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STUDIES ON ANTI-HIV ACTIVITY OF *PHYLLANTHUS EMBLICA* PLANT EXTRACTS

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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 *invitro* 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 *Phyllanthus emblica* was determined. AQF and HXF fractions show highest inhibition of recombinant HIV-RT (91% and 89% respectively) at 1 mg/mL concentration. Chloroform (CFF) fraction shows highest inhibition of HIV-RT at 0.5 mg/mL and Carbon tetra chloride (CTF) fraction at 0.12 mg/mL concentration. 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. At 0.12 mg/mL and 0.5 concentrations 50% of the HIV-RT activity is inhibited in HXF and CTF fractions respectively. The fruit of *Phyllanthus emblica* extracts are shows anti-HIV-1 activity and this plant has great potential for developing useful drugs.

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 *et al*., 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 (Chinsembu, 2009). Medicinal plants like *Osimum sanctum* (Anuya *et al*., 2010), *Phyllanthus myrtifolium* (Chang *et al*., 1995), *Linocera japonica* (Joshi, 2002), *Rhus chinensis* (Rui-Rui wang *et al*., 2006) and *Jatropha curcas* (Kazhila *et al*., 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 is 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

Preparation of plant extracts

Phyllanthus emblica fruits are collected from Parvatagiri Village of Torrur Mandal, Warangal district, Andhra Pradesh. Voucher specimens were prepared and identified at the Department of Botany, Kakatiya University, Warangal. The *Phyllanthus emblica* fruits were collected and left at room temperature for two weeks to dry, then ground into powder and extraction with Soxhlet techniques with methanol. Obtaining methanolic crude extracts of *Phyllanthus emblica* were then fractionated successively using solvents of increasing polarity, such as, *n*-hexane (HX), carbon tetrachloride (CT) and 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 (39ºC).

Isolation of PBMCs

Peripheral Blood Mononuclear Cells (PBMCs) were collected from the blood of healthy volunteers, by ficol-Hypaque density gradient centrifugation method (Indiveri *et al.,* 1980) by venipuncture and transferred into 15 mL 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 1,000 x 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 1 m ML-glutamine, 100units/ mL penicillin and 100μg/mL streptomycin, 10% inactivated FBS, and adjusted to pH 7.2 by the addition of 15mM HEPES. The PBMC cell density used in the cytotoxicity study was 1 x 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 LC_{50} value was determined from the plotted curve.

Cell viability by MTT Assay

Cell viability was determined by the MTT 3-(4, 5dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide) test method (Mosmann and Tim, 1983). Briefly, MTT (5mg/mL) was dissolved in PBS. PBMC Cells were cultured in 96-well plates (1.0 x 10^4 cells/ well) containing 100μL medium prior to treatment with four fractions of selected plants at 37ºC for 24h. After that, 100μL fresh medium containing various concentrations (0.02, 0.04, 0.09, 0.18, 0.37, 0.75 and 1.5mg/ mL) of fractional extracts were added to each well, and incubated for another 48h. 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 100μL medium, MTT dye solution was added (15μL/ 100μL medium) and the plates were incubated at 37ºC for 4h. After that, 100μL of DMSO were added to each well, and mixed thoroughly. The absorbance was measured at 570nm with a reference wavelength of 630nm. 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

$$\% \text{ Cell viability} = \frac{\text{Mean absorbance in test wells}}{\text{Mean absorbance in control wells}} \times 100$$

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 LC_{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 minutes of pre incubation at 33ºC, 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 colour 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 zidothymidine/Zidovudine) was used as positive control. The inhibitory effect of each substance is expressed as an LC_{50} value *i.e.* the concentration at which 50% of the RT activity is inhibited or the LC_{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 calculates as,

Inhibition (%) = [(A control-A sample) / A control] x 100.

Statistical analysis

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

RESULTS

Percentage yield

The yield of methanol crude extract of *Phyllanthus emblica* was 75 (15%) g. The percentage yield of these fractions of the methanolic extract of *Phyllanthus 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.

Cytotoxicity of extract on PBMC cell

After cells were treated with different fractions of *Phyllanthus 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 (LC_{50}). All the four fractions of *Phyllanthus emblica* were non-cytotoxic till 0.75 mg/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 fig.1. The highest non-cytotoxic concentration (>95% cell viability) of HXF, CTF, CFF and AQF fractions are 0.02, 0.04, 0.02 and 0.02mg/mL respectively.

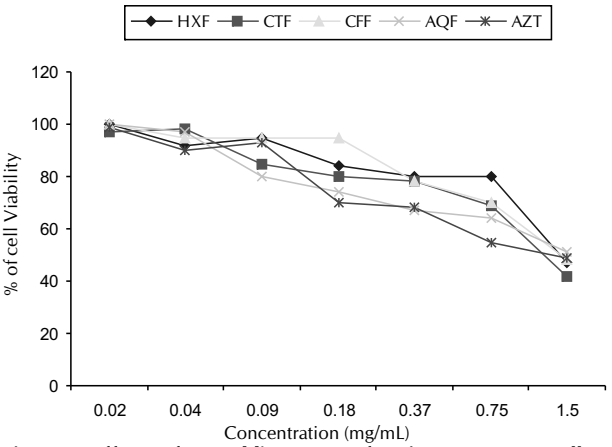

Figure 1: Effects of *P. emblica* extract fractions on PBMC cells

(HXF = n-Hexane fraction, CTF = Carbon tetra chloride fraction, CFF = Chloroform fraction and AQF = Aqueous fraction)

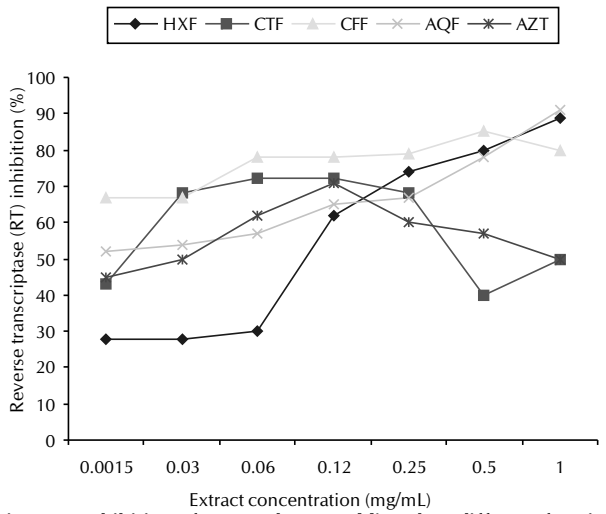

Figure 2: Inhibition of HIV-RT by *P. emblica* plant different fractions at different concentrations

(HXF = n-Hexane fraction, CTF = Carbon tetra chloride fraction, CFF = Chloroform fraction, AQF = Aqueous fraction).

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

Anti-HIV activity

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

DISCUSSION

Over the years, parts of the many medicinal plants have been used for medicine including claims of its antiretroviral potential (Lednicer and Sander, 1991). 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 0.5mg/mL and CTF fraction at 0.12mg/mL concentration (Fig. 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). Our previous investigations established that different medicinal plant extracts inhibit HIV reverse transcriptase in a non-speciûc 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 Raja *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, antifungal and antiviral activity (Gupta and Prakash, 2005; Husain *et al.,* 1992) this report also showed that the medicinal plants possessed an anti-HIV property through inhibition of viral reverse transcriptase activity.

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