BMC Complementary Medicine and Therapies

RESEARCH ARTICLE Open Access

In vitro anti-HIV activity of some Indian medicinal plant extracts

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Abstract

Background: Human Immunodeficiency Virus (HIV) persists to be a significant public health issue worldwide. The current strategy for the treatment of HIV infection, Highly Active Antiretroviral Therapy (HAART), has reduced deaths from AIDS related disease, but it can be an expensive regime for the underdeveloped and developing countries where the supply of drugs is scarce and often not well tolerated, especially in persons undergoing long term treatment. The present therapy also has limitations of development of multidrug resistance, thus there is a need for the discovery of novel anti-HIV compounds from plants as a potential alternative in combating HIV disease.

Methods: Ten Indian medicinal plants were tested for entry and replication inhibition against laboratory adapted strains HIV-1_{IIIB}, HIV-1_{Ada5} and primary isolates HIV-1_{UG070}, HIV-1_{VB59} in TZM-bl cell lines and primary isolates HIV-1_{UG070}, HIV-1_{VB59} in PM1 cell lines. The plant extracts were further evaluated for toxicity in HEC-1A epithelial cell lines by transwell epithelial model.

Results: The methanolic extracts of *Achyranthes aspera, Rosa centifolia* and aqueous extract of *Ficus benghalensis* inhibited laboratory adapted HIV-1 strains (IC_{80} 3.6–118 μg/ml) and primary isolates (IC_{80} 4.8–156 μg/ml) in TZM-bl cells. Methanolic extract of *Strychnos potatorum*, aqueous extract of *Ficus infectoria* and hydroalcoholic extract of *Annona squamosa* inhibited laboratory adapted HIV-1 strains (IC_{80} 4.24–125 μg/ml) and primary isolates (IC_{80} 18–156 μg/ml) in TZM-bl cells. Methanolic extracts of *Achyranthes aspera and Rosa centifolia,* (IC_{80}1-9 μg/ml) further significantly inhibited HIV-1 primary isolates in PM1cells. Methanolic extracts of *Tridax procumbens, Mallotus philippinensis, Annona reticulate,* aqueous extract of *Ficus benghalensis* and hydroalcoholic extract of *Albizzia lebbeck* did not exhibit anti-HIV activity in all the tested strains. Methanolic extract of *Rosa centifolia* also demonstrated to be non-toxic to HEC-1A epithelial cells and maintained epithelial integrity (at 500 μg/ml) when tested in transwell dual-chamber.

Conclusion: These active methanolic extracts of *Achyranthes aspera* and *Rosa centifolia,* could be further subjected to chemical analysis to investigate the active moiety responsible for the anti-HIV activity. Methanolic extract of *Rosa centifolia* was found to be well tolerated maintaining the epithelial integrity of HEC-1A cells in vitro and thus has potential for investigating it further as candidate microbicide.

Keywords: HIV, TZM-b1, PM1, *Achyranthes aspera*, *Rosa centifolia*

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Background

Human Immunodeficiency Virus (HIV) persists to be a significant public health issue worldwide. In 2018, 37.9 million people are living with HIV globally; out of which 36.2 million are adults and 1.7 million are children less than 15 years old. There were 1.7 million new infections and 770,000 people died from AIDS related illness worldwide [1]. The current strategy for the treatment of HIV infection is Highly Active Antiretroviral Therapy (HAART), based on combination of inhibitors of reverse transcriptase and protease. Although HAART has considerably reduced deaths from AIDS related disease, it often has side effects and not well tolerated especially in persons undergoing long term treatment and maintains the risk of developing multidrug resistance [2]. Moreover, HAART is an expensive regime for underdeveloped and developing countries where the drugs are inaccessible to the HIV infected patients. Thus, there is a need for the discovery of novel therapeutic strategies, which identify new anti-HIV compounds from natural sources particularly from medicinal plants.

Natural sources provide a large reservoir for screening of anti-HIV agents with novel structure and antiviral mechanism due to their structural diversity. For the purpose of this study, ten Indian traditional medicinal plants, *Albizzia lebbeck*, *Tridax procumbens, Achyranthes aspera*, *Ficus benghalensis*, *Mallotus philippinensis*, *Rosa centifolia, Strychnos potatorum*, *Annona reticulate*, *Ficus infectoria* and *Annona squamosa* were selected to investigate their in vitro inhibitory activity against entry inhibition/replication of HIV-1 as first step towards identification of potential anti-HIV microbicide. The microbicides provide protection by directly inactivating HIV or preventing HIV from attaching, entering or replicating in susceptible target cells as well as dissemination from target cells present in semen or the host cells that line the vaginal/rectal wall [3]. These plants were selected on the basis of detailed patent survey and scientific articles on the ethnomedicinal usages of the plant genera directly in HIV/AIDS or for symptoms/conditions closely associated with this disease (Table 1).

Plants such as *R. centifolia, S. potatorum, F. infectoria*, *F. benghalensis* and *M. philippinensis* were selected because other species of the same genera have exhibited anti-HIV activity [56–60]. Its traditional use in gonorrhoea and leukeorrhea [61] and suppressive effects on sperm motility [39] further made *S. potatorum,* a plant of choice for this study. Fruit pulp of *A. squamosa* has been reported to inhibit HIV replication significantly in H9 lymphocytes [49] therefore the seeds of *A. squamosa* which have also shown spermicidal property, an additional desirable attribute for a vaginal microbicide [62] was selected for the study. In addition the leaves of other species *A. reticulate* were also selected for assessing the anti-HIV activity.

