EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 4
Issue: 12
516-522
Year: 2017

IN VITRO HIV-1 REVERSE TRANSCRIPTASE INHIBITION OF ANDROGRAPHOLIDE ISOLATED FROM *ANDROGRAPHIS PANICULATA*

Swapna Gurrapu and Estari Mamidala*

Infectious Diseases & Metabolic Disorders Research Lab, Department of Zoology, Kakatiya University, Warangal-506009, (T.S).

***Corresponding Author: Dr. Estari Mamidala**
Infectious Diseases & Metabolic Disorders Research Lab, Department of Zoology, Kakatiya University, Warangal-506009, (T.S).

Article Received on 01/10/2017 **Article Revised on 21/10/2017** **Article Accepted on 11/11/2017**

ABSTRACT
HIV is a major global concern due to its drug resistance and warrants the development of novel antivirals as alternative therapy. In the current study, andrographolide was isolated from *Andrographis paniculata* and HIV-1 reverse transcriptase inhibition was assessed. The crude extracts were prepared from dried areal parts *Andrographis paniculata* in ethanol by maceration method and isolated andrographolide by using column chromatography and HPLC. Peripheral Blood Mononuclear Cells (PBMCs) isolated from healthy donors by ficoll–hypaque density gradient centrifugation method. Cell viability test was performed on andrographolide by MTT assay against PBMC and HIV-1 RT inhibition activity was determined by HIV-1 Reverse Transcriptase (p66) Capture ELISA. In the HIV-1 reverse transcriptase assay, isolated compound showed good inhibitory activity (87%) at 160 µg/ml and MTT assay confirmed that the isolated andrographolide had no cytotoxic activity and IC50 values higher than 100 µg/ml.

KEYWORDS: HIV, *Andrographis paniculata*, reverse transcriptase, MTT assay.

INTRODUCTON
India has one of the oldest, richest and most diverse cultural traditions associated with the use of medicinal plants. Medicinal plants are great importance to the health of individuals and communities in general. The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids, phytosterols and phenolic compounds.[1,2] Many of the indigenous medicinal plants are used as spices and food plants. They also sometimes added to foods meant for pregnant women and nursing mothers for medicinal purposes.[3] Different parts of the medicinal plants like bark, roots, leaves, exudates etc. are used as per medicinal properties for various infectious diseases.[4]

A. paniculata, as a traditional medicinal plant, has been used for centuries to successfully treat respiratory, skin infections, fever, sore throat, herpes, dysentery, lower urinary tract infections and to reduce inflammation and stop diarrhoea.[5] In Ayurvedic medicine, it is used as a bitter tonic and stomachic, for diabetes, debility, hepatitis and as an anthelmintic.[6] However the potential of *A. paniculata* extract or andrographolide to inhibit HIV-1 reverse transcriptase activity has not been determined. Based on previous work A. *paniculata* crude extract showed more potent HIV-1 RT inhibition when compared to other selected medicinal plant extracts. Therefore *A. paniculata* crude extract was selected for isolation of active compound and andrographolide has been purified from *A. paniculata* confirmed by the TLC, Column chromatography, HPLC studies.

MATERIAL AND METHODS
Preparation of plant crude extracts and fractions
The aerial parts of *A. paniculata* were collected in bulk, washed, shade dried at room temperature until they were free from moisture and the above mentioned parts of medicinal plants were subjected to size reduction to get coarse powder (500 gr) was then stored in a clean dry air tight container and extracted by maceration and solvent-solvent fractionation using ethanol, diethyl ether, ethyl acetate and butanol solvents. 500 grams of dried powder was macerated with ethanol for 48 hours. The ethanol extract was filtered and it was finally dried at low room temperature under pressure in a rotary vacuum evaporator (Thermotech, buchi type model th-012).

