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# *In-vitro* testing of anti-HIV activity of some medicinal plants

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Medicinal plants namely *Ocimum sanctum* Linn., *Withania somnifera* Dunal, *Tinospora cordifolia* (Willd.) Miers. ex Hook.f. & Thoms., *Avicennia officinalis* Linn. and *Rhizophora mucronata* Lam. were screened for anti-HIV activity in the present study. *O. sanctum*, *T. cordifolia*, *A. officinalis* and *R. mucronata* showed anti-HIV potential by inhibiting the virus by 2 different mechanisms. Interference with the gp120/CD4 interaction and inhibition of viral Reverse Transcriptase (RT) contributed to the overall anti-viral activity *in vitro*. Among these plants *A. officinalis* and *R. mucronata* are mangrove plants and their medicinal properties are rarely reported.

**Keywords:** Anti-HIV, Medicinal plants, Mangrove plants, *Ocimum sanctum*, *Withania somnifera*, *Tinospora cordifolia*, *Avicennia officinalis* and *Rhizophora mucronata*.

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## Introduction

Human Immunodeficiency Virus (HIV) is the etiological agent of Acquired Immunodeficiency Syndrome (AIDS) that has created a major health care problem not only in India but also globally. The current strategy for the treatment of HIV infection is Highly Active Antiretroviral Therapy (HAART), which is based on combination of inhibitors of reverse transcriptase and protease. Although HAART has reduced death from AIDS-related diseases, it is still an expensive regime, often not well tolerated and leads to drug resistance. In the efforts to develop a vaccine, certain approaches are showing early promise in monkeys. However, the persistence of HIV infection coupled with its high rate of spontaneous mutation raises concerns about eventual viral immune escape. Most importantly, the ability of HIV to establish latent reservoirs early in the course of infection ensures the persistence of this pathogen even in the face of intensive drug therapy and vigorous antiviral immune responses.

Besides, HIV/AIDS affects individuals in prime-working age group which has a large negative effect on domestic as well as national income. Mother to child transmission is resulting in increasing number of orphans and children living with HIV that makes HIV/AIDS more of social, emotional problem than mere economical burden[1].

Thus there is a need for the discovery of novel therapeutic strategies. One of the strategies has been to identify anti-HIV compounds from natural sources, particularly from plants. Traditional medicine has served as a source of alternative medicine, new pharmaceuticals and healthcare products. Considerable research on pharmacognosy, phytochemistry, pharmacology and clinical therapeutics has been carried out on potential Ayurvedic medicinal plants. Numerous molecules have come out of Ayurvedic experimental base, examples include Rauwolfia alkaloids for hypertension, guggulsterons as hypolipidemic agents, picrosides in hepatic protection, phyllanthins as antivirals, curcumine in inflammation, withanolides and many other steroidal lactones and glycosides as immunomodulators[1].

In addition to common terrestrial medicinal plants, mangrove plants are also used in folklore medicine for treatment of several diseases. The term mangrove is used to designate halophytic (salt loving) and salt-resistant marine tidal forests comprising of trees, shrubs, palms and ground ferns. Mangroves were first mentioned in 325 BC, by the Greek explorer Nearchus[2]. Mangroves occur in 121 countries covering 15 million ha worldwide. Asia harbors the largest mangroves in the world and India alone

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contributes for 3% of the global mangrove habitat[3]. They are highly productive ecosystem with various important economic and environmental functions. Apart from this, they also possess medicinal properties and numerous bioactive compounds are derived from mangroves[4].

In present study five plants, viz. *Ocimum sanctum* Linn., *Withania somnifera* Dunal, *Tinospora cordifolia* (Willd.) Miers. ex Hook.f. & Thoms., *Avicennia officinalis* Linn. and *Rhizophora mucronata* Lam., reported in literature as medicinal plants (Table 1)[5-9] were selected to investigate their inhibitory activity against HIV *in vitro*.

