Full Length Research Paper

Inhibition of HIV-1 lentiviral particles infectivity by *Gynostemma pentaphyllum* extracts in a viral vector-based assay

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Three different extracts of *Gynostemma pentaphyllum* (Cucurbitaceae), a medicinal plant used for a variety of ailments in complementary and alternative medicine (CAM) including those caused by viral infections with claims of efficacy against HIV-1 infections were screened. These claims motivated the study in which the inhibition of viral vector infectivity of HeLa cells was assessed flow cytometrically by measuring the expression of green fluorescent protein (GFP) transgene incorporated in the lentiviral vector construct. An infectious VSV-G-pseudotyped, human immunodeficiency virus type 1-based, self-inactivating lentivirus vector particles were generated by transient co-transfection of the vector plasmid (pHIV-1 CSCG), with packaging plasmids encoding tat, rev, gag-pol (pCMVΔR8.2), a VSV-G expression plasmid (pHIT-G) and a secretory alkaline phosphate expression plasmid (pSEAP) all necessary for viral infectivity. The extracts studied were obtained by solvent extraction of the leaf powder of *G. pentaphyllum* with ethyl ether (EG), methanol (MG), and water (AG). The AG, MG and EG were all active against the HIV-1 lentiviral vector and inhibited the early events of the viral replication cycle on HeLa cells in a concentration-dependent manner with a IC_{50} of 6.21 µg AG/ml, 8.32 µg MG/ml and 5.8 µg EG/ml, respectively. The cytotoxicity of the extracts to HeLa cells evaluated in parallel by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay method showed TC_{50} values of 36.77 µg AG/ml, 38.68 µg MG/ml and 41.02 µg EG/ml with selectivity indices (SI) of 5.92, 4.65 and 7.02, respectively. The results of the study show that the extracts of *G. pentaphyllum* possess potent and selective anti-retroviral potentials and could serve as possible source of lead antiviral drugs against HIV.

Key words: Antiviral activity, antiviral screening, *Gynostemma pentaphyllum,* HIV-1, viral vector-based screening.

INTRODUCTION

The introduction of antiretroviral drugs have had an effect on reducing morbidity and mortality, prolonging lives and improving the quality of life (QoL) for many people living with HIV infection (Eisenburg et al., 1998). However, people with HIV/AIDS in developing countries, especially in the sub-Saharan Africa with rampaging HIV/AIDS pandemic, cannot afford the high costs of highly active antiretroviral therapy (HAART). Moreover, HAART has limited effectiveness in some patients, including a complicated dose regimen and the associated debilitating drug toxicities. In addition, there is a problem with cross-resistance among antiretroviral drugs of the same class (Bonfanti et al., 1999; Vella and Palmisano, 2000; Liu, 2007). The therapeutic options of treating HIV/AIDS are

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still limited at present and alternative approaches are needed.

Herbal medicine is one of the mainstreams of complementary and alternative medicine (CAM), and its use has increased during the last decade (Eisenburg et al., 1998; Vickers, 2000; Liu, 2007). Due to the chronic course and the effect of the HIV-related diseases on QoL and the possibility of severe complications and death, people with HIV/AIDS are very likely to seek herbal therapies (Ernst, 1997; Ozsoy and Ernst, 1999; Faragon et al., 2002), particularly in the parts of the world where HAART is not accessible or not economically feasible to sustain the therapy.

Even in developed countries like the United States, surveys have found that HIV-infected people used CAM at a high rate, made frequent visits to CAM providers, incurred substantial expenditures, and have also reported considerable improvement with these treatments (Fairfield et al., 1998; Wootton and Sparber, 2001). It is therefore important to verify claims of antiretroviral effi-cacies of herbal and complementary remedies which could lead to potent and safe alternative therapies or lead molecules for further development.

