

**IN VITRO ANTI-HIV ACTIVITY STUDIES ON ENICOSTEMA  
LITTORALE (LAM), RAYNAL.WHOLE PLANTS**

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**ABSTRACTS**

*Enicostema axillare* (Lam.). Raynal, syn. *Enicostemma littorale* Blume (Family) Gentinaceae is a perennial herb found throughout the greater part of India. Locally it is known as chota chirayita and used in indigenous medicines in the treatment of fevers and as bitter tonic and forms one of the ingredients of many hypoglycemic marketed formulations. In the present study, we evaluated anti-HIV activity of the whole plant. Methanolic extract showed prominent anti-HIV activity.

**KEYWORDS:** *Enicostemma littorale*, Chota chirayita, Anti-HIV. Activity 2.

**INTRODUCTION**

Advances in HIV pharmacotherapy led to the current highly active antiretroviral therapy (HAART), which has had significant impact on HIV/acquired immunodeficiency syndrome (AIDS) in the developed world, and these drugs have acted to prolong survival and to alleviate suffering. However, the incidence of side effects and HIV drug resistance in patients under HAART is high and HIV/AIDS persists as a major cause of morbidity in Western societies and continues to surge unabated in the developing word. Consequently, there remains an urgent need for more potent and conceptually novel antiviral therapeutics to add to current treatment regimens. Over the past decade, the concept of topical microbicides to prevent transmission of HIV has emerged as an important strategy to control the HIV pandemic The increased incidence of HIV infection in women aged 15–49 years in resource-poor countries has emphasized the need to develop female-controlled, efficacious

and safe microbicides for vaginal application. Desirable basic characteristics of Topical microbicides include a high *in vitro* activity against a wide range of HIV-1 strains, a broad activity against other sexually-transmitted pathogens, not-to-low cytotoxicity in *in vitro* assays, stability under likely storage conditions, low cost, and good acceptance in the target population. TZM-bl (JC53-bl) is a genetically engineered HeLa cell line that expresses CD4, CXCR4 and CCR5 and contains Tat-inducible Luc and -Gal reporter genes. Plants of gentianaceae family are a perennial herb found throughout India and are more common in the coastal areas. The plant is used in Folk medicine to treat diabetes mellitus, rheumatism, abdominal swelling, itching and hypoglycemia.<sup>[3-5]</sup> Anticancer.<sup>[6]</sup> activities also have been reported. These reported activities and many of the ethno medical uses of the plant are related to its antioxidant activity. Swertiamarin, alkaloids, steroids, triterpenoids, saponins flavonoids, xanthone.<sup>[4]</sup> And many such compounds have protective effects due to their antioxidant properties.<sup>[7]</sup>

## MATERIALS AND METHODS

The whole plant of *Enicostema littorale* was collected during the Month of March 2010, from Alangulum, Tirunelveli District, Tamil Nadu, South India. The plant was identified and authenticated by professor Dr.P.Jayaraman, Director, National Institute of Herbal 3.

Science (Reg. No of the certificate: PARC/2011/858. The fresh plant material was then dried under shade, and the material was powdered using mechanical grinder and passed through 60 # sieve to get the powder of desired coarseness. Powdered material was preserved in an air tight container.

### Preparation of Extract

The coarse powder (500 gm) was subjected to maceration for 72 hours, followed by exhaustive maceration for 48 hours, by using solvents Chloroform, Ethylacetate and Methanol in the order of increasing by decanting and drying the marc after each extraction. The solvents were recovered by distillation of the extracts at 750°C to 800°C. The extracts were dried under desicator and percentage yield was calculated. These extracts were subjected to preliminary phytochemical screening and anti-HIV activity.

### Cell Lines

PM-1 (T cell line expressing CXCR4 and CCR5 co receptors) and TZM-bl cells (expressing

CD4, CXCR4 and CCR5 co receptors,  $\beta$  galactosidase and luciferase activity in HIV-1 Tat dependent manner) were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (NIH ARRRP). TZM-bl cells were grown in high glucose DMEM with L-glutamine (Invitrogen) supplemented with 100U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 10% FBS. Passages 5-40 were used for experiments with no change in cell behavior. PM-1 cells were maintained at a density of  $1 \times 10^6$ /ml in RPMI 1640 supplemented with 100U/ml penicillin, 100  $\mu$ g/ml streptomycin, 100mM HEPES and 10% FBS(Moregate). Passages 5-30 were used for experiments with no change in cell behavior.