## Materials and Methods

### Collection of the material

#### *Procurement of raw plant material*

Root powder of *W. somnifera* and stem powder of *T. cordifolia* were procured from Zandu Pharmaceuticals, Mumbai. Leaf powder of *O. sanctum* was purchased from Atul Medical Stores, Vile Parle (E), Mumbai and *A. officinalis* was collected from Mahim Creek, Mumbai whereas, *R. mucronata* was collected from Vikroli highway, Mumbai. The leaves were washed, shade-dried and powdered in a grinder mixer. All the plant material was identified and authenticated by Dr. J. M. Pathak, Research Director (Pharmacognosy), Zandu Pharmaceutical, Mumbai.

### Preparation of extracts

#### *Soxhlet extraction using organic solvents*

The plant material (3-47g) was first extracted in Soxhlet apparatus with petroleum ether (230-460 ml) at 60-80ºC) and then with ethanol (230-460 ml) in succession.

#### *Aqueous extraction*

Aqueous extracts were obtained by plain decoction method[10] using material (3-20 g) with distilled water (50-200 ml). All the extracts were made free from solvents and percentage yield of individual extract was calculated. Thus, three extracts of each plant namely, petroleum ether, ethanol (successive) and aqueous were taken for the study.

#### *Reconstitution of extracts for testing*

Petroleum ether and ethanol (successive) extracts after removal of solvents were dissolved in dimethyl sulphoxide (DMSO) (not more than 1%) and then diluted with RPMI 1640 medium, whereas, aqueous extracts after drying were reconstituted in RPMI 1640 medium alone. Each extract was reconstituted to prepare stock of 25 mg/ml. The stock solutions were then filtered through 0.22 µm membrane filter and stored at 4ºC until further use.

### Anti-HIV assays

#### *Reverse Transcriptase (RT) Inhibition Assay*

The HIV reverse transcriptase enzyme inhibition due to each of the extract(s) was determined using HIV RT inhibition assay with slight modification[11]. For this, 25µl of each extract (Final conc. 5mg/ml) was added to the reaction mixture. The reaction mixture (Final volume: 100µl) contained the following: 50mM Tris (*p*H 7.8), 150 mM KCl, 5mM $MgCl_2$, 0.05% NP-40, 0.5mM EGTA, 5 mM DTT, 20 µM dTTP, 0.3 M Glutathione, 2.5µg/ml BSA, 0.5µCi (microcurie) of [$^3$H]TTP, 2.5µg/ml POLY(rA).p(dT).

The reaction was started by addition of 0.5 Units of recombinant reverse transcriptase enzyme (Ambion); mixture was then incubated for 3hr at 37$^o$C and reaction was terminated by the addition of 25µl of

Table 1— Biological activities of medicinal plants under study

| Plant, Family and Vernacular Names | Plant Part Used | Biological activities |
|---|---|---|
| *Ocimum sanctum* Linn. Lamiaceae (Tulas) | Leaves | Immunomodulatory, Anti-tumor, Anti-diabetic, Antimicrobial[5] |
| *Withania somnifera* Dunal Solanaceae (Ashwagandha) | Root | Anti-inflammatory, Anti-tumor, Antioxidant, Immunomodulatory[6] |
| *Tinospora cordifolia* Willd. Menispermaceae (Gulvel) | Stem | Antioxidant, Hepatoprotective, Anti-neoplastic, Immunomodulatory, Antiviral[7] |
| *Avicennia officinalis* Linn. Verbenaceae (Tivar) | Leaves | Aphrodisiac, Anti-protozoal, Anti-tumor, for Bronchial asthma, Stomach and urinary disorders[2,3,8,9] |
| *Rhizophora mucronata* Lam. Rhizophoraceae (Kandel) | Leaves | Astringent, Anti-diabetic, Hemorrhage and angina[3,8,9] |

0.1 M EGTA followed by chilling the mixture on ice. 100µl of each reaction mixture was then spotted uniformly onto circular 2.5cm DE-81 Whatman filters (discs) (B. Patel and Company) and kept at ambient temperature for 15min. The dried filters were washed four times with 5% aqueous $Na_2HPO_4.7H_2O$ followed by two more washing with double distilled water. Finally, the filters were thoroughly dried and subjected to scintillation counting (TRI-CARB 2100TR, Packard). Negative control was set up in parallel and AZT (Azidothymidine/Zidovudine) was used as positive control.