There are already tremendous research efforts and successes in anti-HIV medicinal plants (Williams, 2001; Jassim and Naji, 2003; Esimone et al., 2007, 2009, 2010). Generally, research in anti-viral medicinal plants has been limited by the shortcomings of the available screening techniques. Traditional methods of evaluating antiviral agents have involved one or more of the following *in vitro* techniques: plaque inhibition assay, plaque reduction assay, inhibition of virus-induced cytopathic effect, virus yield reduction assay, end point titre determination assay, reduction or inhibition of the synthesis of virus-specific polypeptides, immunological assays detecting viral antigens, and viral enzyme inhibition-based assays (Vlietinck and Vanden, 1991; Cowan, 1999). While these methods have successfully been employed to screen a large number of diverse antiviral agents, limitations and shortcomings relating to safety, long assay period, cumbersomeness, experimental-biased read-out, high cost, and the inability to distinguish between mere toxic effects of agents on host cells and selective antiviral effects associated with some of these methods has limited their application in high throughput assays of the vast and yet to be screened medicinal plants with potential antiviral activities (Esimone et al., 2005, 2007).

In this study, we have used a modification of a rapid and reproducible vector-based antiviral screening technique as earlier described (Esimone et al, 2005, 2010) to screen three different solvent extracts of the plant *Gynostemma pentaphyllum* (Cucurbitaceae) for anti-retroviral activity. This technique is a novel and a high throughput approach for the screening of medicinal plant extracts for possible antiviral activities (Esimone et al, 2005). The plant *G. pentaphyllum* is one of many medicinal plants claimed to possess antiviral activities in complementary medicine. *G. pentaphyllum* is a perennial climber used for treatment of many disorders. Gype-nosides, a dammarane-type saponin (Liu et al., 2005) isolated from *G. pentaphyllum*, are major bioactive principles which have been reported to have various activities such as anti-ulcer (Rujjanawate et al., 2004), anti-tumour (Chiu et al., 2003, 2004; Chen et al., 2004); cholesterol-reducing effect (Kimura et al., 1983; Huang et al., 2005), immunopotentiating (Zhang et al., 1990; Li and Xing, 1992) and anti-inflammatory (Lin et al., 1993) activities. This study was motivated by the beneficial effects claimed in complementary medicine of this plant extract in viral diseases, including the benefits claimed in HIV infections.

MATERIALS AND METHODS

Collection and extraction of plant materials

The fresh leaves of *G. pentaphyllum* plants were collected from Nibo in Awka south L.G.A, Anambra State, Nigeria. The identity was authenticated by Prof. C. C. Okeke of the Department of Botany, Nnamdi Azikiwe University, Awka. The plant leaves were washed, dried and ground to powder using a mechanical grinder. The methanol extract (MG) and petroleum ether extracts (EG) were obtained by macerating 40 g portions of the plant powder in 200 ml of either methanol or petroleum ether for 48 h at room temperature with intermittent agitation. The solutions were filtered using Whatman no. 1 filter paper and concentrated to dryness *in vacuo* at 40°C. The aqueous extract (AG) was also obtained by macerating a 40 g portion of the plant powder in 400 ml of distilled water at room temperature for 24 h. The resulting solution was concentrated by forced evaporation at 40°C and the remaining solvent removed by freeze drying.

Phytochemical analysis of plant extracts

The extracts (EG, MG and AG) were tested for the presence of alkaloids, flavonoids, tannins, saponins, glycosides, protein, carbohydrate, terpenoids, resins, fats and oil, steroids and reducing sugar using standard phytochemical methods (Evans, 1989; Harborne, 1998).

Cell lines

Human embryonic kidney cells expressing SV40 Large T-antigen (293 T) and Henrietta Lacks cells were propagated in D-10 medium, consisting of Dulbecco's modified Eagle's medium (DMEM) with high glucose, 2 mM L-glutamine and supplemented with 10% heat-inactivated foetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. Tissue culture medium and supplements were purchased from Invitrogen (Karlsruhe, Germany). The cell cultures were maintained in a humidified 5% carbon dioxide (CO_2) atmosphere at 37°C.