### Viruses

The HIV-1 laboratory adapted strains HIV-1 IIIB (X4, subtype B) HIV-1 Ada5 (R5, Subtype B) and the primary isolates HIV-1(UG070 ((X4, Subtype D) was obtained from NIH ARRRP.HIV-1 primary isolate VB59 (R5, Subtype C) is an Indian isolate from the National AIDS Research Institute, Pune. Virus stocks were developed in PM-1/H9 cells and after Quantification of p24 antigen by ELISA (Vironostika), the supernatants were stored at  $-80^{\circ}\text{C}$ . 50% tissue culture infectious dose (TCID<sub>50</sub>) of each isolate was determined in the Appropriate cell line using the Spearman Karber formula (ACTG Lab Man, 25 May 2004).

### Assays in TZM-bl Cell Line

#### Cell Viability Assay

The cell viability of TZM-bl cell line in the presence of compound was analyzed after 48 h of 4 incubation with increasing concentrations (2x dilution) of each compound, using the MTT (3-[4,5-dimethylthiazol-2-yl]- 2,5-diphenyl tetrazolium bromide) assay (Sigma) as previously described (Saidi H *et al* 2008).

#### Measurement of Anti-HIV Activity by Luciferase Gene Reporter Assay HIV-1 Inhibition Assay (Cell Free)

A 96-well plate was seeded with  $1 \times 10^4$  TZM-bl cells per well. Next day, sub toxic concentrations of the test preparation and HIV-1 stock (400 TCID<sub>50</sub>) were pre-incubated for 1 hour at  $37^{\circ}\text{C}$  and then added onto the cells. After 48 hrs, cells were assayed for luciferase activity using Britelite Plus substrate (Perkin Elmer, USA).

#### HIV-1 Replication Inhibition Assay (Cell Associated)

A 96 well plate was seeded with  $1 \times 10^4$  TZM-bl cells per well. Next day, the cells were exposed to the HIV-1 stock (400 TCID<sub>50</sub>) and incubated for 2 hours at  $37^{\circ}\text{C}$ . Sub toxic

concentrations of test preparation were then added onto the cells. After 48 hrs, cells were assayed for luciferase activity using Britelite Plus substrate (Perkin Elmer, USA). 5

### Assays in T Lymphoid (PM-1) Cell: Cell Viability Assay

A series of double dilutions of compound were prepared and then  $5 \times 10^3$  PM-1 cells were added to each well. After five days of incubation the cell viability was determined by trypan blue dye exclusion method (Velleca WM *et al* 1991).

### Confirmation of Anti-HIV Activity Using PM-1 cell line assay

#### HIV-1 Inhibition Assay (Cell Free)

Sub toxic concentrations of the test preparation were incubated with HIV-1 primary isolates (CXCR4 tropic HIV-1UG070 or CCR5 tropic HIV-1VB59) (20 TCID<sub>50</sub>). PM-1-cells were then exposed to virus-test preparation mixture and incubated overnight at 37°C. Next day the cells were washed to remove the un adsorbed virus and then added onto 24 well plates. After five days, the inhibition of virus growth was monitored by p24 ELISA and compared with the virus growth in the absence of drug. Anti-HIV activity was analyzed by measuring supernatant p24 antigen according to the Manufacturer's protocol (Vironostika, Netherland). Dextran sulphate (Sigma, USA) was used as the positive control.

#### HIV-1 Replication Inhibition Assay (Cell Associated)

PM-1 cells were infected with HIV-1 virus stock (20 TCID) and incubated overnight at 37°C. The cells were washed thrice and serial dilutions of test preparations were added onto the cells in 24 well plate. After five days, the inhibition of virus growth was monitored by p24 antigen detection and compared with the virus growth in the absence of drug. Anti-HIV activity was analyzed by measuring supernatant p24 antigen according to the Manufacturer's protocol (Vironostika, Netherland). AZT (Cipla, India) was used as the positive control.