The percentage inhibition was calculated as,

Inhibition (%) = [(CPM of Negative control – CPM of Test)/CPM of Negative control] × 100. Where, CPM is counts per minute.

***Gp120 Binding Inhibition Assay***

Binding of gp120 to CD4 was analyzed using a commercially available gp120 Capture ELISA kit (Immuno Diagnostics, Inc., USA) with slight modification[12]. To determine whether extract(s) could interfere with the binding of CD4 to gp120 by interaction with soluble gp120, each extract (Final conc. 5mg/ml) was mixed with 25ng of purified gp120 in a total volume of 100µl and incubated at room temperature for 1h. This mixture was then added to microtiter plate wells coated with CD4 ligand and incubated at room temperature for 1h. The solutions were aspirated and the wells were washed 3 times with washing buffer. The extent of gp120 binding was assessed by using detector reagent provided in the kit according to the manufacturer's instructions. Negative control was set-up in parallel and heparin was included as positive control.

The percentage inhibition was calculated as,

Inhibition (%) = $[(A_{Control} - A_{Sample}) / A_{Control}] \times 100$. Where, A is Optical Density (OD).

***Time-course study***

In order to investigate the mechanism of inhibition of gp120/CD4 binding, a study was conducted to evaluate the time-course effect by adding each extract before binding and after binding of gp120 to CD4. The assay was performed using commercial gp120 Capture ELISA kit (ImmunoDiagnostics, Inc., USA).

To determine whether the extract(s) could bind to the immobilized CD4 and thus interfere with the interaction with gp120, CD4-coated wells were incubated with 100µl of each of the extract (Final conc. 5mg/ml) for 1h at room temperature. The solutions were aspirated and the extracts-treated wells were then washed 3 times with washing buffer and incubated with 25ng of gp120 for 1h at room temperature. The degree of bound gp120 was measured using detector reagent provided in the kit[12]. In separate experiments, the extracts were added after 1h pre-incubation of gp120 (25ng) in CD4-coated plates. After gp120-preadsorbed plates were washed 3 times with washing buffer, each extract (Final conc. 5mg/ml) was incubated in the plates for another 1h at room temperature, followed by detection of bound gp120 by detector reagent supplied in the kit according to the manufacturer's instructions[13].

## Results and Discussion

Among petroleum ether and ethanol (successive) extracts, *R. mucronata* showed the highest inhibition of RNA Dependent DNA Polymerase (RDDP) function of recombinant HIV-RT, whereas, among aqueous extracts, *O. sanctum* showed the highest inhibition of recombinant HIV-RT (Table 2). Petroleum ether and aqueous extracts of *A. officinalis* showed the highest inhibition of gp120 binding, whereas, among ethanol (successive) extracts, *R. mucronata* showed the highest inhibition followed by *T. cordifolia*. Petroleum ether extract of *O. sanctum* and ethanol (successive) and aqueous extracts of *W. somnifera* showed no inhibition (Table 3).