Taylor et al., [63, 64] reported methanolic extract of *T. procumbens* to exhibit in-vitro anti-Herpes Simplex Virus activity in Vero cells; hence it was selected for investigating its anti-HIV activity. Anticipating the potential of spermicide-based vaginal contraceptives in the reproductive health of women such as Nonoxynol (N-9) and Praneem polyherbal (*Azadirachta indica* leaves, *Sapindus mukerossi* pericarp of fruit and *Mentha citrate* oil) [65]; two plant extracts, methanolic leaf extract of *A. aspera* that has exhibited safety as well as good antifertility property [66] and methanolic pod extract of *A. lebbeck* which has been shown to suppress spermatogenesis and alter the structure and activity of the Sertoli and Leydig cells [4] were considered worthwhile to explore for anti-HIV activity.

Therefore, under the DBT-ICMR sponsored programme (HIV/AIDS and Microbicides, Phase I) developed for screening plant derived HIV microbicidal candidates, we evaluated these 10 plant extracts against 2 CXCR4 (HIV-1_{IIIB}, HIV-1_{UG070}) and 2 CCR5 tropic (HIV-1_{Ada5}, HIV-1_{VB59}) HIV-1 strains.

Methods

Plant materials and extraction

10 plant materials were collected from various parts of India in different seasons. A plant taxonomist at publicly available herbarium, Botanical Survey of India, Pune, India, validated scientific names and classification of these plants. The specimens were also deposited in the herbarium. Table 2 presents ethno-botanical information and solvents used for extraction of the selected plants.

The collected plant materials were cleaned, freed of foreign contaminates and washed with water, first air dried and then dried in an electric oven at 40 °C. The dried plant materials were pulverized in an electric mixer. The plant materials were extracted with various solvents individually by hot continuous Soxhlet extraction method for 18–24 h. After extraction, the extract obtained was filtered through 0.2-μm syringe filter and then concentrated on a rotary evaporator by distilling off the solvent under vacuum at 40 °C. The concentrated extracts was finally lyophilized to obtain free flowing powder and stored in airtight bottles in the refrigerator at 4-8 °C. The extractive yields of the individual extracts are recorded in Table 2. Powder was reconstituted in DMSO for final concentration of extract 10 mg/ml and stored at -20 °C until tested for anti-HIV1 activity.

Preliminary phytochemical investigation

Qualitative tests were carried out to ascertain the presence of various phytochemicals in the plants extract of the selected plants using the methods described by Harbourne [67] (Table 3). It involved the appropriate addition of chemicals and reagents to the

Table 1 Ethnomedicinal usages of selected plant materials

Sr No	Botanical name	Common name	Family	Conventional use and published reports
1	*Albizzia lebbeck*	Shirisha	Mimosaceae	Bark: Anti-oxidant, anti-fertility, anti- microbial activity Seeds: Anti-inflammatory activity [4, 5].
2	*Tridax procumbens*	Ghamra	Asteraceae	Whole plant: Anti-microbial Flowers, Leaves: Anti septic, insecticidal, parasiticidal, anti-Cancer Activity Aerial parts: Hepatoprotective Leaves: Hypotensive, anti- diabetic, immunomodulating activity [6–12].
3	*Achyranthes aspera*	Apaamaarga	Amaranthaceae	Whole plant: Nephroprotective, hypolepidemic activity. Roots: anti-oxidant, spermicidal, activity Leaves: anti-oxidant, anti-fertility, anti-depressant, anti-cancer, anti- microbial activity Aerial parts: Hepatoprotective activity Seeds: Anti- microbial activity [13–22].
4	*Ficus benghalensis*	Vad	Moraceae	Whole plant: anthelmentic, anti-bacterial activity. Bark: Anti-inflammatory, anti-bacterial activity. Aerial roots: anti-oxidant, anti-diabetic, immunomodulatory activity [23–27].
5	*Ficus infectoria*	Pilkhan		Bark and Leaves: anti-oxidant, anti-hyperlipidemic, hypoglycemic activity [28].
6	*Mallotus philippinensis*	Kamala	Euphorbiaceae	Seeds: Anti-fertility activity. Stembark: anti-oxidant, anti-tumor activity, anti- bacterial. Fruits: anti-inflammatory, immunoregulatory, anti-proliferative activity. Leaves: Hepatoprotective activity Roots: Anti-leukaemic activity [29–36].
7	*Rosa centifolia*	Gulab	Rosaceae	Leaves: treating wounds, ophthalmia, hepatopathy, hemorrhoids and anti-microbial, Flowers: cardio tonic, anti-inflammatory, anti-asthmatic, anti-bronchitic, anti-diarrheal, dysmenorrheal, urinary tract infections, anti-tussive activity [37, 38].
8	*Strychnos potatorum*	Nirmali	Loganiaceae	Plant: Anti-diabetic, anti- microbial activity Seeds: Contraceptive, diuretic, anti-inflammatory, hepatoprotective, antioxidant, antiarthritic activity [39–45].
9	*Annona reticulate*	Ramphal	Annonaceae	Leaves: Anti-oxidant, anti- inflammatory, anti-helmentic activity. Seeds: Anti-cancer [46–48].
10	*Annona squamosa*	Sitafal		Bark: Anti-malarial activity Seeds: Anti-tumor activity Twigs: Anti-ulcer activity Leaves: anti- oxidant,hepatoprotective, anti- bacterial activity Fruit pulp: Anti-HIV activity [49–55].