## RESULTS

**Solvent (Methanol) -No toxicity observed up to highest concentration tested.**

#### Anti-HIV Result

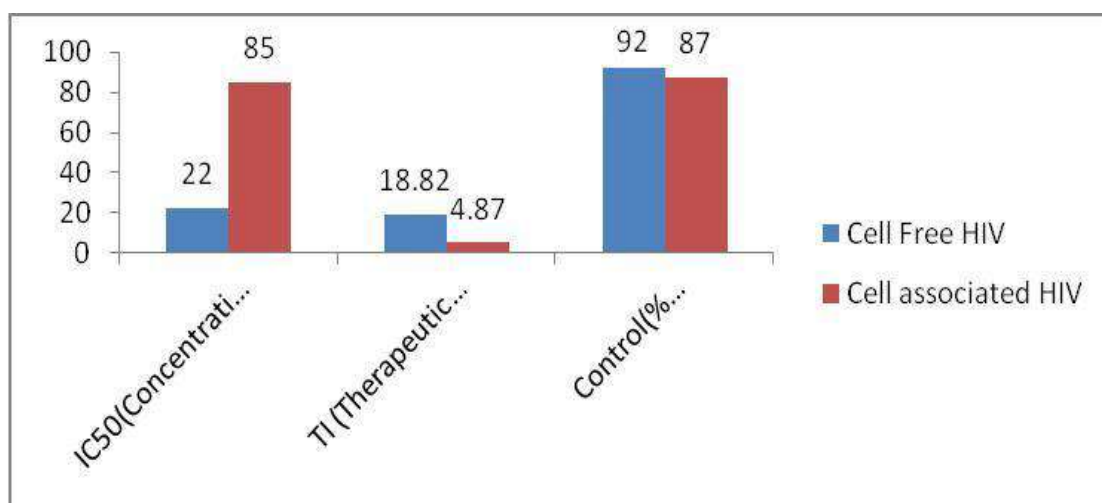
HIV-1 Strain	Cell Free Assay				Cell Associated Assay			
	IIIB	Ada5	UG070	VB59	IIIB	Ada5	UG070	VB59
IC <sub>50</sub> (Concentration showing 50% inhibition)	22	18	8.69	32.59	85	156	62.5	136
TI (Therapeutic Index)	18.82	22.99	47.63	12.70	4.87	2.65	6.62	3.04
Control (% inhibition)	92	87	86	90	87	78	86	76

### Control

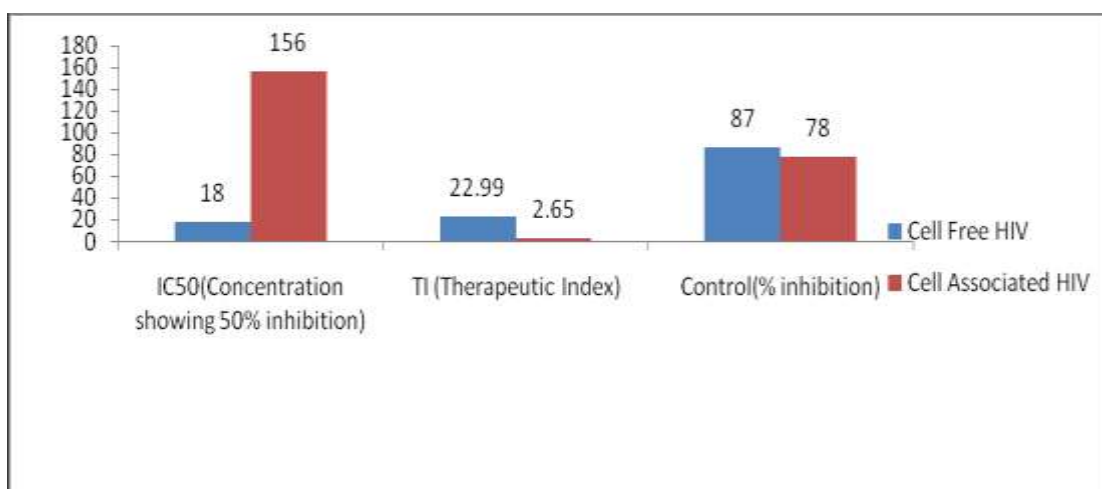
Cell free-Dextran Sulfate 7.8 µg/ml, Cell associated-AZT 31.25µM

Concentration showing 50% cytotoxicity (CC<sub>50</sub>) value for the extracted plant material was 413.98µg/ml. No cytotoxicity was observed for methanol in the same concentration range.

