

Evaluation of *in vitro* inhibitory effect of selected plants and *Shilajit* on HIV-Reverse Transcriptase

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Three tropical medicinal plants, *Ocimum sanctum* Linn., *Withania somnifera* Dunal and *Tinospora cordifolia* (Willd.) Miers ex Hook. f. & Thoms. and two mangrove medicinal plants, *Avicennia officinalis* Linn. and *Rhizophora mucronata* Lam. were taken up in the present study along with *Shilajit*. Three extracts, petroleum ether, ethanol (successive) and aqueous were prepared from each plant, whereas, only 2 extracts of *Shilajit*, ethanol (successive) and aqueous were included for the *in vitro* study. Thus a total of 17 extracts were evaluated for their effect on HIV-reverse transcriptase of 2 clinical isolates, designated as non-antiretroviral therapy (non-ART, from drug naïve patient) and antiretroviral therapy (ART, from drug-treated patient), by reverse transcriptase inhibition assay. The virus stocks were prepared by standard co-cultivation method. Eleven of the 17 extracts showed more than or equal to 50% ($\geq 50\%$) inhibition of non-ART isolate whereas, only 9 of the 17 extracts showed $\geq 50\%$ inhibition of ART isolate. In general, *Shilajit* extracts showed $\geq 50\%$ inhibition of both the clinical isolates. Furthermore, different combinations of aqueous extracts were assessed for their effect on reverse transcriptase of non-ART isolate. Combination of *A. officinalis* and *Shilajit* showed the highest inhibition of viral enzyme. However, the lowest inhibition was noted with combination of *W. somnifera* and *T. cordifolia*.

Keywords: Anti-HIV, HIV-reverse transcriptase (RT), Mangroves, Medicinal plants, RT-inhibition, *Shilajit*.

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Introduction

Acquired Immuno Deficiency Syndrome (AIDS) is a devastating human disease as there is no vaccine, adverse effects of currently approved Anti-HIV drugs, emergence of drug resistance and latent phase of the virus. Hence there is urgent need of safe, effective and economical alternative. Several natural products, mostly of plant origin have been shown to possess promising activities that could assist in the prevention and/or amelioration of the disease¹. Although, majority of natural drugs are derived from plant and animal origins, a few of them, obtained from mineral sources, like *Shilajit*, are of paramount significance as pharmaceutical aids². *Shilajit* is a pale-brown to blackish-brown exudation from rocks in Himalayan ranges of Indian subcontinent. It is also found in Nepal, Bhutan, Tibet and China³. Numerous traditional uses of *Shilajit* have been reported, a number of which have been verified by modern scientific evaluation such as anti-ulcerogenic, anti-inflammatory, antidiabetic, anti-allergic and immunomodulatory activities to name a

few⁴. Besides *Shilajit*, plants included in the present study include, *Ocimum sanctum* Linn., *Withania somnifera* Dunal, *Tinospora cordifolia* (Willd.) Miers ex Hook. f. & Thoms., *Avicennia officinalis* Linn. and *Rhizophora mucronata* Lam. The biological activities of *O. sanctum* include immunomodulatory, antitumour, antidiabetic and antimicrobial⁵, that of *W. somnifera* include, anti-inflammatory, antitumour, antioxidant and immunomodulatory⁶, whereas, that of *T. cordifolia* include, antioxidant, hepatoprotective, anti-neoplastic, immunomodulatory and antiviral⁷. *A. officinalis* and *R. mucronata* belong to mangrove plants. The term mangrove is used to describe halophytic and salt-resistant marine tidal plants⁸ which are also used in folklore medicine for treatment of several diseases⁹. Some of the biological properties of *A. officinalis* consist of aphrodisiac¹⁰, astringent¹⁰, antitumour¹¹, antiprotozoal¹² to name a few. While, the medicinal uses of *R. mucronata* comprise, anti-diabetic, astringent, for hemorrhage and angina¹³. Thus many of these natural products have other medicinal values as well, which can deal with both the virus and the various disorders that characterize HIV/AIDS.

