (1.0×10^4 cells/well) containing 100 µl medium prior to treatment with four fractions of selected plants at 37°C for 24 h. After that, 100 µl fresh medium containing various concentrations (0.02, 0.04, 0.09, 0.18, 0.37, 0.75, and 1.5 mg/ml) of fractional extracts were added to each well, and incubated for another 48 h. Diluted fractional extracts solutions were freshly prepared in DMSO. 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 color that is to a high number of viable cells able to metabolize MTT salts. The fractional absorbance was calculated by the following formula

% Cell viability =

$$\frac{\text{Mean absorbance in test wells}}{\text{Mean absorbance in control wells}} \times 100.$$

(iv) HIV-1 reverse transcriptase inhibition assay

The HIV reverse transcriptase enzyme inhibition due to each fraction was determined using HIV-RT inhibition assay (Xingwu *et al.*, 1996; Ekstrand *et al.*, 1996) by using of Retro Sys HIV-1 RT activity kit (Innovagen, Sweden). When determining IC_{50} values the substances that are to be analysed are serially diluted. The diluted substances are then added to a plate with reaction mixture. After 30 min 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 reaction is stopped by washing the plate. 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

color reaction. 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/Zidovudine) was used as positive control. The inhibitory effect of each substance is expressed as an IC_{50} value i.e. the concentration at which 50% of the RT activity is inhibited or the IC_{50} value is the substance concentration giving a 50% inhibition of the RT activity and is determined with the aid of the obtained graph. The percentage inhibition of HIV-1 RT was calculated as,

$$\text{Inhibition (\%)} = \frac{[(A \text{ control} - A \text{ sample}) / A \text{ control}] \times 100.$$

(v) Statistical analysis

For statistical analysis, the results of anti-HIV-1 RT activity were expressed as means \pm SD of three determinations. The IC_{50} values were calculated using the Microsoft Excel program. Results were considered significant if the p -values were less than 0.05.

Results and Discussion

(a) Percentage yield

The yield of methanol crude extract of *P. emblica* was 75g (15%). The percentage yield of these

fractions of the methanolic extract of *P. emblica* were showed in the Table 1. The CTF fractions obtained highest yield (2.9%) when compared to other fractions. 0.8% yield obtained in AQF which is lowest.

Table 1: The percentage of yield obtained from crude methanol extract.

S. No.	Fractions	Yield (%)
1	HXF	1.8
2	CTF	2.9
3	CFF	1.6
4	AQF	0.8

(b) Cytotoxicity of extract on PBMC cell

After cells were treated with different fractions of *P. emblica* at various concentrations for 48h, the cytotoxic effects were investigated using the MTT assay. Cytotoxicity of each extract fraction was determined by an inhibitory concentration at 50% growth (IC_{50}). All the four fractions of *P. emblica* were non-cytotoxic till 0.75mg/ml concentration in PBMC cells. AQF fraction is non-cytotoxic even at 1.5mg/ml (51% cell viability). The highest non-cytotoxic concentration at which more than 95% cells were viable was calculated for each of the fraction in PBMC cells. The results are showed in Figure 1. The highest non-cytotoxic concentration (>95% cell viability) of HXF, CTF, CFF, and AQF fractions are 0.02, 0.04, 0.02, and 0.02 mg/ml respectively.

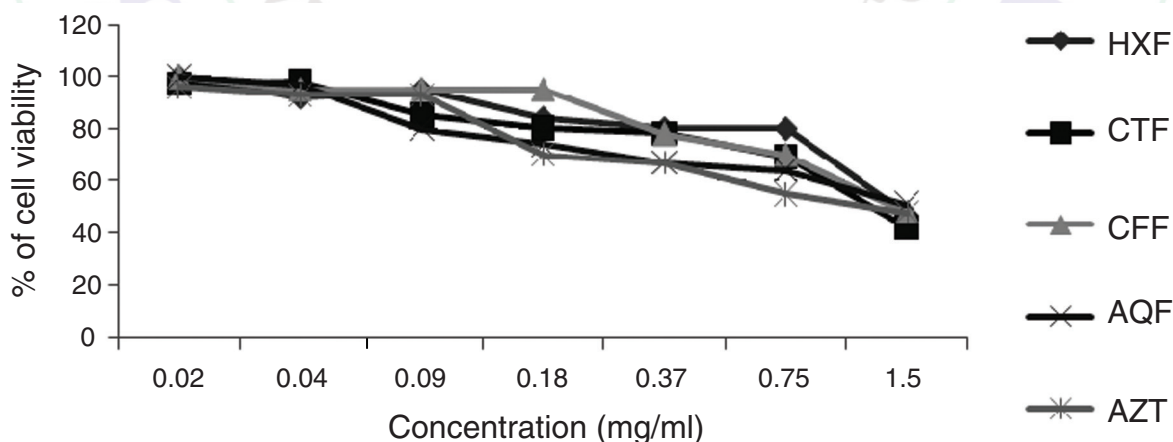


Figure 1: Effects of *P. emblica* extract fractions on PBMC cells.