Table 2— Inhibition of HIV-RT by plant extracts

| Name of the Plants | Reverse Transcriptase (RT) Inhibition (%) [Mean ± SD] | | |
|---|---|---|---|
| | Petroleum ether extract | Ethanol extract (Successive) | Aqueous extract |
| *O. sanctum* | 79.92 ± 1.22 | 76.22 ± 0.96 | 85.04 ± 0.04 |
| *W. somnifera* | 68.71 ± 1.23 | 46.19 ± 1.77 | 32.30 ± 5.50 |
| *T. cordifolia* | 64.48 ± 0.50 | 54.29 ± 1.24 | 66.03 ± 0.88 |
| *A. officinalis* | 74.79 ± 3.47 | 82.00 ± 0.26 | 82.18 ± 1.72 |
| *R. mucronata* | 87.07 ± 1.85 | 84.74 ± 0.05 | 82.48 ± 1.93 |
| AZT [control] (0.0016 mg/ml) | 71.04 ± 1.94 | | |

Note: Inhibition ≥50% is considered as significant.

When immobilized CD4 were pre-treated with the extracts, among petroleum ether, ethanol (successive) and aqueous extracts, *R. mucronata* showed the highest inhibition of gp120/CD4 interaction by binding to immobilized CD4. Whereas, when the extracts were added after gp120/CD4 interaction in separate experiments, among aqueous extracts, *R. mucronata* blocked gp120/CD4 interaction with the highest inhibition by displacing pre-adsorbed gp120. Among petroleum ether extracts, *T. cordifolia* and among ethanol (successive) extracts, *A. officinalis* showed the highest inhibition of gp120/CD4 interaction by displacing the pre-adsorbed gp120 (Table 4).

In view of magnitude of AIDS pandemic, absence of protective vaccine, paucity of non-toxic therapy and resistance developed by the virus to different anti-HIV therapeutic drugs, there is an urgent need for the development of new, specific and non-toxic drugs. To obtain lead molecules for this drug development, a number of plant products are being screened all over the world by scientists. Some of these plant-derived anti-HIV compounds like glycyrrhizin, papaverine, tricosanthin, castanospermin, etc. have been used in a limited number of patients suffering from AIDS. Some more plant products are in clinical trials and many more hope to follow this to give new drugs and lead molecules for drug-development for treatment of HIV infection[14]. It is evident, therefore, that plants could be useful sources or leads for the discovery of novel anti-HIV compounds. Non-polar solvent, petroleum ether and two polar solvents, ethanol and water were used for extraction since these solvents are reported to give a wide range of bioactive chemical constituents. Certain phytochemicals have been shown to exhibit anti-HIV activity[15].

Antiviral research has been focused on compounds that interfere with various parts of the viral life-cycle such as proteins encoded by the virus itself. HIV-Reverse Transcriptase (RT), performs 3 principle

Table 3— Gp120 binding inhibition by plant extracts

| Name of the plants | Gp120 binding inhibition (%) [Mean ± SD] Petroleum ether extract | Ethanol extract (Successive) | Aqueous extract |
|---|---|---|---|
| *O. sanctum* | 0 | 36.85 ± 0.29 | 21.24 ± 0.88 |
| *W. somnifera* | 1.12 ± 0.63 | 0 | 0 |
| *T. cordifolia* | 78.54 ± 0.16 | 80.22 ± 0.38 | 41.91 ± 0.57 |
| *A. officinalis* | 81.01 ± 0.30 | 78.65 ± 0.23 | 69.33 ± 0.37 |
| *R. mucronata* | 69.33 ± 0.22 | 81.69 ± 0.13 | 60.11 ± 0.69 |
| Heparin [control] (12.5 Units) | 75.96 ± 0.25 | | |