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In the present study, seventeen extracts were prepared from *O. sanctum*, *W. somnifera*, *T. cordifolia*, *A. officinalis*, *R. mucronata* and *Shilajit* were evaluated for specific inhibitory activity against HIV using HIV-reverse transcriptase inhibition assay. Most of these extracts showed potent inhibitory activity against recombinant HIV-reverse transcriptase in our earlier study^{14,15}. Hence these extracts were evaluated for their effect on reverse transcriptase of 2 clinical isolates *in vitro*.

Materials and Methods

Collection of the material

Root powder of *Withania somnifera* and stem powder of *Tinospora cordifolia* were procured from Zandu Pharmaceuticals, Mumbai. Leaf powder of *Ocimum sanctum* was purchased from Atul Medical Stores, Mumbai. *Avicennia officinalis* was collected from Mahim Creek, Mumbai, whereas, *Rhizophora mucronata* was collected from Vikroli highway, Mumbai. The leaves were washed, shade-dried and powdered in a grinder mixer. *Shilajit* was purchased from Vaidya (Dr) Rajeev K. Kanitkar, Ayurvedic physician, Mumbai (Plate 1). All the material was identified and authenticated by Dr J. M. Pathak, Research Director (Pharmacognosy), Zandu Pharmaceuticals, Mumbai.

Phytochemical analysis of the material

Qualitative phytochemical analysis of *O. sanctum*, *W. somnifera*, *T. cordifolia* and *A. officinalis* was carried out for the presence of resins, alkaloids, tannins, saponins, flavonoids, anthraquinone, cardiac glycosides, steroidal ring, steroidal terpenes and carbohydrates to name a few¹⁶. In case of *R. mucronata*, as the material was not obtained in sufficient quantity, phytochemical analysis was not possible. Hence, it was subjected to biological testing only.

Preparation of extracts

The plant material (3-47 g) was first extracted in Soxhlet apparatus with petroleum ether (60-80°C, 230-460 ml) and then with ethanol (230-460 ml) in succession. Aqueous extracts were obtained by plain decoction method¹⁷ 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 (Table 1).

Petroleum ether and ethanol extracts after removal of solvents were dissolved in dimethyl sulfoxide (DMSO) (not more than 1%) and then diluted with RPMI (Roswell Park Memorial Institute) 1640 medium, whereas, aqueous extracts after drying on boiling water bath were reconstituted in RPMI 1640 medium alone. Each extract was reconstituted to