Generation of HIV-1 viral vectors transferring green fluorescent protein (GFP) genes

Infectious HIV lentiviral particles transferring the GFP gene were generated by transient co-transfection of GFP-expressing vector constructs based on HIV-1 (pHIV-CSCG, 5 µg), *Vesicular stomatitis*
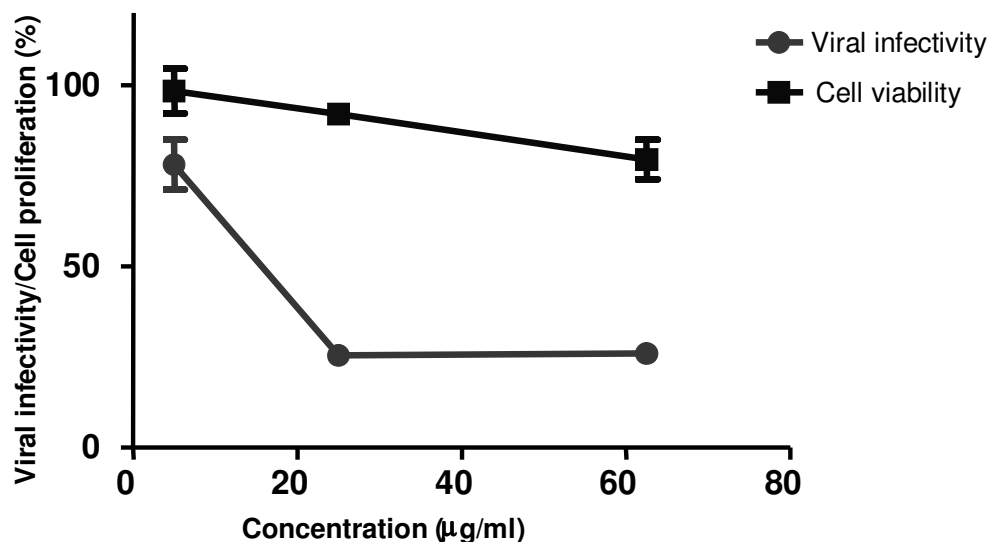
Figure 1. Inhibitory effect of aqueous extract of *Gynostemma pentaphyllum* against HIV-1 viral vector.

virus- G (VSV-G) protein expression plasmid (pHIT-G, 2 µg), a secretory alkaline phosphate expression plasmid (pSEAP, 0.1 µg) and the HIV-1 codon-optimised gag-pol, tat, rev expression plasmids pCMVΔR8.2 using polyethyleneimine (PEI) transfection protocol (Wagner et al., 2000) in 293T cells. The cells were sub-cultured and transfected during the log growth phase in a T-75 cell culture flask using a 1:1 ratio of DNA/PEI. Supernatant containing the viral particles were harvested after 48 h of transfection.

Vector-based antiviral assay

The effect of the test extracts on HIV-1 vector infectivity was determined according to a previously described technique (Wagner et al., 2000; Esimone et al., 2005). HeLa cells were plated in triplicates into 96-well plates at a density of 6000 cells/well and incubated overnight. Various concentrations of the test extracts (at twice the final concentrations indicated) were prepared in DMEM (without FBS) such that the final dimethyl sulfoxide (DMSO) concentration does not exceed 0.5%. These various concentrations were pre-incubated with equal volumes of the HIV-1 vector supernatant (100 µL) for 30 min at 37°C. Culture medium from the HeLa cell monolayers was replaced with 50 µL of the above vector/drug mixtures in triplicates and incubated for 2 h at 37°C + 5% CO_2. Fresh D-10 medium (50 µL) containing the drugs at the final concentrations stated were then added and the culture incubated for two days. Control wells (containing vector alone but without drug) received D-10 medium containing 0.5% DMSO instead. The number of infected cells expressing the GFP was determined by FACS acquisition (FACScalibur[TM] flow cytometer, Becton Dickinson). The FACS data was analysed with FCS Express V3 software®.