Note**:** Inhibition ≥50% is considered as significant

Table 4— Effect of plant extracts on gp120/CD4 interaction (Time-course study)

| Name of the plants | Extracts | Gp120/CD4 interaction inhibition (%) [Mean ± SD] Addition of extracts before interaction | Addition of extracts after interaction |
|---|---|---|---|
| | Petroleum ether | 68.09 ± 0.63 | 7.75 ± 1.53 |
| *O. sanctum* | Ethanol successive | 78.20 ± 0.23 | 74.27 ± 0.42 |
| | Aqueous | 78.43 ± 0.15 | 90.67 ± 0.07 |
| | Petroleum ether | 75.73 ± 0.25 | 37.75 ± 0.52 |
| *W. somnifera* | Ethanol successive | 67.30 ± 0.31 | 69.44 ± 0.21 |
| | Aqueous | 68.54 ± 0.38 | 87.53 ± 0.08 |
| | Petroleum ether | 76.18 ± 0.33 | 87.42 ± 0.17 |
| *T. cordifolia* | Ethanol successive | 64.04 ± 0.42 | 69.10 ± 0.30 |
| | Aqueous | 78.88 ± 0.39 | 35.62 ± 0.78 |
| | Petroleum ether | 81.01 ± 0.37 | 83.71 ± 0.28 |
| *A. officinalis* | Ethanol successive | 83.03 ± 0.28 | 91.35 ± 0.14 |
| | Aqueous | 55.28 ± 0.47 | 88.65 ± 0.16 |
| | Petroleum ether | 86.74 ± 0.17 | 85.73 ± 0.10 |
| *R. mucronata* | Ethanol successive | 87.53 ± 0.17 | 88.65 ± 0.33 |
| | Aqueous | 90.11 ± 0.07 | 94.61 ± 0.04 |
| Control | Heparin (12.5 Units) | 87.64 ± 0.17 | 58.09 ± 0.46 |

Note: Inhibition ≥50% is considered as significant.

functions: (i) The polymerase domain transcribes viral genomic RNA to viral DNA, a process referred to as the RNA-dependent-DNA-polymerase (RDDP) activity; (ii) In the course of reverse transcription an intermediary RNA/DNA hybrid is formed. RT through its ribonuclease H (RNase H) domain degrades the RNA component of the hybrid; (iii) RT carries out DNA-dependent DNA polymerization activities, producing complementary DNA strands. The completion of each of these processes is required for the formation of a competent viral DNA capable of integrating into the genome of the infected cell. The function of RT is, therefore, essential for replication of HIV and is a suitable target for chemotherapeutic intervention. Extracts of 41 medicinal plants used in Egyptian folk medicine have been evaluated for their HIV-1 RT inhibitory effects and putranjivain A was identified from *Phyllanthus emblica* Linn. (= *Emblica officinalis* Gaertn.) as a potent inhibitory substance against HIV-RT[16].

In the present study, the assay was optimized and standardized with respect to various experimental parameters and then applied to test the HIV-RT inhibitory activity of the different extracts. Most studies considered inhibition ≥ 50% as significant. Since all extracts were crude extracts and not the fractionated or purified active moieties, it was preferred using not too high or not too low concentration of the extracts, viz. 5mg/ml. At this concentration, all the extracts showed significant inhibition of recombinant HIV-RT except ethanol (successive) and aqueous extracts of *W. somnifera*. Thus 13 of 15 extracts showed potent inhibition of RDDP function of HIV-RT. AZT was included as positive control that showed 71.04% inhibition. The binding of gp120 to CD4 is also a critical step in HIV infection, as the outer envelope glycoprotein gp120 of HIV mediates viral attachment to the cell-surface glycoprotein CD4 in the initial phase of HIV infection. Two agents that have been shown to potently inhibit the adsorption of HIV to $CD4^+$ cells and inhibit HIV infection *in vitro* are the sulfated polysaccharides, dextran sulfate and heparin[17].

The effects of different extracts on gp120/CD4 interaction were examined in the present study, using a gp120 capture ELISA kit. This was determined by pre-incubation of test compounds with the soluble gp120 before addition to immobilized CD4. The study revealed that, only 8 of 15 extracts showed significant inhibition (≥ 50% inhibition) of gp120/CD4 interaction. These extracts that inhibit this interaction after preincubation with gp120, may bind tightly to gp120 thereby inactivating them and preventing the interaction between gp120 and CD4. Similar activity has been shown by Cyanovirin-N (CV-N), a novel virucidal protein isolated from cultures of cyanobacterium (blue-green alga) *Nostoc ellipsosporum*[18]. The reciprocal experiments were then performed to measure the ability of 15 extracts to inhibit gp120/CD4 interaction by binding to immobilized CD4. In the present study, all the 15 extracts showed significant inhibition of this interaction by binding to immobilized CD4[18].