Legend: Details of plants selected and their reported conventional use

concentrated extract of the plant material in a test tube. The changes in the appearance of the colour, as the case may be, confirmed the presence of alkaloids, flavanoids, tannins, steroids and saponins.

Cells, viral strain and culture conditions

TZM-bl (recombinant HeLa cells expressing high levels CD4 receptor, CXCR4 and CCR5 co-receptors) and PM1 cells (Clonal derivative of HUT 78) were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (NIH ARRRP). The HEC-1A (human endometrial adenocarcinoma) cell line was kindly provided by Dr. R. Fichorova (Associate Professor, Brigham and Women's Hospital, Boston, USA) and National Institute of Virology, Pune, respectively.

The TZM-bl cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, USA) and PM1 and HEC-1A cells in RPMI-1640 (Sigma-Aldrich, USA), supplemented with 10% heat inactivated fetal bovine serum (FBS, Moregate Biotech, Australia) and standard antibiotic-antimycotic cocktail.

Table 2 Procurement, authentication and solvents used for extraction of plant material

Sr. No.	Plant Name	Part of plant	Authentication No.	Solvent for extraction	% yield (± SD)
1	*Albizzia lebbeck*	Whole pods	RR 3794	Hydroalcohol	17.20 (±0.32)
2	*Tridax procumbens*	Aerial parts	KK1	Methanol	7.2 (±1.32)
3	*Achyranthes aspera*	Aerial parts	PADAAP1	Methanol	10.74 (±0.02)
4	*Ficus benghalensis*	Leaves	PADFB1	Water	7.56 (±0.52)
5	*Mallotus philippinensis*	Leaves	MPADP12	Methanol	5.72 (± 0.09)
6	*Rosa centifolia*	Leaves	ROAP1	Methanol	9.46 (± 0.56)
7	*Strychnos potatorum*	Seeds	SPAP2	Methanol	15.00 (±0.43)
8	*Annona reticulata*	Leaves	APAR1	Methanol	7.89 (±0.07)
9	*Ficus infectoria*	Leaves	APF1	Water	19.08 (±0.02)
10	*Annona squamosa*	Seeds	SS1/ 2008	Hydroalcohol	10.87 (±0.15)

Legend: Procurement, authentication no. & extraction details of the plants parts used for the study

The laboratory adapted HIV-1 strains [HIV-1IIIB (X4, subtype B), HIV-1Ada5 (R5, subtype B)] and the primary isolate HIV-1UG070 (X4, Subtype D) were procured from National Institutes of Health-AIDS Research and Reference Reagent Program, while the Indian isolate HIV-1VB59 (R5, subtype C) was obtained from the National AIDS Research Institute (NARI), Pune. Phytohemagglutinin-P (5 μg/ml, Sigma Aldrich, USA) activated peripheral blood mononuclear cells (PBMC) derived from healthy donors were used for the growth of all the viral strains. HIV-1 p24 antigen detection kit (Vironostika HIV-1 Antigen, Netherlands) was used to determine the virus production in cell culture supernatants. Samples of viral culture supernatants free form cells were obtained by centrifugation and further filtered and finally stored at -70oC for further use. Spearman Karber formula was used to ascertain the 50% tissue culture infectivity dose (TCID50) of each virus stock in both TZM-bl and PM1 cells [68].

Anti HIV1 assays

Determination of cytotoxicity in the uninfected TZM-bl and PM1 cell lines

The cytotoxicity of the extracts was determined in uninfected TZM-bl cells using colorimetric assay that measures the reduction of a yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase (Sigma Aldrich, USA) [69]. Briefly, two-fold dilutions of the extracts were prepared, added to 96 well plates pre-seeded with TZM-bl cells (10,000 cells/well) in quadruplicate and incubated for 48 h at 37 °C. The MTT (20 μl, 5 mg/ml) solution was added and the plates were incubated

Table 3 Phytochemical screening of selected plant extracts

Sr. No.	Plant Extracts	Steriods	Saponins	Flavanoids	Alkaloids	Tannins/ Phenolic Compounds
1	*A. lebbeck*	+++	+	+	++++	++
2	*T. procumbens*	–	–	+	–	–
3	*A. aspera*	+++	+	+	++++	++++
4	*F. benghalensis*	+++	+	+	++++	+++
5	*M. philippinensis*	–	+	+	++++	++
6	*R. centifolia*	–	+	+	++++	++
7	*S. potaotorum*	+++	+	–	++++	++
8	*A. reticulate*	+++	–	+	++++	+++
9	*F. infectoria*	++	+	++	++++	++++
10	*A. squamosa*	–	+	+	++++	++