The obtained ethanol crude extract (10.05 grams) was suspended in water and extracted with diethyl ether by solvent-solvent fractionation method. Diethyl ether fraction was collected and concentrated (1.05 gr). The

separated aqueous layer was extracted with ethyl acetate. The ethyl acetate fraction was collected and concentrated under reduced pressure (1.8 gr). The separated aqueous layer extracted with butanol and collected butanol fraction and it was concentrated (0.66 gr) and labelled all the solvent fractions. The collected fractions of diethyl ether, ethyl acetate, and butanol were screened for HIV-1 reverse transcriptase inhibition activity. Diethyl ether and ethyl acetate fractions were not active against HIV-1 RT. The butanol fraction was showed HIV-1 RT inhibition and this fraction was selected for further isolation process. Column chromatography, TLC and HPLC were used for isolation of active compound.

Isolation of active compound

Thin layer chromatographic studies

Thin layer chromatography (TLC) profile with other physicochemical parameter can be good tool for standardization and validation of plants. TLC profile is simple and effective method for determination of the solvent system. TLC as per conventional one dimensional ascending method using silica gel 60F254, 7X6 cm (Merck) were cut with ordinary household scissors. Plate markings were made with soft pencil. Glass capillaries were used to spot the sample on TLC volume 1µl by using capillary at distance of 1 cm at 1 track. To find which solvent system is suitable for separation of compounds, the five solvent systems were selected. These are solvent system I (chloroform : methanol-1:9), solvent system II (chloroform : methanol-3:7), solvent system III (chloroform : methanol–1:1), solvent system IV (chloroform : methanol-7:3) and solvent system V (chloroform : methanol-9 : 1). The butanol fraction was applied on pre-coated TLC plates and developed in TLC chamber. The developed plates were air dried and iodine vapors were used to detect the bands on the TLC plates. In this study, the TLC chromatograms were developed in five eluent systems of different polarity. Finally solvent system III (chloroform: methanol-9:1), was selected for column chromatography.

Column chromatographic studies

Column chromatography is a purification technique used to isolate compounds from a mixture. In column chromatography, the stationary phase is a solid adsorbent and the mobile phase is a solvent that is added to the top and flows down through the column. Separation is achieved based on the polar and non-polar interactions among the compounds, the solvent and the solid stationary phase. Usually Silica or Alumina is used as the solid phase in order to setup the column. In this experiment, Silica was used as the solid medium.

The column can be prepared using a column chromatography flask. Glass wool was inserted at the bottom of the flask to prevent the silica from escaping the column. The mixture of chloroform: methanol in the volume ratio (9:1) used in TLC was used as the mobile phase for column chromatography. 6 gm of plant active crude butanol fraction was added to 60 gm of silica gel and ground using a ceramic mortar and pestle to a fine powdered form. This was then added to a packed column (silica gel packed with selected mobile phase). The selected mobile phase was continuously poured to the top with the aid of a dropper. The bottom outlet of the column was opened, allowing the eluent to flow through the column. As the eluent passed down the column, the compound fraction moved down the column. The separated fraction flowed out of the column where the different elutes were collected in separate test tubes. This was repeated until all the dissolved extract was adsorbed on to the silica gel.

HPLC (High performance Liquid Chromatography)

Sample preparation for HPLC

Pooled fractions (Fraction-II) which showed HIV-1 RT inhibition was dissolved in HPLC grade Methanol (Carbinol). This was sonicated and then passed through what man Nylon Membrane Filter (0.45µm & 47mm diameter) before injecting it into the column.

Preparative HPLC

The HPLC analyses was conducted on a Shimadzu liquid chromatograph system (Shimadzu Corp, Kyoto, Japan) equipped with a quaternary pump, a vacuum degasser, an autosampler, a tunable UV-vis detector. For preparative, the Shimadzu Prep LC System equipped with a 7.8 mm id, RP18 semi-preparative column, coupled with a UV detector and a sample loop of 200 µL capacity. Methanol: water (1:1) was selected as mobile phase and 5 ml/min flow rate was selected. The fractions were detected at 254 nm of wavelength which was selected based on the UV spectra. 100 µL sample was injected. Results was acquired and processed by the Shimadzu Lab Solution software (Shimadzu Corp). The different three peaks were recorded with different retention times.