Cytotoxicity assay

The cytotoxicity assay was performed in parallel to the antiviral screening using the MTT assay method as previously described (Esimone et al., 2005) on HeLa cells. In the MTT assay, cells were seeded onto a 96-well plate at a concentration of 10^4 cells/well and a volume of 100 µL per well. A volume of 100 µL of the different concentrations of the test extracts (5, 25 and 62.5 µg/ml) were added to culture wells in triplicate. Culture medium without any drug was used as the "no-drug" control. After incubation at 37°C under 5% CO_2 for two days, a solution of MTT (3 mg/ml, 50 µL per well) was added to each well and further incubated at 37°C + 5% CO_2 for 4 h to allow formazan formation. After this time, the medium was removed and 150 µL of DMSO was used to dissolve the resulted blue formazan crystals in living cells. The optical density was determined at 550 nm using a multi-well microtiter plate reader (Tecan, Austria). Each single value of the triplicates was expressed as percentage of the mean of triplicates of the "no-drug" control cultures and the mean and standard deviation of the percentage values were calculated for each triplicate. The concentration of 50% cellular toxicity (TC_{50}) of the test extracts was calculated by non-linear regression.

RESULTS

The FACS analysis of viral infectivity show that the aqueous extract of *G. pentaphyllum* (AG) was active against the HIV-1 lentiviral vector and inhibited the early events of the viral replication cycle on HeLa cells in a concentration-dependent manner (5 to 62.5 µg/ml) (Figure 1). Inhibition of viral vector infectivity of HeLa cells was about 72% compared to the untreated control cells obtained at 62.5 µg AG/ml. A concentration of 6.21 µg AG/ml inhibited 50% of the viral vector infectivity (IC_{50}). The 50% cytotoxic concentration (TC_{50}) was 36.77 µg AG/ml in the assay performed by MTT assay in parallel with the study on viral vector infectivity inhibition. For the aqueous extract, this meant an inhibition selectivity index (SI) of 5.92 (Table 2).