Legend: presence or absence of phytochemical components in plant extracts by different methods
-: Absent, ++++: Present in large proportion, +++: Present in good proportion, ++: Present in moderate proportion, +: Present in low proportion
Tests for Steroid: 1. Salkowski Reaction 2. Liebermann- Burchard Reaction 3. Liebermann's Reaction
Tests for Saponins: 1. Foam Test
Tests for Flavanoids: 1. Shinoda Test 2. Lead acetate Test
Test for Alkaloids: 1. Dragendorff's Test 2. Mayer's Test 3. Hager's Test 4. Wagner's Test
Test for Tannins and Phenolic compounds: 1. 5% Ferric Chloride 2. Dilute Iodine Solution 3. Lead acetate solution 4. Dilute Potassium permanganate solution

further for 4 h. The supernatant was removed, 200 μl of DMSO was added, the plates were incubated for 1 h and the absorbance was read at 550 nm and 630 nm. The percent viability was calculated by comparing cell viability in the absence of extract using following formula and the results were expressed as CC_{50} (50% cytotoxic concentration).

$$\%\text{Cell Viability} = [\text{OD test extract}/\text{Average OD control}] \times 100$$

The cytotoxicity in uninfected PM1 cells of all the extracts was determined in a similar manner by using a similar dilution scheme and procedure as mentioned above for TZM-bl cells. The cell viability was determined by the trypan blue dye exclusion assay (Sigma Aldrich, USA) and the results were expressed as CC_{50} [70].

Preliminary screening for anti-HIV1 activity against laboratory adapted strains in TZM-bl cell lines

The anti-HIV1 activity was tested against Cell-free (CF) and Cell-associated (CA) X4 tropic (HIV-1_{IIIB}) and R5 tropic (HIV-1_{Ada5}) laboratory adapted strains in TZM-b1 cell lines.

In cell free assay, the viral stocks (400 $TCID_{50}$) were pre-treated in duplicate with sub toxic concentrations of the extracts/fractions for 1 h, at 37 °C prior to addition onto the TZM-bl cells (10,000 cells/well). While in cell-associated assay, the cells (10,000 cells/well) were pre-infected with the viral stocks (400 $TCID_{50}$) for 2 h at 37 °C before exposure to the extracts/fractions [71]. After 48 h, the supernatant was collected and luciferase activity was determined using Britelite plus (Perkin Elmer, USA). Dextran Sulphate (Sigma Aldrich, USA) and Azidothymidine (AZT, CIPLA, India) were used as positive controls for cell free and cell associated assays respectively. The results were expressed as IC_{50} (50% inhibitory concentration), IC_{80} (80% inhibitory concentration) and Therapeutic Index ($TI=CC_{50}/IC_{50}$).

Confirmation of anti-HIV1 activity against primary isolates in TZM-bl and PM1 cell lines

The anti-HIV1 activity was tested against Cell-free (CF) and Cell-associated (CA) X4 tropic (HIV-1_{UG070}) and R5 tropic (HIV-1_{VB59}) primary isolates in TZM-b1 and PM1 cell lines.

The procedure for anti-HIV1 activity against primary isolates in TZM-bl cell lines followed was same as mentioned above for anti-HIV1 activity against laboratory adapted strains. The results were expressed as percentage inhibition calculated using following equation

$$\%\text{Inhibition} = 1-[(\text{luminescence in presence of the test extract or fraction} - \text{luminescence of uninfected control cells}/\text{luminescence of cells infected with virus} - \text{luminescence of uninfected control cells})] \times 100$$

The results were expressed as IC_{80} (80% inhibitory concentration).

The anti-HIV activity against primary isolates was also evaluated in PM1 cell lines using 24-well plate (Corning, USA). In the cell free assay, 20 $TCID_{50}$ the viral stock (HIV-1_{UG070} and HIV-1_{VB59}) was pre-treated with sub toxic concentrations of the extracts/fractions, before addition onto the cells ($5x10^4$cells/well). Whereas, in the cell associated assay, the PM1 cells ($5x10^4$cells/well) were pre-infected with 20 $TCID_{50}$ of the viral stock and then exposed to the extracts/fractions [72]. The virus growth was monitored by Vironostika®p24 antigen ELISA (Biomerieux, France). Dextran sulphate and AZT were used as positive controls for cell free and cell associated assays respectively. The percent inhibition was calculated by comparing activity in absence of the extracts/fractions/control drug using the formula mentioned above and the results were expressed as IC_{80}.

Toxicity testing using Transwell epithelial model

Cytotoxicity assay

The toxicity of selected plant extract was determined in HEC-1A using similar protocol as described for TZM-bl cells, only with a difference of the read out system, i.e. LDH cytotoxicity detection kit (Roche Diagnostics, Germany).

Determination of epithelial integrity in Transwell dual-chamber system

The epithelial integrity was determined as described by Gali et al., [73]. Briefly, HEC-1A cells (1×10^5/100 μl) were cultured for 7 days on the apical chamber of a Laminin coated dual-chamber Transwell® system (growth area: $0.3cm^2$, pore size: 3.0 μm) (Corning Costar Corp, USA). After 7 days incubation, two-fold serial dilutions of test preparations (100 μl) were added on to the HEC-1A cells and incubated for 24 h (37 °C, 5% CO_2). The test preparations were removed and 100 μl of a 1/20 dilution of yellow-green fluorescent microspheres (FluoSpheres® sulphate microspheres, Molecular Probes Europe NV, Netherlands) were added in the apical chamber. After 24 h, 100 μl of medium was harvested from the basal chamber and the fluorescence was measured using a fluorometer (Perkin Elmer, USA). Untreated HEC-1A cells and 1% Nonoxynol-9 were used as controls for measuring percent transmission.