Analytical HPLC

For analytical the Shimadzu liquid chromatograph system (Shimadzu Corp, Kyoto, Japan) equipped with a Shim-Pack VP-ODS C18 column (250 mm × 4.6 mm, 5 µm), coupled with UV detector and sample loop of 20uL capacity. The mobile phase consisted of Methanol/water (1:1) at a flow rate of 1 mL/min. The sample injection volume was 20 µL and the analyses were monitored with the UV-Vis detector at 254 nm. Results were acquired and processed by the Shimadzu Lab Solution software (Shimadzu Corp) and chromatogram was recorded.

Anti-HIV activity of isolated compound

The HIV reverse transcriptase enzyme inhibition due to purified compound was determined using HIV-1 Reverse Transcriptase (p66) Capture ELISA test (Immunno Diagnostics, Inc, USA). 100 µL of different concentrations of crude extract sample and standard drug-AZT were added to respective wells of microplate. 10 fold serial dilutions were made (1 mg/mL to 31.5 µg/mL). 100 µL of HIV-1 Reverse Transcriptase (p66) added to all wells. Without compound was used as blank and AZT was used as standard. The mixture of samples

and HIV-1 RT (p66) were incubated at room temperature for 1 hour. Washed the plate three times with wash buffer. 100 µL of Anti-p66 Monoclonal Antibody (detector reagent) was added to all wells and incubated the plate in dark for 1 hour. After one hour incubation at dark, the plate washed three times with washing buffer. 100 µL of TMB substrate was added to each well and incubated the plate for 30 minutes up to formation of blue colour. Added 100 µL of stop solution to all wells and read the absorbance at 450 nm with using microplate elisa reader. The HIV-1 RT inhibition efficiency was calculated with compared to the blank control and calculate the percentage of inhibition by using following equation:

% of HIV-1 RT inhibition = 100 – Sample OD / Control OD x 100

Cytotoxicity Screening by MTT assay

Cell viability was evaluated by the MTT 3-(4, 5 dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide) test method. MTT (5 mg/ml) was dissolved in PBS. The solution was filtered through a 0.2 m filter and stored at 2 - 8°C.

PBMC cells (10^3 cells/well) were seeded in 100 µL RPMI-1640 medium in 96-well culture plates prior to the treatment with isolated compound and standard drug AZT and were incubated in a humidified atmosphere with 5% CO_2, 37°C, for 24 h (overnight) or until total adhesion to surface. For MTT assay the medium from the wells was removed carefully after incubation. Each well was washed with MEM (w/o) FCS for 2-3 times. After that, 100 µl fresh medium containing serially dilutions of isolated compound of A. *paniculata* aerial or AZT were added to each well, and incubated for another 48 hrs. Diluted isolated compound or AZT solutions were freshly prepared in DMSO prior to each experiment. AZT treated PBMC and DMSO treated PBMC were used as positive and negative controls respectively. For MTT assay, 10 µl MTT (5 mg/ml) was added into each well to generate formazan, and then cells were incubated in humidified atmosphere with 5% CO_2 at 37°C for 4 h. After removing the supernatant, 100 µl DMSO was added to dissolve the purple crystal resulting from MTT reduction. The extent of MTT reduction to formazan within cells was measured by absorbance at 595 nm by a microplate reader (Robonik, Mumbai). 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.

Absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation. Conversely, a higher absorbance rate indicates an increase in cell proliferation. Rarely, an increase in proliferation may be offset by cell death. Each experiment was performed in triplicates for three times. The percentage of proliferation was calculated by the following formula: % Cell viability = (OD sample – OD control) / OD control × 100

Determination of IC_{50}

The effect of isolated compound was expressed by IC_{50} values. IC_{50}, the concentration of compound required to inhibit 50% cell growth, was determined by plotting a graph (dose-response curves) of Log (concentration of Extract) vs % cell viability. The IC_{50} value was determined from the plotted curve.

