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Research Article

Methanol and Aqueous Extracts of *Ocimum kilimandscharicum* (Karpuratulasi) Inhibits HIV-1 Reverse Transcriptase *in Vitro*

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ABSTRACT

33 Indian medicinal plants documented, in Ayurveda and Siddha literature, to have antimicrobial, blood purifying and immunomodulatory properties were subjected to solvent extraction. The extracts were tested for MMLV-RT and HIV-1 RT inhibitory activity and T cell proliferation property. *In-vitro* safety studies were carried out on the extracts showing anti HIV/immunomodulatory potential. The methanol and aqueous extracts of *Ocimum kilimandscharicum*, and methanol extract of *Pterocarpus marsupium* had significant MMLV -RT inhibitor activity. The methanol and aqueous extracts of *Ocimum kilimandscharicum*, aqueous extract of *Pueraria tuberose* and methanol extract of *Pterocarpus marsupium* inhibited HIV-1RT at an MIC range from 25-100µg/ml. The methanol and aqueous extract of *Rubia cordifolia*, methanol extract and aqueous extract of *Ocimum kilimandscharicum* and aqueous extract of *Plectranthus amboinicus* were immunostimulatory and non-cytotoxic at these levels.

Keywords:

INTRODUCTION

Inhibitors for reverse transcriptase (RT), an enzyme involved in the RNA dependent DNA synthesis of retroviruses is a major target of HIV antiviral research[1-4]. HIV/AIDS being an immunosuppressive disease, identification of immunostimulants that support and enhance the defense mechanism of the body is another important area of anti HIV research[5-9]. With the shift of AIDS epicenters to the developing and under developed world like Asia and Sub-Saharan Africa, there is a paradigm shift towards exploration of cost effective and safer drugs from the traditional systems of medicine. One of the priority areas has been the search for anti-HIV-1 compounds from natural products of terrestrial and marine origin[10-13]. Extracts of blue green algae *Lyngbya langerheinii*, *Phormidium tenue*, brown seaweed *Fucus vesiculosis* and green algae *Spiruluna platensis* have previously been documented to have anti HIV properties[14]. The aqueous extracts of *Prunella vulgaris* has been shown to inhibit of HIV-1 infectivity[15]. A number of studies have identified plants with immunostimulatory, antiviral, antibacterial, anticancer, antiasthmatic and anti-inflammatory activities[16-20]. Immunomodulatory potentials of picroliv from plant *P.kurroa* and NK cell stimulation by *Phyllanthus emblica* have been well documented[21,22]. In the present study an attempt has been made to explore anti-HIV drug potentials from 33 Indian Medicinal plants. The parts of plants were selected on the basis of citations in literatures of Indian system of medicine and analyzed by adopting internationally acceptable study design for pre-clinical laboratory evaluation of anti-HIV drug potentials from natural products[23-26].

MATERIALS AND METHODS

Selection of Plants

The parts of plants with documented antimicrobial, blood purifying and immunomodulatory potentials as cited in Ayurveda, Siddha literatures and in Glossary of Indian medicinal plants were used in this study[23,24,26]. These plants were collected from the states of Tamil Nadu and Kerala. The ease of obtaining and / or local availability of the plants also formed criteria for selection (Table 1).

Preparation of extracts

Parts of plants documented in literature were washed in running water and air dried in shade. Sequential extraction was carried out in environmental shaker at 37˚C for 2 hours followed by heating at 60˚C in a water bath for one hour. The extracts so obtained were kept at 4˚C overnight followed by dehydration in vaccuspin dryer. The partially dried extracts were sealed and stored at –70˚C until used for biological tests.

