

STUDIES OF ANTI-VIRAL ACTIVITY AND CYTOTOXICITY OF SPHAERANTHUS INDICUS AND COLDENIA PROCUMBENS

Dhanapal Venkatachalam

Department of Pharmacognosy, Sanjo College of Pharmaceutical Studies, Velappara, Palakkad, Kerala – 678 702

Abstract

The presence of drug-resistant HIV is a major global concern and warrants the development of novel anti-virals as alternative and inexpensive therapy. In the current study, previously unreported anti-viral activity of aqueous extracts of two indigenous plants used in ayurveda in India, like *Sphaeranthus indicus*. *Coldenia procumbens*, against replication of HIV-1 (III B) and HIV-2 (Rod) in MT-4 cells was assigned. These extracts tested were relatively nontoxic to human lymphocytic MT-4 cells, these extracts exhibited anti-HIV activity in an invitro MTT assay. The aqueous extract of *Sphaeranthus indicus* inhibited the HIV-1 replication at IC₅₀ of 52.35µg/ml, whereas this extract does not inhibit the HIV-2 replication in MT-4 cells. No cytotoxicity observed at 125µg/ml. The maximum inhibition of this extract is 110.5%. The aqueous extract of *Coldenia procumbens* inhibited both HIV-1 and HIV-2 replication at comparable IC₅₀ values, namely 32.10 and 41.60 respectively. No cytotoxicity was observed at 125µg/ml. The maximum inhibition of this extract in HIV-1 replication is 141% and HIV-2 replication is 114.5%. The above results showed that aqueous extract of *Coldenia procumbens* more potent anti-HIV activity than *Sphaeranthus indicus*. In this study, the aqueous extracts of *Sphaeranthus indicus* and *Coldenia procumbens* showed antiviral properties that may be useful in the treatment of patients with AIDS.

Keywords:

Anti-HIV activity, Aqueous extracts Sphaeranthus indicus, Coldenia procumbens.

Introduction

The control of viral diseases has been the subject of intense scientific endeavour, with special attention being devoted to those having retroviruses as etiological agents, including acquired immunodeficiency syndrome (AIDS). AIDS is a pandemic immunosuppressive disease which results in life-threatening opportunistic infections and malignancies. Since human immunodeficiency virus (HIV) is the main cause of this disease,¹⁻² many compounds have been studied for their inhibitory effects on HIV replication *in vitro*. HIV has two main targets *in vivo*: CD4 lymphocytes and tissue macrophages. Treatments aimed at the control of HIV replication in both cell types are envisaged. According to De Clercq,³ the replicative cycle of HIV comprises ten steps that could be considered suitable targets for chemotherapeutic intervention. A number of laboratories are actively involved in the development of antiviral agents that interfere with HIV at different stages of viral replication.⁴⁻⁵ According to the different stages at which the compounds interfere with the IV replication cycle, these compounds were assigned to any one of these ten classes of IV inhibitors. However only three groups of drugs (nucleoside and non-nucleoside analogs of reverse transcriptase and protease inhibitors) have been approved for general application, although prolonged use of these agents is limited because of their toxicity and the development of drug resistance. The high mutation rate of HIV frequently results in the rapid development of resistance towards the drugs used,⁶ and an attempt has been made to circumvent this problem by using a combination of drugs. AIDS treatment may require combination of compounds that shows synergistic antiviral effects to prevent the emergence of drug-resistant HIV

mutants. Combination of three or more drugs reduces the viral load to undetectable levels and results in the prolonged survival of treated patients.

Valuable drugs from traditionally used medicinal plants can be obtained by having through phytotherapeutic approach towards the current drug development. Many of the plant products have been used by patients with AIDS without even having any scientific proof about the anti-HIV activity it possess. Scientific evaluations have been started in those medicinal plants. Many studies provides information about the inhibitory activity of selected plants against HIV.⁷⁻⁹ A wide array of plant derived compounds have been explained to show anti-HIV activity like alkaloids, lignans flavonoids coumarins and terpene.¹⁰⁻¹² Many compounds of plant origin have been identified that inhibit different stages in the replication cycle of HIV¹³⁻¹⁶. The leaves of *Sphaeranthus indicus* (Linn.) which was traditionally used in Ayurveda for various illness like epilepsy, mental illness, jaundice, diabetes, and AIDS. The reports exhibited that it is also used for hypertensive, immunomodulatory, antioxidant, anti-inflammatory, bronchodilator, anti-hyperglycemic and hepato protective. It grows in rice fields, dry waste places and cultivated lands in tropical parts of India.¹⁷ *Coldenia procumbens* 1-6 Linn. (Family Boraginaceae) is abundantly available in most parts of India and has been used by locals for number of medicinal purposes. In villages, the fresh leaves are ground and applied to rheumatic swelling. The whole plant used in external application of causing suppuration of boils. The leaves are also used to cure fever, piles and scorpion sting and viral infections.¹⁸⁻¹⁹ The present study is designed to find the antiviral activity of Aqueous extracts of *Sphaeranthus indicus* (SI) and *Coldenia procumbens* (CP) against the replication of HIV-1(IIB) and HIV -2 (Rod). The cytotoxicity of SI and CP is also tested against mock-infected MT-4.cells