Statistical analysis

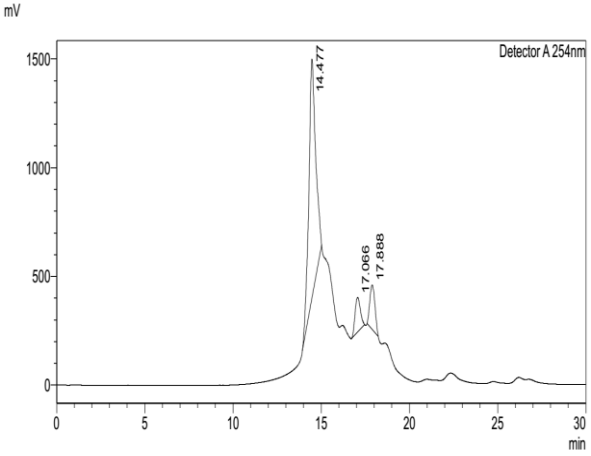
Results were expressed as the mean ± SD of values obtained in triplicate from three independent experiments. Statistical analysis was done using EXCEL (DATA ANALYSIS) for determining ANOVA. A p value of < 0.01 was considered statistically highly significant, $p < 0.05$ in significant.

RESULTS

HPLC analysis

HPLC is a relatively simple technique and is ideal for the rapid comparative study of plant samples. This method is an excellent technique for quality control of drug analysis (Zhang *et al*., 2007). HPLC provides reliable separation of substances even with closed structures. RP-HPLC approach is more popular in the analysis of complex extracts due to its better compatibility and precision (Wang, 2008). To check the purity, the column collected ten fractions were checked in HPLC using Shim-Pack VP-ODS C18 column (250 mm × 4.6 mm, 5 µm), coupled with UV detector and sample loop of 20uL capacity. The mobile phase consisted of Methanol/water (7:3) at a flow rate of 1 mL/min. The sample injection volume was 20 µL and the analyses were monitored with the UV-Vis detector at 230 nm.

Based on HPLC chromatograms of ten fractions and TLC Rf values the ten fractions are reduced into three fractions (Fr-I, Fr-II and Fr-III). To purify the Fr-II, the sample was analyzed in preparative HPLC. Therefore analysis of RP-HPLC spectrum of the column eluted active fraction (Fr-II) of *A. paniculata* was carried out and the result is shown in Figure-1. Three major peaks were recorded in preparative HPLC with different retention times. Several minor peaks were also observed. The retention time and area of the major peaks are shown in Figure-1. From the data, it can be observed that the first peak with a retention time of 14.477 minutes was the biggest peak, followed by peak 2 (17.066 minutes) and peak 3 (17.888 minutes). The peak at 14 minutes was high concentrated and collected several times to obtain more quantity. The collected fractions are pooled and concentrated and the purity was checked by analytical HPLC.

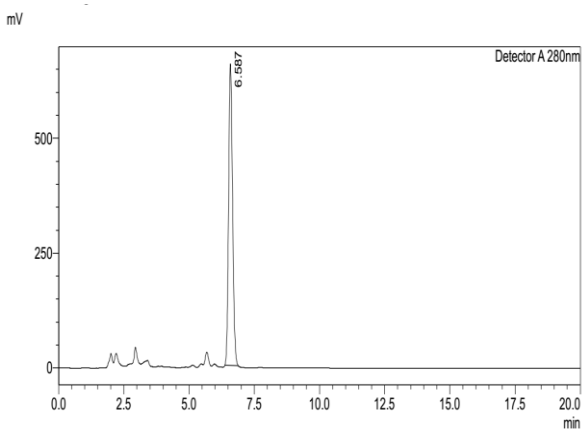


Detector A 254nm

Peak#	Ret. Time	Area	Height
1	14.477	29570626	1097002
2	17.066	3357877	158953
3	17.888	4178459	205316
Total		37106962	1461271

Figure-1. HPLC (preparative) chromatogram of active Fr-II (Pooled fractions of 3-7).

In analytical HPLC, the single peak obtained at retention time of 2.9 minutes and this analytical chromatogram indicates the purity of compound. Therefore the chromatogram revealed that, the isolated compound was pure and this compound was further processed for HIV-1 RT inhibition.



<Peak Table>

Detector A 280nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	6.587	6884840	656129	0.000	mg/L	M	RT:6.587
Total		6884840	656129				

Figure-2. HPLC (analytical) chromatogram of fraction collected from prep-HPLC.