Isotopic reverse transcriptase inhibition assay

The reverse transcriptase activity was calculated using uptake of radioactive isotope, tritiated thymidine triphosphate, according to the previously described protocol with modifications[27,28]. Briefly, known concentrations of extract were added to a reaction mix containing 50mM Tris HCl , 75mM KCl, 10mM $MgCl_2$, 5mM DTT BSA, Poly r(A) d(T)[12-18] (10 µ g/ml)followed by MMLV RT or HIV-1 RT (Amersham). RT minus control acted as negative control. AZT was used as inhibitory control. After incubation at 37ºC for half an hour reaction was stopped with EDTA. The contents of the tube

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Table 1: Taxonomical status of plants selected for the study.

S. No.	Botanical Name	Family
1.	*Amaranthus spinosus* Linn	Amaranthaceae
2.	*Aporosa lindleyana*	Euphorbiaceae
3.	*Argyreia nervosa*	Convolvulaceae
4.	*Asparagus racemosus* Willd.	Liliaceae
5.	*Baliospermum montanum*	Euphorbiaceae
6.	*Boerhaavia diffusa*	Nyctaginaceae
7.	*Eclipta prostrata*	Asteraceae
8.	*Euphorbia ligularia*	Euphorbiaceae
9.	*Ficus microcarpa*	Moraceae
10.	*Gmelina arborea*	Verbenaceae
11.	*Gymnema sylvestre*	Asclepiadaceae
12.	*Hemidesmus indicus*	Asclepiadaceae
13.	*Hedyotis corymbosa*	Rubiaceae
14.	*Ipomoea mauritiana*	Convolvulaceae
15.	*Mesua nagassarium*	Clusiaceae
16.	*Mussaenda frondosa*	Rubiaceae
17.	*Nyctanthes arbor- tristis*	Oleaceae
18.	*Ocimum kilimandscharicum*	Lamiaceae
19.	*Operculina turpethum*	Convolvulaceae
20.	*Oxalis corniculata*	Oxalidaceae
21.	*Pinus roxburghii*s arg.	Pinaceae
22.	*Plectranthus amboinicus*	Lamiaceae
23.	*Plumbago indica*	Plumbaginaceae
24.	*Pterocarpus marsupium*	Fabaceae
25.	*Pueraria tuberosa*	Fabaceae
26.	*Rubia cordifolia*	Rubiaceae
27.	*Salacia reticulata*	Hippocrateaceae
28.	*Sida cordata*	Malvaceae
29.	*Sida rhombifolia*	Malvaceae
30.	*Sida rhombifolia Linn ssp. fetusa*	Malvaceae
31.	*Symplocos cochinchinensis*	Symplococaceae
32.	*Tinospora cordifolia*	Menispermaceae
33.	*Wedelia chinensis*	Asteraceae

were spotted on DE-81 paper. DNA was precipitated with TCA and washed in ethanol. Radioactivity was measured in scintillation counter (Packard TRI CARB-2100 TR, USA). A 50% or more reduction in the radioisotope uptake count between the control and test was taken as positive inhibitory activity.

Calculation of inhibitory ratio

The percentage relative inhibitory ratio (%IR) of the incorporation of ^{3}H dTTP into a polymer fraction by RT in the presence of the sample was calculated as follows:

$$\%IR = \frac{cpm_{(complete\ system)} - cpm_{(complete\ system-RT)}}{cpm_{(complete\ system-sample)} - cpm_{(complete\ system-RT)}} \times 100$$

cpm = count per minute.

T cell proliferation

Peripheral blood mononuclear cells (PBMC) was isolated from whole blood using Histopaque-1077, viable cells enumerated using Trypan blue. Cells were diluted in Minimum Essential Media supplemented with fetal calf serum (GIBCO) and seeded to microtitre wells. Phytohaemagglutinin (PHA) 50µg / ml acted as positive control. Suboptimal concentration of PHA (20µ g/ml) was added to test wells. Known concentration of test extracts were used to determine the T cell proliferation after incubation for 48 hours in a humidified 37°C, 5% CO_2 incubator. MTT assay was carried out as per previously described[29] and plates were read immediately at 540nm.

$$\%\ Viability = \left\{ 1 - \frac{OD_{Test}}{OD_{Control}} \right\} X\ 100$$

In-vitro Cytotoxicity

The extracts were resuspended in Minimum Essential Media (GIBCO) and serial diluted. Known concentrations of the extract were added to appropriate wells seeded with Vero cells and incubated at 37˚C.Changes in cell morphology and viability were recorded on day 1,3,5and7.