Materials and methods

Plant material

The fresh leaves of *Sphaeranthus indicus* Linn and *Coldenia procumbens* were collected from Coimbatore district, TamilNadu, India, identified and by authenticated by Dr. P Jayraman, Director of plant Anatomy Research Centre Chennai. A voucher herbarium specimen number SCOPS/SI/CP/01&2 was also preserved in the Sanjo College of pharmaceutical studies, Palakkad. The collected leaves were dried in shade and powdered to coarse consistency in cutter mill. The powder was passed through 60 # mesh particle size and stored in an airtight container at room temperature.

Preparation of Extract

The powdered leaves were subjected to batch extraction in Soxhlet apparatus. The solvent used as water. The powdered leaves were evenly packed in Soxhlet extractor for extraction with solvent. The temperature was maintained on an electric heating mantle with thermostat control. Appearance of brown solvent in the siphon tube was taken as the termination of extraction. The filtrate was concentrated using a rotary evaporator at low temperature (40-45 °C) and pressure and percentage yield was calculated.²⁰

Chemicals and Reagents

RPMI 1640 DM medium, Foetal calf serum (FCS), 3-(4,5-dimethylthiazo 1,2-yl) 2,5-diphenyl tetrazolium bromide (MTT), Gentamycin, Phosphate buffer saline, Acidified Isopropanol.

Cell Cultures

The MT-4 cells were grown in RPMI – 1640 DM (Dutch Modification) medium, supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS) and 20µg/ml gentamycin. The cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air, every 3 – 4 days; cells were spun down and seeded at 3 x 10⁵ cells / ml in new cell culture flasks. At regular time intervals, the MT-4 cells were analyzed for the presence of mycoplasma and consistently found to be mycoplasma free.

Virus

HIV – 1 (Strain HIV – III_B LAI) and HIV – 2 (Strain LAV – 2 ROD) were obtained from the culture supernatant of HIV – 1 or HIV – 2 infected MT – 4 cell lines. The virus titer of the supernatant was determined in MT-4 cells. The virus stocks were stored at -70°C until used.

Anti HIV assay²¹

Flat bottom, 96 well plastic microtiter plates were filled with 100µl of complete medium using a titertek multi drop dispenser. This eight channel dispenser could fill a micro titer tray in less than 10 seconds subsequently, stock solutions (10x final test concentration) of the aqueous extracts were added in 25µl volumes to two series of triplicate wells so as to allow simultaneous evaluation of their effects on HIV and mock – infected cells. Serial five fold dilutions were made directly in the microtiter trays using a Biomek 1000 robot. Untreated control HIV and mock-infected cell samples were included for each compound. 50µl of HIV at 100 CCID₅₀ medium was added to either infected or mock infected part of a micro titer tray. Exponentially growing MT-4 cells were centrifuged for 5 minutes at 140 ×g and the supernatants were discarded.

The MT-4 cells were resuspended at 6 x 10⁵ cells / ml in a flask which was connected with an autoclavable dispensing cassette of Titertek Multi drop dispenser. Under slight magnetic stirring 50 µl volumes were then transferred to the microtiter tray wells. The outer row wells were filled with 200µl of medium. The cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. The cells remained in contact with the test compounds during the whole incubation period. Five days after infection the viability of mock and HIV-infected, cells were examined spectrophotometrically by the MTT method.

MTT assay²²⁻²³

The MTT assay is based on the reduction of the yellow coloured 3 – (4, 5 – dimethylthiazo 1-2-yl) -2, 5 – diphenyltetrazolium bromide (MTT) by mitochondrial dehydrogenase of metabolically active cells to a blue formazan which can be measured spectrophotometrically. Therefore, to each well of the microtiter plates, 20µl of a solution of MTT (7.5mg/ml) in phosphate buffered saline was added using the Titertek Multidrop dispenser. The trays were further incubated at 37°C in a CO₂ incubator for 1 hour. A fixed volume of medium (150 µl) was then removed from each cup using M96 washer (ICN flow) without disturbing the MT – 4 cell clusters containing the formazan crystals. Solubilization of the formazan crystals was achieved by adding 100 µl 10% (v/v) Triton x-100 in acidified isopropanol (2ml concentrated HCl, Per 500 ml solvent) using the M96 washer. Complete dissolution of the formazan crystals could be obtained after the trays had been placed on a plate shaker for 10min. Finally, the absorbances were read in a eight – channel computer – controlled photometer (Multiscan MCC, ICN flow) at two wavelengths (540 and 690 nm). The absorbance measured at 690nm was automatically subtracted from the absorbance at 540nm, so as to eliminate the effects of non-specific absorption. Blanking was carried out directly on the microtiter plates with the first column wells which contained all reagents except the MT-4 cells.