Anti-HIV activity of isolated compound from *A. paniculata*

The anti-HIV activity was measured using HIV-1 Reverse Transcriptase (p66) Capture ELISA test and the IC50 values were determined. The results of the screening are shown in **Figure3**. At the beginning of the study it was hypothesized that compounds present in the aerial part of the plant could be active against HIV-1 RT. At 125 ug/mL concentration the isolated compound of *A. paniculata* showed more potent HIV-1 RT inhibition while standard drug shows at 250 µg/mL. The activity of HIV-1 RT inhibition is increased up to 125 µg/mL from 31.2 µg/mL and after that the inhibition activity was decreasing. The standard drug inhibition activity also increasing up to 250 µg/ml concentration, but after up to 1000 µg/mL the activity was slightly decreasing. These results concludes that the isolated compound from *A. paniculata* shows, 92% of HIV-1 RT inhibition at 125 µg/mL with IC50 of 772 µg/mL while the standard drug shows 93% of HIV-1 RT inhibition at 250 µg/mL with IC50 of <31.2 µg/mL. Therefore the isolated compound shows anti-HIV activity nearly similar to used standard drug (AZT) showed in figure.

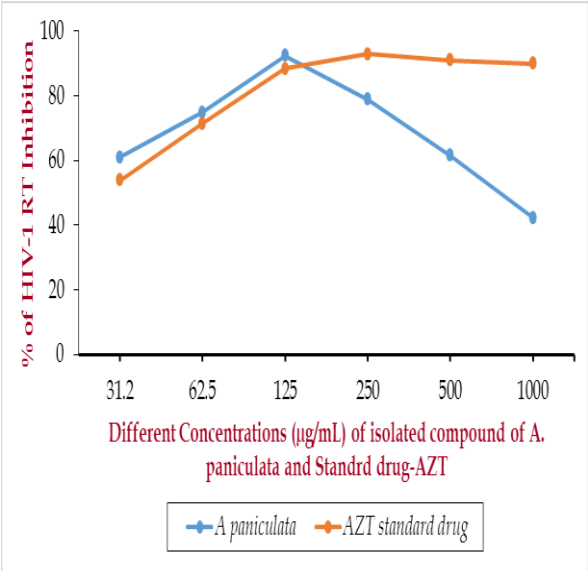


Figure-3. The HIV-1 RT inhibition of isolated compound from *A. paniculata* using.

HIV-1 Reverse Transcriptase (p66) Capture ELISA

By isolating and identifying these bioactive compounds new drugs can be formulated to treat various diseases and disorders. The main limitation in the use of traditional remedies is the lack of standardization of raw material, manufacturing progress and the final product.[7] A biomarker on the other hand is a group of chemical compounds which are not unique for that plant material but also correlates with biological efficacy. So the need arises to lay standards by which the right material could be selected and incorporated into the formulation.[8] Medicinal plants are reported to possess a wide range of properties including anti-carcinogenic, anti-inflammatory, antioxidant, antitumor, antimicrobial, immune modulatory, anti-helminthic, antiviral and antibiotic activities.[9,10] Medicinal plants is a table food with incredible medicinal properties. In the previous studies.

TLC and column profiling of all fractions gave an impressive result that directing towards the presence of number of phytochemicals. Various phytochemicals gave different R_f values in different solvent systems. This variation in R_f values of the phytochemicals provides a very important clue in understanding of their polarity and also helps in selection of appropriate solvent system for separation of pure compounds by column chromatography. In this present study the column fractions (Fr-II) showed highest anti-HIV activity thus it can provide lead molecules to compare with the Fr-I and Fr-III fractions which could be useful substrate for the synthesis of new broad spectrum for anti-HIV activity. Based on highest HIV-RT inhibition, the Fr-II was purified with HPLC and obtained single compound. This compound was more potent HIV-1 RT inhibition activity compared to the crude and column fraction. Therefore, this study concludes that, the isolated compound could be a potential source of natural molecules having great importance as therapeutic agent and preventing the viral diseases.