Enzyme Amplified Sensitivity Immunoassay (EASIA) based Cytokine Quantitation

Heparinized blood drawn from healthy donor was used for separation of Peripheral blood mononuclear cells (PBMC) using histopaque –1077. Cells resuspended in RPMI media (GIBCO) with antibiotics and seeded on microtitre plates. Cells were incubated with known concentration of extracts. Supernatants from test and control wells were collected and stored at -70C.Human IFN gamma *EASIA* kits procured from (Biosource Europa S.A.) were used for quantization of respective cytokines in the supernatant as per manufacturer's instructions.

RESULTS

A total of 221 extracts obtained from 33 Indian medicinal plants were used for the study. The scientific nomenclature and taxonomical status of these plants are depicted in Table 1.

Reverse transcriptase inhibition

The aqueous extract of *Ocimum kilimandscharicum* and methanol extract of *Pterocarpus marsupium* showed 60% and 52% inhibitory ratio (IR) against MMLV-RT at a concentration of 400µg/ml whereas, the methanol and aqueous extracts of *Ocimum kilimandscharicum*, aqueous extract of *Pueraria tuberosa* and the methanol extract of *Pterocarpus marsupium* showed more than 50% inhibition of HIV-1RT at MIC ranging from 25-100µg/ml. Table 2a-b depicts the activity shown by the extracts exhibiting significant RT inhibitory potentials. The inhibitory ratio of extracts showing RT inhibition at 50µg/ml is depicted in Fig1.AZT was used as inhibitory control and showed 75% IR at 1µg/ml.

T cell proliferation and *Quantitation of IFNγ*

Proliferation of T cells significantly increased in the presence of the methanol and aqueous extracts of *Ocimum kilimandscharicum*, aqueous extract of *Plectranthus amboinicus* and the methanol and aqueous extracts of *Rubia cordifolia* at a concentration of 100µg/ml of respective extracts. The five extracts of three plants showing T cell proliferation were subjected to quantitative test using

Table 2a: MIC and percentage activity of extracts found positive for the screening tests.

S. No.	Name of the Plant	MMLV-RT Inhibition	T cell proliferation
1.	*Ocimum kilimandscharicum*	Aq: 400µ g/ ml 60%	M: 100µ g/ml 58% Aq: 100µg/ml 52%
2.	*Plectranthus amboinicus*	Aq:400µ g/ ml 15%	Aq: 100µg/ml 52%
3.	*Pterocarpus marsupium*	M: 400µ g/ml 52%	M:100µg/ ml 10%

M = Methanol, Aq = Aqueous

Table 2b: MIC of extracts showing HIV RT inhibition.

S. No.	Test extract	MIC
1.	*Ocimum kilimandscharicum* (Aq)	50µg/ml
2.	*Ocimum kilimandscharicum* (M)	25µg/ml
3.	*Pueraria tuberosa* (Aq)	100µg/ml
4.	*Pterocarpus marsupium* (M)	100µg/ml

M = Methanol, Aq = Aqueous

Table 3: IFNγ detection by EAISA.

S. No.	Extract	IFNγ (IU) 100µg/ml	50µg/ml
1.	PHA control (50µg/ml)		4.5
2.	*Ocimum kilimandscharicum (M)*	2.5	1.5
3.	*Ocimum kilimandscharicum (Aq)*	2	2
4.	*Plectranthus amboinicus (Aq)*	4.8	2
5.	*Rubia cordifolia (M)*	5	3.3

M = Methanol, Aq = Aqueous

commercially available EAISA kit. The methanol and aqueous extract of *Rubia cordifolia,* methanol and aqueous extract of *Ocimum kilimandscharicum* and aqueous extract of *Plectranthus amboinicus* were found to stimulate cells to produce IFN γ to levels comparable to PHA control. Table 3 depicts the amount of cytokines detected by different concentration of extracts used.