Results

Preliminary phytochemical investigation

The preliminary phytochemical evaluation of plant extracts for the presence of steriods, flavanoids, alkaloids,

saponins, tannins and phenolic acids was done for 10 plants extracts from 8 different families. Steroids were not present in *T. procumbens, M. philippinensis, R. centifolia* and *A. squamosa* extracts and the Saponins in *T. procumbens* and *A. reticulate* extract. Only flavanoids was present in *T. procumbens* extract while it was not present in *S. potaotorum* extract (Table 3).

Determination of cytotoxicity in TZM-bl and PM1 cell lines

Six methanolic extracts, two aqueous extracts and two hydroalcoholic extracts of 10 medicinal plants were examined for their ability to inhibit HIV-1 entry and replication. The in vitro toxicity of these extracts to TZM-bl cells was investigated by MTT assay. Methanolic extracts of, *A. aspera*, hydroalcoholic extract of *A. squamosa* and water extract of *F. benghalensis* tested were relatively non-toxic to TZM-bl cells at a CC_{50} value between 51 and 72 μg/ml. The CC_{50} values of other extracts such as methanolic extract of, *R. centifolia*, *S. potatorum* and aqueous extract of *F. infectoria* were found to be comparatively higher ranging between 118 and 147 μg/ml. However, methanolic extract of *A. reticulate* was found to be toxic at a very low concentration (CC_{50} = 11 μg/ml) as compared to the other extracts (Tables 4 and 5).

Cytotoxicity of plant extracts, *A. aspera, F. benghalensis, R. centifolia, S. potatorum, F. infectoria* and *A. squamosa* showing activity in preliminary anti-HIV-1 assay was carried out in PM1 cells using trypan blue dye exclusion assay. The 50% cytotoxicity was observed at a concentrations ranging from 2.9–46 μg/ml. Aqueous extract of *F. benghalensis* was toxic at a very low concentration as compared to other extracts (Table 5).

Preliminary screening for anti-HIV1 activity against laboratory adapted strains in TZM-bl cell lines

Plant extracts of *A. aspera*, *F. benghalensis*, *R. centifolia*, *S. potatorum*, *F. infectoria* and *A. squamosa* showed inhibition of HIV-1_{IIIB} and HIV-1_{Ada5} laboratory adapted strains in both cell free and cell associated assays. Aqueous extract of *F. infectoria* revealed significant activity against the laboratory adapted strains with estimated IC_{80} in the range of 4.24–125 μg/ml giving TI of 189, 49 and 27 in cell free HIV-1_{IIIB}, HIV-1_{Ada5} and cell associated HIV-1_{IIIB} respectively. This was followed by methanolic extract of *A. aspera* which showed activity with preliminary IC_{80} in the range of 18–35 μg/ml giving TI of 14, 35 and 13 in cell free HIV-1_{IIIB}, HIV-1_{Ada5} and cell associated HIV-1_{IIIB} respectively. Aqueous extract of *F. benghalensis* exhibited activity in both laboratory adapted strains with estimated IC_{80} in the range of 18–35 μg/ml giving TI between12–32. Methanolic extract of *S. potatorum* showed activity with preliminary IC_{80} in the range of 29.17–79.35 μg/ml giving estimated TI of 24 in cell free HIV-1_{Ada5} strain. Methanolic extract of *R. centifolia* and hydroalcoholic extract of *A. squamosa* displayed very low activity (estimated TI in the range of 1–6). Hydroalcoholic extract of *A. lebbeck*, methanolic extract of *T. procumbens*, *M. philippinensis* and *A. reticulate* did not demonstrate any activity against cell free and cell-associated laboratory adapted HIV-1 strains.

Confirmation of anti-HIV activity against primary isolates in TZM-bl and PM1 cell lines

The plant extracts showing activity in preliminary screening against laboratory adapted strains were further screened both cell free and cell associated assays against primary isolates HIV-1_{UG070} and HIV-1_{VB59} in TZM-b1

Table 4 Inhibitory concentrations and therapeutic index of plant extracts against Laboratory adapted HIV-1_{IIIB} and HIV-1_{Ada5} strains in TZM-bl cell lines

Sr. No.	Plant Extract	CC_{50} (μg/ml)	IC50				IC80				Therapeutic Index			
			CF		CA		CF		CA		CF		CA	
			IIIB	Ada5	IIIB	Ada5	IIIB	Ada5	IIIB	Ada5	IIIB	Ada5	IIIB	Ada5
1	*A. lebbeck*	203	No activity											
2	*T. procumbens*	62	No activity											
3	*A. aspera*	69	8.3	2.3	4.1	28.4	18	21	26	35	14	35	13	3
4	*F. benghalensis*	72	6	6	5.2	2.25	9.6	9.6	8.32	3.6	12	12	14	32
5	*M. philippinensis*	71	No activity											
6	*R. centifolia*	132	13.6	24.8	51.9	75.4	30.4	45.2	96.1	118	5	6	1	1
7	*S. potatorum*	124	4.97	3.65	18.51	17.67	29.17	35.89	79.35	78.43	10	24	8	7
8	*A. reticulata*	11	No activity											
9	*F. infectoria*	147	1.18	2.97	4.97	87.38	4.24	8.6	52.49	> 125	189	49	27	2
10	*A. squamosa*	51	No activity		23	11.3	No activity		27	20	2	4	2	3