The methanol extract of *G. pentaphyllum* (MG) showed inhibition of 50% of viral infectivity (IC_{50}) at a concentration of 8.32 µg MG/ml, while cytotoxicity of 50% of the cells (TC_{50}) was at 38.68 µg MG/ml and thus giving
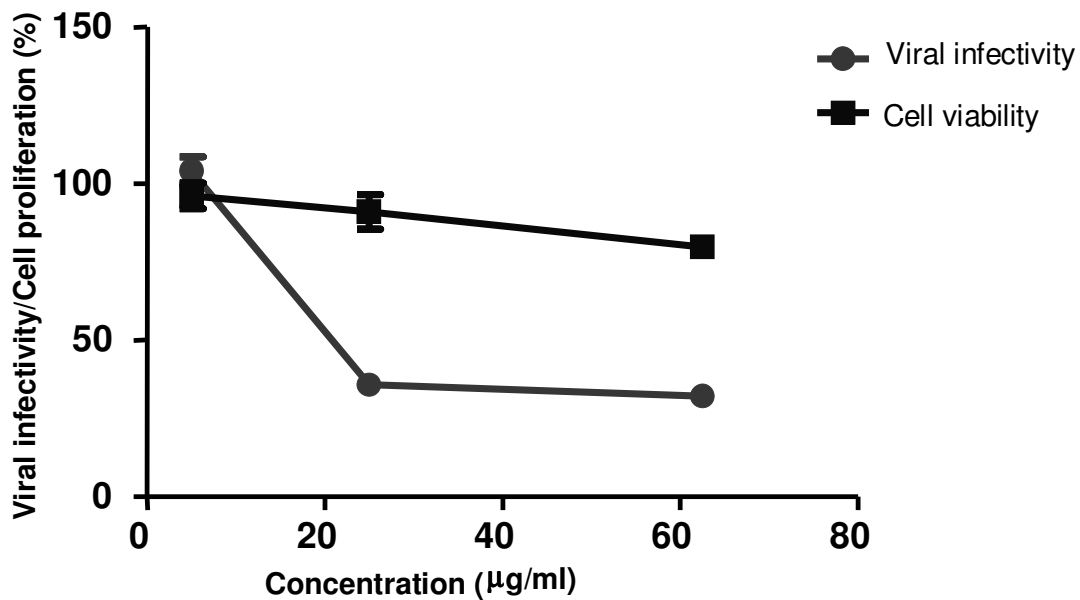
Figure 2. Inhibitory effect of methanolic extract of *Gynostemma pentaphyllum* against HIV-1 vector.
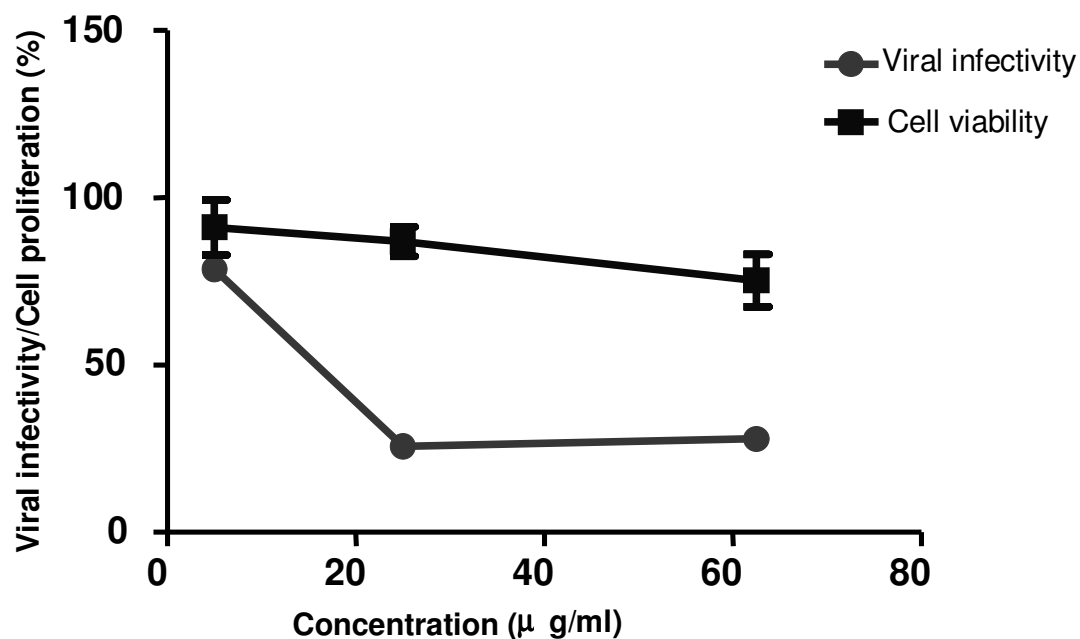

Figure 3. Inhibitory effect of ether extract of *Gynostemma pentaphyllum* against HIV-1 vector.

a selectivity index of 4.65 (Table 2). The MG showed less inhibition of the lentiviral vectors than the AG inhibiting the viral infectivity of HeLa cells only at the concentrations above 25 µg/ml. Inhibition of viral vector infectivity of HeLa cells was about 65% compared to the untreated control cells obtained at 62.5 µg MG/ml and the cells were still 83% viable at this concentration (Figure 2). Moreover, the ether extract of *G. pentaphyllum* (EG)

inhibited 50% of viral infectivity (IC_{50}) of HeLa cells at a concentration of 5.8 µg EG/ml, while 41.02 µg EG/ml was cytotoxic to 50% of the cells. This implied a viral inhibition selectivity index of 7.024 (Table 2). The percentage inhibitions of viral infectivity of EG (5, 25 and 62.5 µg/ml) were 28, 75 and 70%, respectively. Also, the cells were still viable by as much as 75% for the 62.5 µg EG/ml concentration studies (Figure 3).

Table 1. Relative abundance of different phytochemical constituents of *Gynostemma pentaphyllum* extracts.