In-vitro cytotoxicity

The extracts showing RT inhibition and / or Immunomodulatory potentials were tested for cytotoxicity on Vero cell lines. $TCTD_{50}$ was determined by observing a marked change in morphology of cells, disruption of cell monolayer and cell death. The extracts of *Plectranthus amboinicus, Rubia cordifolia* and *Baliospermum montanum* were found to be non-toxic above 2 mg/ml concentration. The extracts of *Hedyotis corymbosa* and *Gmelina arborea* Roxb were cytotoxic at <10µg/ml. (Table 4.)

Table 4: Assessment of Tissue culture toxicity dose 50 ($TCTD_{50}$).

S. No.	Extract	Toxicity level
1.	*Aporosa lindleyana(Aqueous)*	100 µg/ml
2.	*Aporosa lindleyana(Methanol)*	100 µg/ml
3.	*Baliospermum montanum (Methanol)*	>2000 µg/ml
4.	*Baliospermum montanum (Aqueous)*	>2000 µg/ml
5.	*Gymnema sylvestre(Chloroform)*	1000 µg/ml
6.	*Gymnema sylvestre(Methanol)*	1000 µg/ml
7.	*Gmelina arborea Roxb.(Aqueous)*	10 µg/ml
8.	*Hedyotis corymbosa (Chloroform)*	10 µg/ml
9.	*Hedyotis corymbosa(Aqueous)*	50 µg/ml
10.	*Ocimum kilimandscharicum(Methanol)*	500 µg/ml
11.	*Ocimum kilimandscharicum (Aqueous)*	500 µg/ml
12.	*Plectranthus amboinicus(Aqueous)*	>2000 µg/ml
13.	*Pterocarpus marsupium(Methanol)*	1000 µg/ml
14.	*Pueraria tuberosa(Aqueous)*	500 µg/ml
15.	*Rubia cordifolia (Aqueous)*	>2000 µg/ml
16.	*Rubia cordifolia(Methanol)*	>2000 µg/ml

M=Methanol, Aq= Aqueous, C=Chloroform

DISCUSSION

The quest to explore possible anti HIV leads from natural sources has led to identification of *in vivo* anti HIV activity in marine sponge *Stellatta sp,* tropical plants of the genera *Erythrina,* fungi in the genera *Fusarium* and *Alternaria*[30]. A natural reverse transcriptase inhibitor Calanolide A was isolated from the terrestrial plant *Calophyllum lanigerum*[31]. Among higher plants *Euodia roxburghiana*[32], *Ancistrocladus korupensis*[33] and the methanol extract of *Phyllanthus emblica*[34] have been reported to have potential HIV RT inhibitory activity. The extracts of *Ocimum gratissimum, Ficus polita, Clausena anisata, Alchornea cordifolia, Elaeophorbia drupifera* with HIV-1 reverse transcriptase inhibitor activity with EC_{50} values of <0.01–0.03 mg/ml have been reported[35]. Recently, we have reported the HIV-1 protease inhibition by PA-9 flavinoid extract from Plectranthus amboinicus[36]. In the present study 33 Indian medicinal plants were analyzed for their anti HIV properties. The extracts of *Ocimum kilimandscharicum, Gmelina arborea Roxb, Pureria tuberosa* and *Pterocarpus marsupium* have shown significant HIV-RT inhibition properties. With the available literature no other study is available to show the anti HIV properties of the above plants except in *Ocimum gratissimum.* However the species analyzed in our study is *Ocimum kilimandscharicum.* In our present study MMLV-RT inhibition was compared with HIV-RT inhibition by isotopic methods. While the MMLV- RT inhibition study had shown only two out of the 33 medicinal plants analyzed to have RT inhibitory potentials the HIV specific