CC_{50}–50% cytotoxic concentration, IC_{50}–50% inhibitory concentration
IC_{80}–80% inhibitory concentration, CF- Cell Free, CA- Cell Associated

Table 5 Inhibitory concentrations of plant extracts against Primary isolates HIV-1UG070 and HIV-1VB59 in TZM-bl and PM1 cell lines

Extracts/ Fractions/ Controls		TZM-bl assay					PM-1 assay				
		CC_{50} (μg/ml)	IC_{80}(μg/ml)				CC_{50} (μg/ml)	IC_{80} (μg/ml)			
			CF HIV-1		CA HIV-1			CF HIV-1		CA HIV-1	
			UG 070	VB 59	UG 070	VB 59		UG 070	VB 59	UG 070	VB 59
A. aspera		69	4.8	< 19.53	26	53	10.7	2.6	8.4	1	1.6
F. benghalensis		72	< 78	< 156	< 156	< 78	2.9	3.5	2.9	NA	NA
R. centifolia		132	17	33.5	60.5	> 125	20	3.6	6.8	2.2	9
S. potatorum		124	< 31.25	80	60	105	46	29	NA	6	8.3
F. infectoria		147	18	22	42	73	28	2	29	NA	NA
A. squamosa		51	27	27	25	26	15.12	NA	NA	0.8	0.8
Control	DS	5553	6.43	4.5	–	–	4978	9.18	16.54	–	–
	AZT	782	–	–	8.01	18.70	998.5	–	–	8244.35	12,079.33

CC_{50} 50% cytotoxic concentration, *IC_{80}* 80% inhibitory concentration, *DS* Dextran Sulphate, *AZT* Azidothymidine, *CF* Cell Free, *CA* Cell Associated
Legend: The plant extracts showing 80% inhibition of HIV-1 primary isolates (UG070 & VB59) in TZM-bl cell line by cell free and cell associated assay with positive controls dextran sulphate and azidothymidine respectively

and PM1 cell lines for confirmation of their anti-HIV1 activity.

In TZM-b1 cell lines methanolic extract of *A. aspera* and hydroalcoholic extract of *A. squamosa* exhibited a very good activity with lowest estimated IC_{80} of 4.8–53 μg/ml and 25–27 μg/ml respectively against primary isolates of HIV-1 strains. This was followed by aqueous extract of *F. infectoria*, methanolic extract of *S. potatorum* and *R. centifolia* and aqueous extract of *F. benghalensis* with preliminary IC_{80} in the range of 18–73 μg/ml, < 31.25–105 μg/ml, 17- > 125 μg/ml, and < 78- < 156 μg/ml respectively (Table 5).

In PM1 cell lines, methanolic extract of *A. aspera* and *R. centifolia* showed activity with estimated IC_{80} ranging 1–9 μg/ml. Aqueous extract of *F. benghalensis* exhibited activity at preliminary IC_{80} of 2.9–3.5 μg/ml in cell free assay. Methanolic extract of *S. potatorum* exhibited activity at IC_{80} of 6–29 μg/ml except for HIV-1_{VB59} cell free assay. The hydroalcoholic extract of *A. squamosa* showed very good activity at lower concentration 0.8 μg/ml in cell associated assay. Aqueous extract of *F. infectoria* showed activity in cell free assay at a concentration ranging 2–29 μg/ml (Table 5). The representative dose response bar graphs for *A. aspera and R. centifolia*, in cell free and cell associated assays for TZMb1 and PM1 are given in Additional file 1: (Figures S1 to S5).

Toxicity testing using Transwell epithelial model

Amongst the three extracts which showed highest activity, methanolic extract of *R. centifolia* exhibited least toxicity both inTZM-b1 and PM1 cell lines, therefore it was further tested for in vitro activity against HEC-1A cells and demonstrated minimal cytotoxicity with CC_{50} of 1443 μg/ml. Epithelial integrity of HEC-1A in Transwell dual-chamber system was maintained with only 1% relative fluorescence (percent of positive control) detected after treatment with 500 μg/ml of methanolic extract of *R. centifolia.* At higher concentration of 1000 μg/ml integrity was affected with 24% leaked fluorescence relative to positive control (Fig. 1).

Discussion

Natural products continue to be major sources of innovative therapeutic agents for treatment of infectious diseases, and their exploration has been one of the most successful strategies for the discovery of medicines. The development of new microbicides as preventive interventions is a promising area in AIDS research [3]. They could be valuable addition in prevention of sexual transmission of HIV-1 and could be an important way to reduce the number of cases of HIV infection globally [74, 75]. Currently available anti-HIV drugs are chemically synthesized and are often limited by side effects and emergence of drug resistance [76].

In order to find such potential anti-HIV agents from natural sources, ten traditional medicinal plants from India were studied for their inhibitory effects against laboratory adapted strains HIV-1 IIIB, HIV-1Ada5 and primary isolates HIV-1UGO70, HIV-1VB59 in TZM-b1 and primary isolates HIV-1UGO70, HIV-1VB59 in PM1 cell lines. HIV viruses can spread in the body via either a cell-free (virus floating free in plasma) mode or a cell associated (virus particles that remain attached to or within the host cell after replication) mode involving direct cell-cell contact. Hence all the selected plant extracts were evaluated to depict their mechanism of action, whether they will act as an entry inhibitor or at the HIV replication stage [77].