Phytoconstituent	Extract		
	EG	AG	MG
Saponins	+	+++	++
Alkaloids	++++	-	+
Glycosides	-	+++	+++
Tannins	-	++	+
Carbohydrates	-	+++	+++
Reducing sugar	-	++	++
Flavonoids	++++	-	+++
Resins	++	-	+
Steroids	-	-	-
Terpenoids	-	-	-
Fats and oil	+	-	-
Acidic compounds	-	+++	-
Proteins	-	+	+++

AG, Aqueous extract of *Gynostemma pentaphyllum;* EG, ether extract of *G. pentaphyllum;* MG, methanolic extract of *G. pentaphyllum*.
-, Not present; +, present in small concentration; ++, present in moderately high concentration; +++, present in very high concentration; ++++, abundantly present.

DISCUSSION

The need for the discovery of novel anti-viral agents is increasingly felt due to many shortcomings of the existing drugs, which includes their inherent cytotoxicity to the host cells, the ease with which resistance are developed and the emergence of new viral variants which are not susceptible. In this study, we used a novel vector-based assay technique to screen lipophilic and polar solvent extracts of the plant, *G. pentaphyllum*. A VSV-G-pseudotyped, human immunodeficiency virus type 1-based, self-inactivating lentivirus vector that expressed GFP under the control of cytomegalovirus promoter was constructed and used as viral infective particles on HeLa cells. The infectious vector particles were generated by transient co-transfection of the vector plasmid (pHIV-1 CSCG) with packaging plasmids encoding tat, rev, gag-pol (pCMVΔR8.2), a VSV-G expression plasmid (pHIT-G) and a secretory alkaline phosphate expression plasmid (pSEAP), all necessary for viral infectivity. Transient supply of the packaging plasmids and the self-inactivation of the vector by deletion of the U3 region in the 3[1]-LTR ensured that the resulting lentiviral vector was only capable of a single round of replication, which makes the viral vectors comparatively safer than their parent wild retroviral virus. The infectious vector particles additionally differed from the wild-type HIV-1 virus in that they lacked some of the HIV-1 accessory genes such as nef, vif, vpu and vpr. Due to the transgene (in this case, green fluorescent protein) being integrated into the genome of target cells in the process of infection, vector infectivity in the presence or absence of various concentrations of the extracts is easily determined flow cytometrically as a function of the amount of GFP expression. In this system, GFP expression is driven by an internal CMV promoter and occurs after integration. The implication is that inhibitors of late stages in viral replication cycle such as inhibitors of new viral assemblage and inhibitors of budding are not detected by the screening assay used in the study.

The results of the study show that the extracts inhibited early events in viral replication cycle. Although HIV-1 has been shown to transduce both dividing and non-dividing cells (Mendoza et al., 1997; Merluzzi et al., 1990; Pannell, 1992), the cell cycle-dependence of productive HIV-1 and other retroviral infection is well established (De Clercq, 2000; Raff and Glover, 1988; Yang et al., 1999). Using the Rous sarcoma virus (an avian retrovirus) as a prototype retrovirus, it was shown that arrest of cells at the G0 phase resulted in failure of reverse transcription (and hence blockage of productive infection) (Zack et al., 1990; Esimone et al., 2007), while arrest of cells during the S phase did not affect the reverse transcription or integration process (Fritsch and Temin, 1977; Zack et al., 1990). The selectivity index of the *G. pentaphyllum* extracts (4.65 to 7.02) against the HIV-1 lentiviral vector is sufficiently large to ascertain that the antiviral effects are not simply due to the cytotoxicity of the extracts.

Phytochemical analysis of the extracts of *G. pentaphyllum* showed generally, the presence of sapo-nins, alkaloids, glycosides, tannins, carbohydrates, flavonoids, resins, acidic compounds and proteins. These phytoconstituents were present in varying degrees in the different solvent extracts of *G. pentaphyllum* (Table 1). Although, the scope of the work is not yet enough to associate the antiviral activity of *G. pentaphyllum* extracts

Table 2. Antiviral selectivity indices of *Gynostemma pentaphyllum* extracts against HIV-1 lentiviral vector.