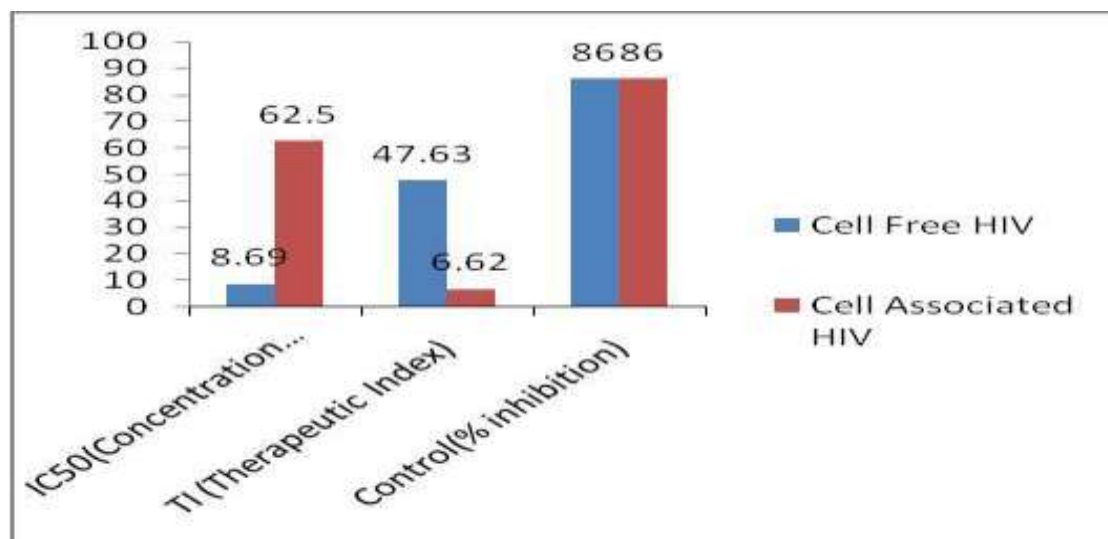
Compound was evaluated for activity against cell frees (CF) and Cell associated (CA) CXCR4 and CCR5 tropic HIV-1 in TZM-bl cells. The compound effectively inhibited CF lab adapted strains (IIIB & Ada5) and primary isolates (UG070 & VB 59) of HIV-1 (IC<sub>50</sub> range: 8.69- 32.59 µg/ml, TI range: 12.70-47.63), than CA HIV-1 (IC<sub>50</sub>: 62.5-156 µg/ml, TI: 2.65-6.62). The positive control used for cell free assay is Dextran sulphate (CC<sub>50</sub> value- 4978, IC<sub>50</sub> range: 2.5- 5.9 µg/ml, TI range: 840- 2,154) whereas for cell associated assay is AZT (CC<sub>50</sub> value- 872, IC<sub>50</sub> range: 0.004-0.026 µM, TI range: 34,352-1, 98,182) for all tested HIV-1 isolates.



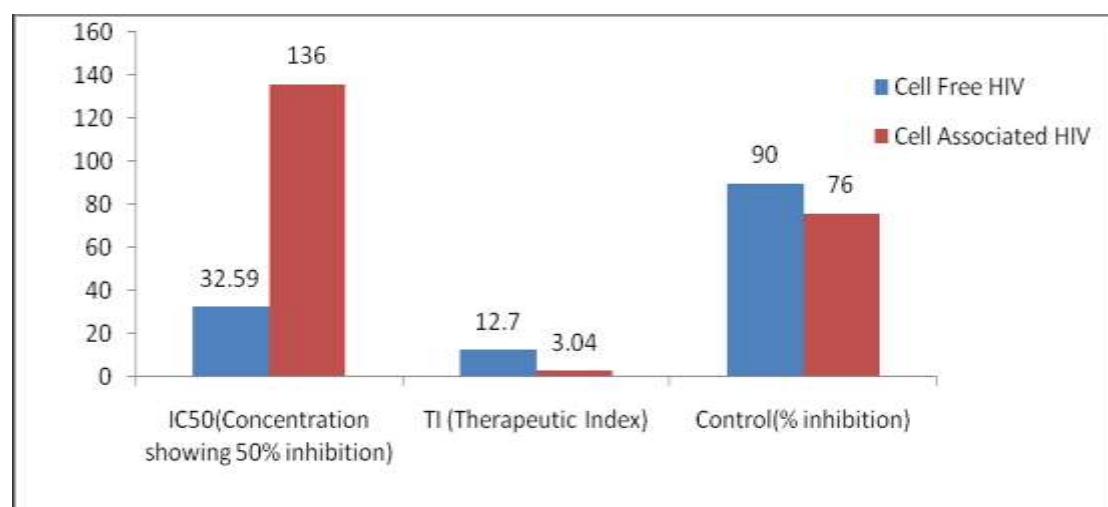
**Graph.1: *Enicostemma littorale* activity against cell free and cell associated HIV-1 IIIB in TZM-bl cell line**