Many scientists such as[11-16], have shown that methanolic extracts from medicinal plants are more inhibitory than the aqueous extracts in screening for anti-HIV activity. However in this study the butanol extracts of *A. paniculata* showed the highest anti-HIV activity. This is in keeping with studies of[17]; and[18] who reported that phytochemicals extracted with butanol and water are more commonly effective inhibitors of HIV virus. The results suggest that the separation of polar and non-polar compounds can increase the chance of finding highly active anti-viral compounds with low cytotoxicity (Cos et al., 2004). The isolated compound from *A. paniculata* showed strong anti-HIV activity with IC_{50} of 772 µg/mL and this plant is used traditionally as a treatment of viral diseases and the different compounds are isolated, but there is no literature of its anti HIV activity. Therefore, it may be the first report of this isolated compound which showed HIV-1 RT inhibition.

Isolation of PBMC cells

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 from Kakatiya University health centre and blood sample were diluted at 1:1 ratio with PBS). Centrifuged at 1,000 x g for 30 min. 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 and centrifuged at 400 x g. The supernatant was then removed and the PBMC's were resuspended in RPMI 1640 medium.

Cytotoxicity of active compound on PBMC cells

In vitro assay of cytotoxic activity of isolated compound of *A. paniculata* aerial parts against PBMC cells at different concentrations was evaluated by MTT assay. MTT assay is based on the metabolic reduction of MTT into formazan crystals on treatment with PBMC cells. The inhibitory activities of isolated compound was compared with the standard drug AZT. Cell proliferation of compound was determined by an inhibitory concentration at 50% growth (IC_{50}). The PBMC cell viability percentage were found to be at different concentration of isolated compound (Table-1 and Figure-4). Cytotoxic activity at the different concentrations of 15.6 µg, 31.25 µg, 62.5 µg, 125 µg, 250 µg, 500 µg and 1000 µg/ml showed effective inhibition against PBMC cells. Therefore, data indicate that cytotoxicity of isolated compound was influenced by their concentration. Increased percentage of inhibition by suppressing viability was observed from Figure-4 that a gradually increase in percentage in all the treatments. However at 250 µg/ml of tested drug AZT shows 48.10 ± 1.05 cell viability against PBMC cells was observed whereas isolated compound only crossed 50% inhibition at 250 µg/ml. Therefore, the percentage of 50% inhibition concentration (IC_{50}) is 248 µg/ml.

This study revealed that, at 250 µg/mL the isolated compound showed highest HIV-1 RT inhibition and at 15.6 µg/mL the cell viability percentage is highest. The 50% cell viability showed less toxicity of isolated compound. The correct statement must be that the MTT bioassay is used as a parameter for mitochondrial or glycolytic metabolic activity of living cells and if that activity of experimental and control cells is the same, it could be supposed that any decrease of metabolic activity in experimental wells could suggest a decreased cell viability or inhibition of cell proliferation. Cytotoxicity screening models provide important preliminary data to help selecting plant extracts with potential antineoplastic properties for future work.[19,20] Extracts of *A. paniculata* reduce the risk of cancer due to the presence of flavonoids.[21] In this study the isolated compound from *A. paniculata* showed less toxicity at lowest concentration and also showed HIV-1 RT inhibition.

Table-1. Cell proliferative effect of isolated active compound on PBMC cells.

Concentration (µg/mL)	% of Cell viability	
	Isolated Compound	Standard Drug (AZT)
15.6	96.35 ± 0.13***	91.07 ± 1.07
31.25	91.0 ± 0.27**	88.15 ± 0.87
62.5	81.05 ± 0.86**	77.08 ±1.02
125	67.71 ± 1.05*	66.5 ± 0.87
250	51.5 ± 0.96*	48.10 ± 1.05
500	32.6 ± 0.85**	36.5 ± 0.85
1000	19.6 ± 0.56*	21.9 ± 0.21
IC_{50}	248 (µg/mL)*	251 (µg/mL)