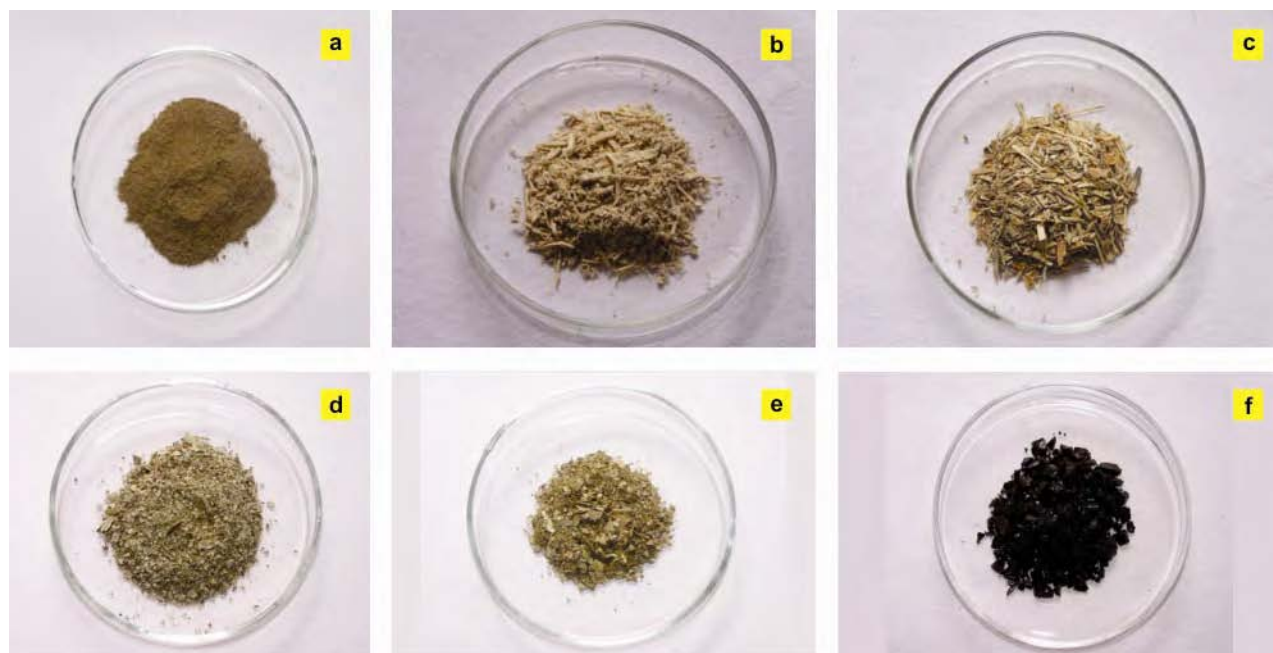


Plate 1 (a-e) — Powder samples of plants and Shilajit: a-Leaf powder of *Ocimum sanctum*, b-Root powder of *Withania somnifera*, c-Stem powder of *Tinospora cordifolia*, d-Leaf powder of *Avicennia officinalis*, e-Leaf powder of *Rhizophora mucronata*, f-Granules of pure *Shilajit*

Table 1 — Percentage yield of various extracts

Plants	Yield (%)		
	Petroleum ether extract	Ethanol extract (Successive)	Aqueous extract
<i>Ocimum sanctum</i>	1.68	6.88	4.4
<i>Withania somnifera</i>	0.47	7.08	7.0
<i>Tinospora cordifolia</i>	1.15	4.63	5.8
<i>Avicennia officinalis</i>	1.47	14.73	16.0
<i>Rhizophora mucronata</i>	5.98	19.60	24.77
<i>Shilajit</i>	0.07	17.44	66.0

prepare stock of 25 mg/ml. The stock solutions were then filtered through 0.22 µm membrane filter and stored at 4°C until further use.

Preparation of virus stock

For preparation of virus stocks, 10 ml of blood samples were collected in EDTA vacutainer from known HIV seropositive drug-naïve (non-ART) and drug-treated (ART) individuals, where ART stands for antiretroviral therapy. Written consent was obtained before blood collection. Similarly, 10 ml of blood sample was collected in EDTA vacutainer from the healthy individual. The rapid HIV card test (PMC medical Pvt. Ltd.) was performed in order to confirm the sero-status of the patients.

Separation of peripheral blood mononuclear cells (PBMCs) was carried out using density gradient centrifugation as per the protocol mentioned¹⁸. The PBMCs of the HIV-infected patients were co-cultivated with that of the PHA-stimulated PBMCs isolated from healthy individual¹⁸. The culture supernatant was collected at regular time period and stored at -70°C as a source of HIV for the study.

Assessment of Anti-HIV activity

a) Effect on clinical isolates:

Reverse transcriptase inhibition assay¹⁹ was carried out to determine the activity of each extract against two different clinical samples. The reverse transcriptase of *in vitro* cultivated virus stocks from blood samples of HIV seropositive non-ART and ART individuals was considered for this study. The assay was carried out with slight modification. For this, 25 µl of each extract (Final concentration 5 mg/ml) was added to the reaction mixture.

The reaction mixture (100 µl) contained the following: 50 mM Tris (pH 7.8), 150 mM KCl, 5 mM MgCl₂, 0.05% NP-40, 0.5 mM EGTA, 5 mM DTT, 20 µM dTTP, 0.3 M glutathione, 2.5 µg/ml

BSA, 0.5 µCi (microcurie) of ³H-TTP, 2.5 µg/ml POLY(rA).p(dT)¹²⁻¹⁸.