**Graph 2: *Enicostemma littorale* activity against cell free and cell associated HIV-1 Ada5 in TZM-bl cell line.**



**Graph .3** *Enicostemma littorale* activity against cell free and cell associated HIV-1 UG070 in TZM-bl cell line.



**Graph .4** *Enicostemma littorale* activity against cell free and cell associated HIV-1 VB59 in TZM-bl cell line

#### Cytotoxicity and Anti-HIV Result in PM-1 assays

**Cytotoxicity Result** -Concentration showing 50% cytotoxicity (CC<sub>50</sub>) =117 µg

#### Anti-HIV Result

HIV-1 Strain	Cell Free Assay		Cell Associated Assay	
	UG070	VB59	UG070	VB 59
IC <sub>80</sub> (Concentration showing 80% inhibition)	5.6	28	Value could not be calculated because of cytotoxicity	Value could not be calculated because of cytotoxicity
Control (% inhibition)	100	100	100	100

Control: Cell free-Dextran Sulfate 78µg/ml, Cell associated AZT 78 µM

CC<sub>50</sub>: Values determined by fitting appropriate trend line to the data.

IC<sub>80</sub>: Values determined by fitting appropriate trend line to the data.



The compound was tested in PM-1 cell line for confirmation of its activity against primary isolates of CCR5 tropic HIV-1VB59 and CXCR4 tropic HIV-1UG070. The CC50 value for the extract was 117µg/ml. The compound showed inhibition of CF HIV-1(IC80: 5.6-28 µg/ml), whereas IC 80 value could not be calculated for CA HIV-1 due to toxicity. The positive control Dextran sulphate showed 100% inhibition at 78 µg/ml where as the AZT showed 100% inhibition at 78µM of tested HIV-1 isolates.

## DISCUSSION

The purpose of standardized extraction procedures for crude drugs (medicinal plant parts) is to attain the therapeutically desired portions and to eliminate unwanted material by treatment with a selective solvent known as menstrum. The extract thus obtained, after standardization, may be used as medicinal agent as such in the form of tinctures or fluid extracts or further processed to be incorporated in any dosage form such as tablets and capsules. These products contain complex mixture of many medicinal plant metabolites, such as alkaloids, glycosides, terpenoids, Tannin, flavonoids and lignans. *Enicostema littorale* Blume is a plant with a number of antioxidative phytochemicals, which include alkaloids, catechins, saponins, sterols, triterpenoids, phenolic acids, flavonoids and xanthenes. It also contains minerals like iron, potassium, sodium, calcium, magnesium, silica, phosphate, chloride, sulphate and carbonate (Murali B *et al.*, 2002). Flavonoids abundant in plant kingdom, has proved a lot of pharmacological activities, such as antioxidant, anti-According to ethno-botanical claim this plant is used in typhoid fever, dropsy, malaria and skin diseases. As plant contains phenolic and terpenoids compounds, present study has undertaken to evaluate anti-HIV activity. Methanol extracts of anti-HIV activity is determined using In-vitro studies such as TZM-bl assay and PM-1 assay. The above product induced cytopathic effects in cell culture can be monitored by an increase in cellular viability. In vitro cytotoxic effects of *Enicostemma littorale* in TZM-bl and PM-1 cell lines are recorded. The cytotoxicity increases with increase in concentration of *Enicostemma littorale* (CC50 value in TZM-bl cells-413.98 µg/ml and in PM-1 cells-117µg/ml). The values show that *Enicostemma littorale* extract is more cytotoxic to PM-1 cells than TZM-bl cells. Furthermore percent inhibition of HIV-1 and therapeutic index of product calculated for concentrations showing viability of both TZM-bl and PM-1 cells above 50%. Only cell free HIV-1 primary isolates were inhibited upto 80% by compound (IC80- 5.6 and 28 ug/ml, but not cell associated primary isolates, due to toxicity for higher concentrations of compound. This indicates that product is more active against cell

free than cell associated HIV-1. Further experiments in culture conditions mimicking in vivo environment of HIV-1 infection will help in deciding product efficacy.

## CONCLUSION

The prominent antimicrobial activity may be due to presence of higher content of tannins, phenolic acid, flavanoid, terpenoids, and glycoside. Further scope involves isolation and identification of different constituents responsible for these activities.

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