**** P< 0.05, ** p < 0.01,***p < 0.001** value are considered statistically significant.*

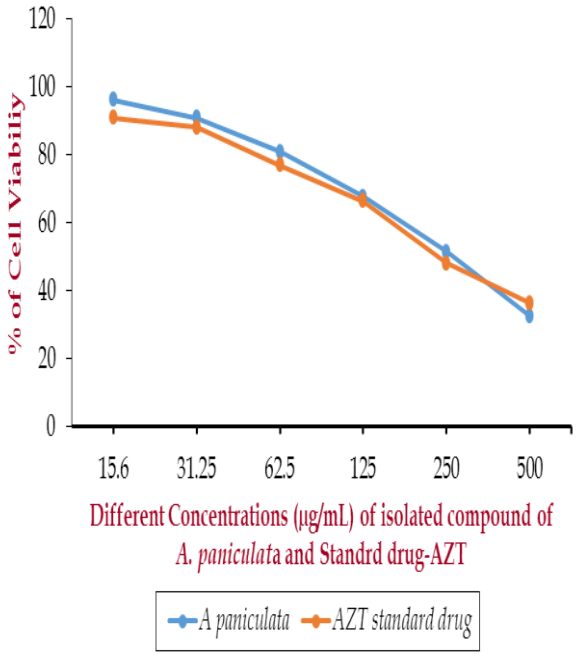


Figure-4. Cell proliferative effect of isolated active compound against PBMC cells.

Phytomedicines have been used since ancient times to treat various infections but clinical studies are limited.[22] Safety is a major requirement for an antiviral agent and in the search for new drugs it is important to consider possible secondary effects. The minimal cytotoxicity observed in the extracts investigated may be due to the presence of cytoprotective components. These cytoprotective (chemical compounds) components of plants have been reported in other studies on plant extracts.[23]

CONCLUSION

The compound isolated from *A. paniculata* seemed to have the potential to act as a source of useful drugs and also to improve the health status of the consumers as a result of the presence of active compound that are vital for good health. The single compound from aerial parts of this plant can provide lead molecules which could be useful substrate for the synthesis of new broad spectrum for anti-HIV and the treatment of infections caused by the pathogenic organisms. Therefore it can be concluded that this concentration of active compound could be possibly used safely against HIV. Based on this results, the isolated compound of *A. paniculata* potentially to be developed as herbal medicine which replace the standard antiretroviral (AZT) drugs against PBMC cells.

REFERENCES

1. H.O. Edeoga., D. E. Okwu. And B.O Mbaebie. Phytochemical constituents of some Nigerian medicinal plants. *African Journal of Biotechnology. 2005;* 4(7): 685-688.
2. Swapna Gurrapu and Estari Mamidala, 2016. Medicinal Plants Used by Traditional Medicine Practitioners in the Management of HIV/AIDS-Related Diseases in Tribal Areas of Adilabad District, Telangana Region. *The Ame J Sci & Med Res*., 2(1): 239-45.
3. Hill A.F. (1952). Economic Botany. Textbook of useful plants and plant products. 2nd edn. McGraw-Hill Book Company Inc, New York.
4. Gurrapu S, Mamidala E. *In vitro* antibacterial activity of alkaloids isolated from leaves of *Eclipta alba* against human pathogenic bacteria. Pharmacog J. 2017; 9(4): 573-7.
5. Techadmrongsin, Y., Dechatiwongse Na Ayudhya, T. and Punyarajun, S. Harvesting, post-harvesting handing and storing of crude drug. Department of Medical Sciences, Nonthaburi, Thailand (Thai language), 1999.
6. Mills, S. and Bone, K. Principles and practice of phytotherapy. Churchill Livingstone, Edinburgh. 2000; 262-268.
7. Manikandan M, et al. (2012) Cell population based mass spectrometry using platinum nanodots for algal and fungal studies. *Biosens Bioelectron*, 35(1): 493-7.
8. Mariswamy, Y., W.E. Gnaraj and M. Johnson, 2011. Chromatographic finger print analysis of steroids in *Aerva lanata* L by HPTLC technique. Asian Pac. J. Trop. Biomed., 1: 428-433.
9. Rajkumar S, et al. Multiple myeloma: 2011 update on diagnosis, risk-stratification and management Am J Hematol, 2011; 86: 57.