All data represent the average values for a minimum of three wells. The 50% cytotoxic concentration (CC₅₀) was defined as the concentration of compound that reduced the absorbance (OD₅₄₀) of the mock-infected control sample by 50%. The percent protection achieved by the compounds in HIV-infected cells was calculated by the following

$$\text{formula. } \frac{(\text{OD}_T)_{\text{HIV}} - (\text{OD}_C)_{\text{HIV}}}{(\text{OD}_C)_{\text{mock}} - (\text{OD}_C)_{\text{HIV}}} \text{ expressed in \%}$$

Whereby (OD_T) HIV is the optical density measured with a given concentration of the test compound in HIV infected cells; (OD_C) mock is the optical density measured for the control untreated mock infected cells; all OD values determined at 540nm. The dose achieving 50% protection according to the above formula was defined as IC₅₀.

Results and Discussion
Table-1 Anti HIV activity and cytotoxicity of S.indicus and C.procumbens

Compound	Strain	IC ₅₀ µg/ml	CC ₅₀ µg/ml	SI CC ₅₀ / IC ₅₀	Max protection %	Average IC ₅₀ µg/ml	S.D.	Average CC ₅₀ µg/ml
AESI	III _B	52.2	>125	>2	117			
		52.5	>125	>2	104	52.35	0.21	>125
	ROD	> 125	>125	X1	27			
		> 125	>125	X1	17			>125
AACP	III _B	38.3	>125	<1	135			
		25.9	>125	<1	147	32.10	8.77	>125
	ROD	44.1	>125	<1	112			
		39.1	>125	<1	117	41.60	3.54	>125

AESI- Aqueous extract of *Sphaeranthus indicus*. AACP- Aqueous extract of *Coldenia procumbens*, IC₅₀- 50 % Effective concentration, CC₅₀- 50% Cytotoxic concentration, SI-Selectivity index(CC₅₀/IC₅₀)

The in vitro anti-HIV activity of aqueous extracts of leaves of *Sphaeranthus indicus* and *Coldenia procumbens* was shown in (Table 1). These extracts showed anti HIV activity in MT-4 cells by MTT assay. The aqueous extract of *Sphaeranthus indicus* inhibited the HIV-1 replication at IC₅₀ of 52.35µg/ml, whereas this extract does not inhibit the HIV-2 replication in MT-4 cells. No cytotoxicity observed at 125µg/ml. The maximum inhibition of this extract is 110.5%. The aqueous extract of *Coldenia procumbens* inhibited both HIV-1 and HIV-2 replication at comparable IC₅₀ values, namely 32.10 and 41.60 respectively. No cytotoxicity was observed at 125µg/ml. The maximum inhibition of this extract in HIV-1 replication is 141% and HIV-2 replication is 114.5%. The above results showed that aqueous extract of *Coldenia procumbens* more potent anti-HIV activity than *Sphaeranthus indicus*. In these experiments, macrophages were infected with HIV at a high titer, and active extract was added at different times during viral infections. The extract inhibited HIV replication under conditions in which it was added at the same time as the viral infection. On the other hand, when extract was added only during viral adsorption, a slight decrease in the kinetics of HIV replication was observed, although the active extract inhibited the virus replication even after penetration of the virus into the cells. Differences observed when the extract was added only after adsorption or was maintained thereafter were not significant, and detectable p24 levels were very close to the threshold of the technique. Thus, these findings indicate that the main target for antiviral activity of an aqueous extracts *Sphaeranthus indicus* and *Coldenia procumbens* could be estimated to be the early steps of virus replication, including virus-cell attachment, virus-cell fusion and cell-to-cell fusion. In a preliminary chemical characterization, the active compounds appeared to be of high relative molecular weight, probably due to the presence of polysaccharides and flavonoids. These type of compounds have been reported in the literature as a potent inhibitor of different enveloped viruses, including HIV.²⁴⁻²⁷

Conclusion

Our studies indicate that the aqueous extracts of *Sphaeranthus indicus* and *Coldenia procumbens* contains antiviral activity that may be useful in the treatment of patients with AIDS. These extracts inhibited HIV replication without producing cytotoxicity. This study therefore concludes that the medicinal plant possesses anti-HIV activity. We are undertaking further studies on the purification of bioactive compounds from the *Sphaeranthus indicus* and *Coldenia procumbens* and on the various modes of their action. The study thus, scientifically confirms the traditional use of the medicinal plant in AIDS.

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