Parameter	AG	EG	MG
TC_{50} (µg/ml)	36.77	41.02	38.68
IC_{50} (µg/ml)	6.21	5.84	8.32
Selectivity index (TC_{50}/IC_{50})	5.921	7.024	4.649

AG, Aqueous extract of *Gynostemma pentaphyllum;* EG, ether extract of *G. pentaphyllum;* MG, methanolic extract of *G. pentaphyllum*; IC_{50}, concentration of extract that inhibited viral infectivity (cytopathic effect) by 50%; TC_{50}, concentration of extract that is cytotoxic to 50% of the cells.

to a particular phyto-constituent, the antiviral activities may be attributed to some phytoconstituents contained in the extracts such as the tannins, which has been found in previous studies to prevent a variety of viral infections (Serafini et al., 1994, Nonaka et al., 1990). Tannins have also been demonstrated to inhibit viral reverse trans-criptase (Nonaka et al., 1990). Similarly, flavonoids have been shown to exhibit inhibitory effects against viruses including HIV and respiratory syncytial virus (Li et al., 2000). *G. pentaphyllum* is also rich in saponins which have been reported to be responsible for most of the observed activities of the plant (Cui et al., 1999).

In addition, the result of the vector-based antiviral assay show that the extracts had potent antiviral activity against the HIV-I lentiviral vector. The cytotoxicity studies conducted in parallel demonstrated that the viral vector infectivity of the extracts is not due to a general/metabolic toxicity of the extracts on the cells, but that the extracts selectively inhibited viral infectivity. The pattern of inhibition of the HIV lentiviral vector suggests that the extracts either directly interacted with the vector particles inhibiting the envelop protein or that it interacted with some host cells-derived components of the viral particle which could be the lipid membrane derived from the cell or the cellular membrane proteins that are frequently incorporated in lentiviral particles during budding (Gould et al., 2003). In previous studies, correlations between anti-HIV-I vector activity and anti-wild type HIV-I activity have been demonstrated. In one of such studies, Steinstraesser et al. (2005) demonstrated in an assay that the porcine defensin (protegrin-I) showed more than three-fold higher activity against the wild type HIV-I than against the corresponding lentiviral vector. It was also shown that some antiviral medicinal plant extracts showed up to ten-fold higher activities against wild type lentiviruses as against the corresponding lentiviral vector particles (Esimone et al., 2005). This is very instructive and points to the reliability of the high through-put viral vector particles-based assay in the screening of putative substances for antiviral activities. It also suggested that the extracts screened in this study using the lentiviral HIV-I vector could possibly show higher antiviral activities when used against wild type HIV-I virus.

For the pilot screening of potential anti-HIV medicinal plants, the vector-based assay used in this study offers multiple advantages. First, it reduces the risk of working with the wild type HIV-1 while retaining the sensitivity and reliability of the assay. Fear of the risks associated with working with wild type HIV-1 viruses has deterred many investigators from evaluating potential anti-HIV medicinal plant extracts, especially in less developed countries with limited resources and ill-equipped laboratories. Secondly, pseudo-typing of the vector with the VSV-G envelop ensures a wider cell tropism. Therefore, antiviral screening is not only confined to cell lines that express CD4 and/or CCR5 receptors (Esimone et al., 2007), but the viral vector can infect a wide range of other cell lines. Third, the assay is very rapid. Reading was obtained in about two days as opposed to 5 to 10 days for many conventional anti-HIV screening techniques. In addition, reading is simple and highly reproducible as opposed to the cumbersome procedure of enumerating HIV-induced cytopathic effects. In this study, the flow cytometric estimation of viral infectivity was simple and devoid of experimental bias and therefore reproducible. Finally, it is possible to make a clear distinction between direct antiviral effect of plant extract and the mere cytotoxic effects of the extract to target cell lines.

Conclusion

The results of the study show that the extracts of *G. pentaphyllum* possess potent and selective inhibitory activity against HIV-1 lentiviral vector particles infectivity. This supported the claims of efficacy in complementary and alternative medicine in the use of the extracts against different viral infections, including claims of activity against HIV infections. Extracts of *G. pentaphyllum* could be a possible source of lead antiviral drugs against HIV and should be further explored and harnessed.

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