10. Abbasi AM., Khan MA., Ahmad M., Zafar M., Jahan S. and Sultana S. (2010). Ethnopharmacological application of medicinal plants to cure skin diseases and in folk cosmetics among the tribal communities of North-West Frontier Province. *Pak J Ethnopharmacol*. 128: 322-335.
11. Tommasi N.D., Piacente S., Rastrelli L., Mahmood N. and Pizza. 1998. Anti-HIV activity directed fractionation of the extracts of Margyricarpus setosus. Pharmaceutical Biology. 36(1): 29–32.
12. Bedoya L.M., Palomino S.S., Abad M.J., Bermejo P. and Alcami J. Anti-HIV activity of medicinal plant extracts. Journal of Ethanopharmacology. 2001; 7; 1: 113-116.
13. Rukunga G.M., Kofi-Tsepko M.W., Kurokawa M., Kageyama S., Mungai GM., an Muli J.M. 2002. Evaluation of the HIV-1 transcriptase inhibitory properties of extracts from some medicinal plants in Kenya. African Journal of Health Sciences. 9(2): 81-90.
14. Bessong P.O., Obi C.L., Igumbor E., Andreola M.L., Litvak S. In vitro activity of three selected South African medicinal plants against human immunodeficiency virus type 1 reverse transcriptase. African Journal of Biotechnology. 2004; 3; 10: 555-559.
15. Bessong P.O., Obi C.L., Andréola M.L., Rojas L.B., Pouységu L., Igumbor E., Meyer J.J.M., Quideau S., and Litvak S. Evaluation of selected South African medicinal plants for inhibitory properties against human immunodeficiency virus type one reverse transcriptase and integrase, Journal of Ethnopharmacology., 2005; 99;1: 83-91.
16. Bessong P.O., Rojas L.B., Obi L.C., Tshisikawe P.M., and Igunbor E.O. Further screening of Venda medicinal plants for activity against HIV type 1 reverse transcriptase and integrase, African Journal of Biotechnology., 2006; 5; 6: 526-528.
17. Locher C.P., Witvrouw M., De Bethune M.P., Burch M.T., Mower H.F., Davis H., Lasure A., Pauwels R., Clercq E.D., and Vlietinck A.J. Antiviral activity of Hawaiian medicinal plants against human immunodeficiency Virus Type -1 (HIV-1). Journal of Phytomedicine. 1996; 2(3): 259-264.
18. Bot Y.S., Mgbojkwe L.O, Nwosu C., Abimiku A., Dadik J., and Damshak D. Screening of the fruit pulp extract of Momordica balsamina for anti-HIV property. African Journal of Biotechnology. 2007; 6(1): 47-52.
19. Cardellina JH. Fuller RW. Gamble WR, Westergard C. and Boswell J. Evolving strategies for the selection de-replication and prioritization of anti-tumor and HIV-inhibitory natural products extracts. In: Bohlin L, Bruhn JG. (eds.) *Bioassay methods in natural product research and development.* Dordrecht: Kluwer Academic Publisher. 1999; 25-36.
20. Baskar AA., Ignacimuthu S., Paulraj GM. and Numair KSA. Chemopreventive potential of β sitosterol in experimental colon cancer model-an in vitro and in vivo study. *BMC Complement Altern Med., 2010;* 10: 2-10.
21. Chattopadhyay D. and Naik TN. Antivirals of ethnomedicinal origin: Structure-activity relationship and scope. *Mini-Reviews in Medicinal Chemistry*, 7(3): 275–301.
22. Tayal S., Duggal S., Bandyopadhyay P., Aggarwal A. and Tandon S. Cytoprotective role of the aqueous extract of *Terminalia chebula* on renal epithelial cells. *International Braz J Urol*., 2012; 38(2): 204–213.
23. Venkanna Lunavath. and Estari Mamidala. Studies on anti-HIV activity of *phyllanthus emblica* plant extracts. *The Bioscan*., 2012; 7(3): 451-454.