(HXF = n-hexane fraction, CTF = carbon tetrachloride fraction, CFF = chloroform fraction, AQF = aqueous fraction, and AZT = zidovudine).

(c) Anti-HIV activity

Inhibition of HIV-RT by *P. emblica* plant extract fractions were presented in Figure 2. AQF and HXF fractions show highest inhibition of recombinant HIV-RT (91% and 89% respectively) at 1 mg/ml concentration. CFF fraction shows highest inhibition of HIV-RT at 0.5 mg/ml and CTF fraction at 0.12 mg/ml concentration. The IC_{50} of the CFF and AQF fractions are more than 100. At 0.12 mg/ml and 0.5 concentrations 50% of the HIV-RT activity is inhibited in HXF and CTF fractions respectively.

Over the years, parts of the many medicinal plants have been used for medicine including claims of its antiretroviral potential (Indiveri *et al.*, 1980). Anti-HIV agent which could possibly inhibit the early stages of the HIV replicative cycle would be very useful in treating HIV infection. Our results demonstrate that compared to the standard anti-HIV drug AZT, a CFF fraction of *P. emblica* shows highest inhibition of HIV-RT at 0.5 mg/ml and CTF fraction at 0.12 mg/ml concentration (Figure 2). These data were in good agreement with the results of one previous study on the inhibition of HIV infection by medicinal plant extracts (Mahmood *et al.*, 1993; Chinsembu and Hedimbi, 2010). Our previous investigations established that different medicinal plant extracts inhibit HIV reverse transcriptase in a non-specific manner (Venkanna and Estari, 2012).

The strongest inhibitory action against HIV-1 RT was found in *P. emblica*. The results

showed that this plants contained anti-HIV properties, which was in accordance with previous reports in which the different plants *A. calamus* L. and *P. indica* L. exhibited potent antiviral activity against the Herpes simplex viruses HSV-1 and HSV-2 (Elaya *et al.*, 2009; Akanitapichat *et al.*, 2002). *A. sativum* was reported to be effective against HIV infection by inhibiting virus replication (Harris *et al.*, 2001; Wang *et al.*, 2004) specifically by interfering with viral reverse transcriptase activity. *O. sanctum* L. was found to demonstrate antibacterial, anti-fungal, and antiviral activity (Gupta *et al.*, 2005; Mahmood *et al.*, 1993). This report also showed that the medicinal plants possessed an anti-HIV property through inhibition of viral reverse transcriptase activity.

Conclusion

Experimental results thus suggested that the *P. emblica* plant extracts which have been tested in the present study exert their anti-HIV activity via inhibition of HIV reverse transcriptase activity. Thus the present study down seems to justify the traditional use of plant for the treatment of infectious disease of viral origin. However, in order to assess the usefulness of this herb, it is necessary to isolate the active principle(s) from the crude and fractions, identify them and study their mechanism of action.

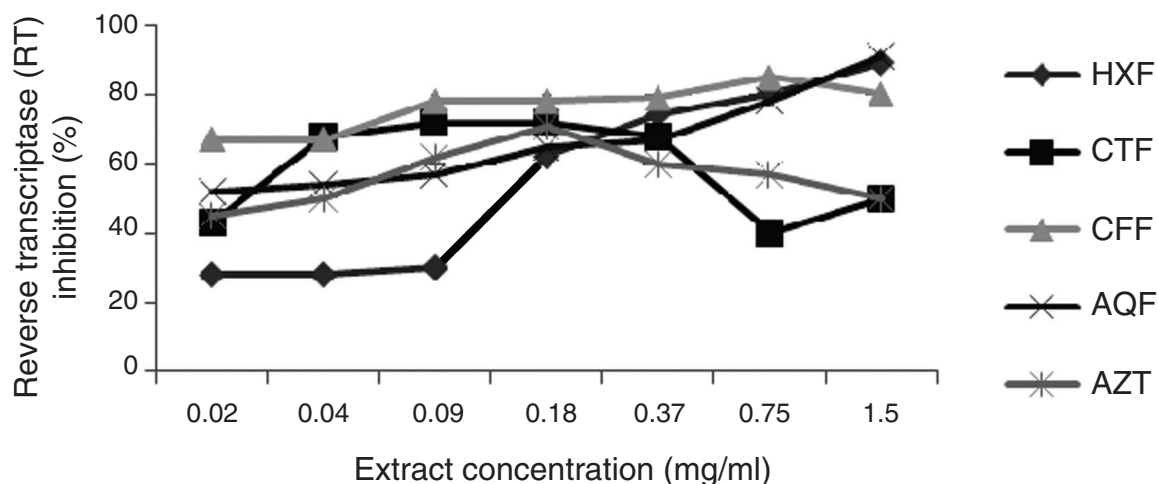


Figure 2: Inhibition of HIV-RT by *P. emblica* plant different fractions at different concentrations. (HXF = n-hexane fraction, CTF = carbon tetrachloride fraction, CFF = chloroform fraction, AQF = aqueous fraction, and AZT = zidovudine).

Ethical Approval

The study was approved by the ethics committee of Department of Zoology, Kakatiya University.

Conflict of Interests

None.

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