Further, the test compounds were added after 60min pre-incubation of gp120 in CD4-coated plates. The study revealed that, twelve of fifteen extracts significantly blocked the gp120/CD4 interaction. These extracts competed with gp120 for binding to CD4 immobilized on a solid phase and displaced gp120 preadsorbed to CD4. Similar activity has been reported with a group of small molecule Stibonic acid-containing compounds (NSC 13778) that competitively inhibited gp120 binding to CD4[8]. Heparin was used as positive control that showed the highest inhibition of 87.64% when preincubated with immobilized CD4. It showed 75.96% inhibition when preincubated with gp120 and 58.09% inhibition when added after gp120/CD4 interaction.

In general, mangrove plants namely, *A. officinalis* and *R. mucronata* showed effective inhibition of gp120/CD4 interaction by binding to both gp120 and to CD4 ligand. Similar activity has been demonstrated by oligonucleotides composed entirely of Guanosine and Thymidine (GTOs)[12]. All the 3 extracts of *A. officinalis* and *R. mucronata* also potently inhibited this interaction by displacing gp120 preadsorbed to immobilized CD4. All the 3 extracts of *O. sanctum* and *W. somnifera* potently inhibited gp120/CD4 interaction by binding to CD4 (but not to gp120). Similar activity has been exhibited by Niphatevirin, a novel anti-HIV protein isolated from aqueous extracts of the Caribbean sponge *Niphates erecta*. Niphatevirin bound to CD4 in a manner that prevented the binding of gp120, but did not directly bind gp120[19]. Extracts of *O. sanctum* and *W. somnifera* also competitively inhibited gp120 binding to CD4 except petroleum ether extract of *O. sanctum* and *W. somnifera*. Among *T. cordifolia* extracts, only petroleum ether and ethanol (successive) extracts

significantly inhibited gp120/CD4 interaction by binding to gp120 and by competitive inhibition. All the 3 extracts of *T. cordifolia* also inhibited this interaction by binding to immobilized CD4.

A variety of tannins are capable of inhibiting HIV-RT. They may also inhibit HIV replication in cell culture by additional or other mechanisms, probably by interfering with HIV cell interactions. Galloylquinates and Galloylshikimates (tannins) isolated from *Castanopsis hystrix* A. DC. and Caffeoylquinates (tannin) isolated from *Lonicera japonica* Thunb. showed HIV-RT inhibition activity[20]. Also, it has been shown that the tannins chebulic acid and punicalin were able to block the binding of HIV rgp120 to CD4 and that these compounds were not toxic to the stimulated human peripheral blood lymphocytes[21].

The sulfated polysaccharides are believed to function by destabilizing the glycoprotein complex. They are also reported to inhibit the activity of reverse transcriptase. A study has revealed a new HIV-RT inhibitor extracted and purified from red alga *Schizymenia pacifica*. The chromatographic behaviour and chemical properties of this sea algal extract suggest that it is a sulfated polysaccharide composed of galactose, sulfonate and 3,6-anhydrogalactose[22].

## Conclusion

Experimental results thus suggested that most of the plant extracts which have been tested in the present study exert their anti-HIV activity via multiple mechanisms of action, viz. interference with the gp120/CD4 interaction and inhibition of viral RT. Thus the present study does seem to justify the traditional use of plants for the treatment of infectious diseases of viral origin. However, in order to assess the usefulness of these herbs, it is necessary to isolate the active principle(s) from the crude extracts, identify them and study their mechanism of action.

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