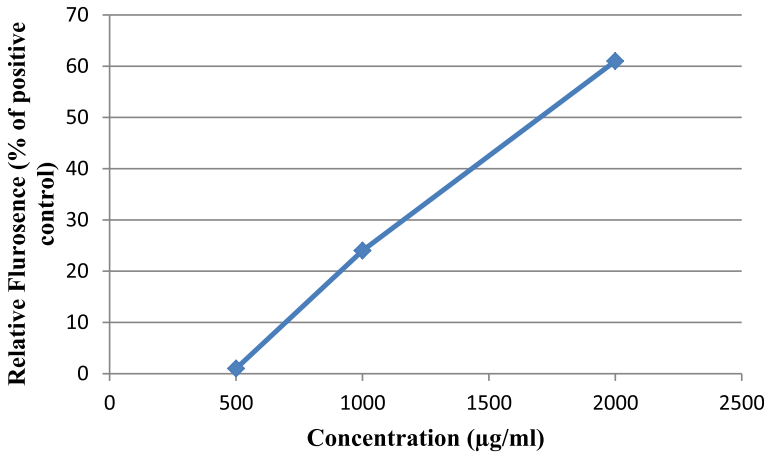

Fig. 1 Plot of Relative Fluroscence (%) Vs Concentration (µg/ml) determining epithelial integrity of *R. centifolia* by measuring permeability to FluoSpheres using the Dual-Transwell Epithelial Model

The selected plant extracts were subjected to high throughput (cost-effective, quick and reproducible) TZM-bl assay model which is useful for preliminary screening allowing screening of large number of products against HIV [70, 78]. The results presented here indicate that the methanolic extracts of aerial parts of *A. aspera,* leaves of *R. centifolia* and seeds of *S. potatorum* and aqueous extract of leaves of *F. benghalensis* and *F. infectoria* possess anti-HIV properties of therapeutic interest inhibiting HIV-1 virus at an estimated TI of 3–35, 1–6, 7–24,12–32, and 2–189 respectively against laboratory adapted strains and at a very low preliminary IC_{80} ranging from 4.8–26, 17–125, 31–105, 78–156 and 18–73 µg/ml respectively against primary isolates using TZM-b1 assay.

The lead extracts were also confirmed for anti-HIV activity in PM1 cell line which supports persistent HIV-1 infection [72]. The PM1 cell line have been reported to be comparable to peripheral blood mononuclear cells (PBMCs) for culturing of any of the HIV-1 strains and subtypes and thus provide a valuable research tool for studying new anti-HIV therapies [79]. This cell line has been previously used for studying the anti-HIV1 properties of the polyherbal cream Basant [80]. Hence PM-1 was used for confirming the anti-HIV activity of the methanolic extracts of aerial parts of *A. aspera* and leaves of *R. centifolia* which showed anti-HIV activity (IC_{80}) ranging between 1 and 8.4 and 2.2–6.8 µg/ml respectively. These extracts may potentially inhibit the entry and also inhibit HIV-1 replication if the virus enters the vaginal cells. However further work on more replicates and wider concentration range studies are required for confirmation. Future studies on PBMCs for qualifying the results should also be considered.

Our earlier work has shown that methanolic extract of *R. centifolia* has also shown activity against four strains of *N. gonorrhoeae* [81]. It substantially lacks cytotoxicity even at high concentrations (CC_{50} greater than 1 mg/ml) when tested in vitro on HEC-1A cell line (endometrial origin) and maintained its epithelial integrity when studied in Transwell model at concentrations up to 500 µg/ml thus showing potential for investigating it further as candidate anti-HIV microbicide.

As per the literature these extracts have not been further analyzed chemically, although the active components such as oleanolic acid and pomolic acid isolated from *Rosa wodsii* leaves the other species of *Rosa,* have been reported to inhibit HIV replication in acutely infected H9 cell growth at IC_{50} of 40 µg/ml [56, 57]. The literature indicates the phytosteroids, polyphenols and saponins present in the methanolic leaf extract of *A. aspera* are responsible for its anti-fertility effect [66] and methanolic root extract possess anti-herpes virus activity at EC_{50} of 64.4 µg/ml for HSV-1 and 72.8 µg/ml for HSV-2 [82]

The other selected medicinal plants extract showed anti-HIV activity against at least any one of the assay model except for hydroalcoholic extract of whole pods of *A. lebbeck*, methanolic extract of aerial parts of *T. procumbens*, methanolic extract of leaves of *M. philippinenis* and methanolic extract of leaves of *A. reticulate*, they were incapable of showing anti-HIV1 activity against cell free and cell associated HIV-1_{IIIB}, HIV-1_{Ada5} laboratory adapted strains and HIV-1_{UG070}, HIV-1_{VB59} primary isolates in TZM-b1 and PM1 cell lines. It's worth mentioning that these plants were selected on basis of their sub species showing activity against other strains and primary isolates of HIV and the same species having contraceptive and activity related to this infectious

disease. The inactivity of these plants against our test strains and primary isolates of HIV does not prove that they do not possess anti-HIV1 activity. These plants can be taken further for the activity against other strains and primary isolates of HIV virus using other anti-HIV assays.