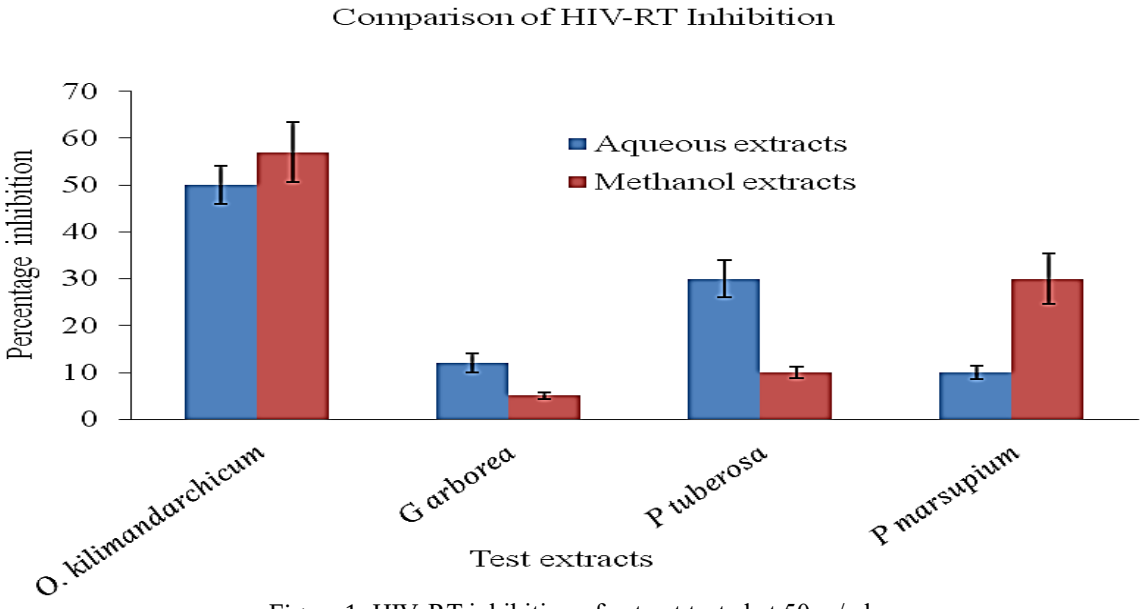

Figure 1: HIV-RT inhibition of extract tested at 50µg/ml.

RT inhibition assay has revealed five plants to possess HIV-1 RT inhibitory properties. Studies have shown that natural herbal drugs are not always harmless. Traditional remedies leading to hepatotoxicity have been widely reported[37,38]. Hence safety studies have formed an integral component in drug development. At present, continuous cell lines like Vero, Hep 2 and He La are used to assess the cytotoxic effects of Indian medicinal plant preparations[39]. In the present study, 16 extracts of ten medicinal plants, which showed either antiviral and/or T cell proliferation, were assessed for their tissue toxicity using Vero, cell line. In our study the $TCTD_{50}$ dose varied from 10µg/ml to 2mg/ml. This study has brought out certain original observations which are hitherto unreported in literature on *Ocimum kilimandscharicum* and further in depth studies are warranted to confirm anti-HIV drug potential. Virus growth inhibitory studies using the bioactive fractions of *Ocimum kilimandscharicum* shall be the most confirmatory procedure to prove the HIV specific inhibitory activity of *Ocimum kilimandscharicum*. Thymidine uptake methodologies and /or flour metric assays for T cell enumeration should also be performed to confirm immunomodulatory potential of *Ocimum kilimandscharicum*. The structural elucidation of the reproducible bioactive fraction will help with large scale production of the identified molecule.

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