Some plants extract such as *F. benghalensis, S. potatorum* and *F. infectoria* showed moderate to mild anti–HIV1 activity. These plants extracts had variable activities across the assays presented in this study where the extract exhibited inhibition of one strain of the primary isolates in one assay but did not inhibit the same primary isolates in another assay model. Aqueous extract of leaves of *F. benghalensis* showed anti-HIV1 activity against all HIV-1 laboratory adapted strains and primary isolates using TZM-b1 assay (TI: 12–32, IC_{80}: 78–156 μg/ml) but did not inhibit cell associated primary isolates in PM1 assay. Methanolic extract of seeds of *S. potatorum* showed anti-HIV1 activity against all HIV-1 laboratory adapted strains and primary isolates using TZM-b1 assay (TI: 7–24, IC_{80}: 31.25–105 μg/ml) but was not capable of inhibiting cell free primary isolate HIV-1_{VB59} in PM1 assay. Aqueous extract of leaves of *F. infectoria* showed anti-HIV1 activity against all HIV-1 laboratory adapted strains and primary isolates using TZM-b1 assay (TI: 2–189, IC_{80}: 18–73 μg/ml) but did not inhibit cell associated primary isolates in PM1 assay. Hence these extracts may not altogether be classified as extracts not having anti-HIV1 inhibitory potential.

Hydroalcoholic extract of seeds of *A. squamosa* exhibited activity against all HIV-1 laboratory adapted strains and primary isolates using TZM-b1 assay (TI: 2–4, IC_{80}: 26–27 μg/ml) but was not capable of inhibiting cell free primary isolates in PM1 assay. This plant extract has a some potential to be explored further and may be used supplementary as a replication inhibitor.

Conclusion

To conclude the study, out of 10 plants screened for anti-HIV activity using TZM-b1 and PM1 assays, methanolic extracts of aerial parts of *A. aspera* and leaves of *R. centifolia* has prospective anti-HIV1 potential as an entry and replication inhibitors. Hence these experimental moieties may have favourable implications on the prevention or management of HIV/AIDS. Additionally methanolic extract of leaves of *R. centifolia* have shown good safety and maintained the epithelial integrity on HEC-1A cells. Plant extracts are complex mixtures of many compounds. Some compounds may mask the anti-HIV1 potential of plant extract due to their cytotoxicity. Therefore our next step would be isolating the phytoconstituents and increasing the chances to find active anti HIV1 compounds with low cytotoxicity.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12906-020-2816-x.

Additional file 1: Figure S1. Plot of % HIV inhibition Vs Concentration (μg/ml) in TZMb1 (primary isolates) Cell free (CF) and Cell associated (CA) assay for *R. centifolia*. **Figure S2.** Plot of % HIV inhibition Vs Concentration (μg/ml) in TZMb1 (primary isolates) Cell free assay for *A. aspera*. **Figure S3.** Plot of % HIV inhibition Vs Concentration (μg/ml) in TZMb1 (primary isolates) Cell associated assay for *A. aspera*. **Figure S4.** Plot of % HIV inhibition Vs Concentration (μg/ml) in PM1 Cell free assay for *R. centifolia and A. aspera*. **Figure S5.** Plot of % HIV inhibition Vs Concentration (μg/ml) in PM1 Cell associated assay for *R. centifolia* and *A. aspera*.

Abbreviations

AIDS: Acquired Immunodeficiency Syndrome; CA: Cell-associated; CC_{50}: 50% cytotoxic concentration; CF: Cell-free; DMEM: Dulbecco's modified Eagle's medium; DMSO: Dimethyl Sulphoxide; FBS: Fetal Bovine Serum; HAART: Highly Active Antiretroviral Therapy; HEC-1A: Human Endometrial Adenocarcinoma; HIV: Human Immunodeficiency Virus; HSV: Herpes Simplex Virus; IC_{80}: 80% inhibitory concentration; LDH: Lactate Dehydrogenase; MTT: 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide; OD: Optical Density; PBMC: Peripheral Blood Mononuclear Cells; PHA P: Phytohemagglutinin P; RPMI-1640: Roswell Park Memorial Institute1640 Medium; $TCID_{50}$: Median Tissue Culture Infectious Dose; TI: Therapeutic Index

Acknowledgments

The authors would like to thank Sh. V. Chelladurai, Research Officer (Botany), S-III, Survey of Medicinal Plants Unit – Siddha, Palayamkottai (Rtd.) and S.N.D.T. Women's University campus for supplying plant materials and specimens for authentication. The authors would also like to thank Botanical Survey of India for carrying out authentication of the plant specimens.

Authors' contributions

AP participated in the design and coordination of the study, carried out the extraction and standardization studies and drafted the manuscript. NP, MP and AW participated and performed the cytotoxicity and anti-viral assays. NJ and AW participated and performed cytotoxicity and anti-viral assays and edited the manuscript. SK designed the research work and participated in cytotoxicity and anti-viral assays and gave final approval for its publication. KKS designed the research work, overviewed the extraction and standardization studies and edited and revised the manuscript critically for important intellectual content and gave final approval for its publication. All authors read and approved the final manuscript.

Funding

This study was supported by grant BT/PR7965/Med/14/1203/2006 from Department of Biotechnology (DBT, New Delhi) and Indian Council of Medical Research (ICMR, New Delhi).

Availability of data and materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Received: 18 July 2019 Accepted: 15 January 2020
Published online: 06 March 2020

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