The reaction was started by the addition of 10 µl of each of the viral supernatant. The mixture was then incubated for 3 h at 37°C. The reaction was terminated by the addition of 25 µl of 0.1 M EGTA followed by chilling the mixture on ice. Hundred µl of each reaction mixture was then spotted uniformly onto DE-81 Whatman filters (discs) (B. Patel and Company) and kept at ambient temperature for 15 min. The dried filters were washed four times with 5% aqueous Na₂HPO₄.7H₂O followed by two more washing with double distilled water. Finally, the filters were thoroughly dried and subjected to scintillation counting. Negative control without extract was set up in parallel. AZT (Azidothymidine) was included as a standard. The percent inhibition was calculated as, [(CPM of negative control – CPM of test)/CPM of negative control] X 100, where, CPM is counts per minute.

b) Combinatorial effect

To evaluate the combinatorial effect, reverse transcriptase inhibition assay was performed as described earlier and non-ART isolate was used as virus source. For this study, different combinations of aqueous extracts of 5 plants and *Shilajit* were prepared by mixing them in equal proportion (12.5 µl) in separate eppendorf tubes and a mixture of all the 6 extracts (4.2 µl each) together was also prepared to study their effect on HIV-reverse transcriptase. The effect of combinations was determined by comparing the CPM values obtained after scintillation analysis with that of negative control. The result is expressed as percent inhibition as stated earlier.

The data was compared with that of negative control and the statistical analysis of the data was done by one-way ANOVA followed by Dunnett t (2-sided) test using statistical analysis system SPSS (14.0) package. *P*<0.05 was regarded as significant.

Results and Discussion

As shown in Table 1, percent yield of petroleum ether extract of *Shilajit* was very low to pursue further work. Thus, total 17 extracts were subjected to *in vitro* anti-HIV activity. As illustrated in Table 2, ethanol (successive) extract of *O. sanctum* showed the highest inhibition of Non-ART isolate, whereas, aqueous extract of *R. mucronata* showed the highest inhibition of ART isolate. As illustrated in Table 3, combination of *A. officinalis* and *Shilajit* showed

Table 2 — Effect of extracts on Reverse Transcriptase of clinical isolates

Plants	Extracts	Reverse Transcriptase Inhibition (%) [Mean ± SD]	
		Non-ART	ART
<i>Ocimum sanctum</i>	Petroleum	44.01 ± 0.67	24.32 ± 12.78
	Ether		
	Ethanol (Successive)	76.35 ± 0.57	63.24 ± 0.16
	Aqueous	57.49 ± 2.31	58.74 ± 5.71
<i>Withania somnifera</i>	Petroleum	46.41 ± 0.99	17.48 ± 4.50
	Ether		
	Ethanol (Successive)	46.26 ± 0.49	45.05 ± 8.58
	Aqueous	50.00 ± 3.07	16.22 ± 6.01
<i>Tinospora cordifolia</i>	Petroleum	51.80 ± 0.41	16.40 ± 10.34
	Ether		
	Ethanol (Successive)	64.52 ± 1.53	49.73 ± 0.62
	Aqueous	55.84 ± 2.37	65.05 ± 0.09
<i>Avicennia officinalis</i>	Petroleum	27.84 ± 1.51	31.89 ± 6.30
	Ether		
	Ethanol (Successive)	59.73 ± 1.05	65.59 ± 2.98
	Aqueous	43.86 ± 0.17	76.22 ± 2.72
<i>Rhizophora mucronata</i>	Petroleum	30.69 ± 0.73	49.01 ± 6.08
	Ether		
	Ethanol (Successive)	58.23 ± 0.97	60.54 ± 1.85
	Aqueous	70.36 ± 1.06	80.00 ± 3.63
<i>Shilajit</i>	Ethanol (Successive)	56.89 ± 1.94	74.41 ± 0.63
	Aqueous	55.24 ± 2.49	67.03 ± 0.71
Standard	AZT	26.50 ± 2.04	38.02 ± 10.42

Note: Significant ($P < 0.05$) inhibitory activity.

Non-ART- Virus isolated from drug-naïve patient, ART- Virus isolated from drug-treated patient (Indinavir + Stavudine + Nevirapine).

the highest inhibition of viral enzyme followed by the mixture of all the 6 extracts, whereas, the lowest inhibition was noted with combination of *W. somnifera* and *T. cordifolia*.

The development of safe, effective and low-cost anti-HIV agent is among the top global priorities of drug development, since the disease is not yet curable and the mortality is high²⁰.

Traditional knowledge-driven drug development can follow a reverse pharmacology path and reduce time and cost of development²¹. *Rasayana* therapy, described in Ayurveda, consists of nourishing and rejuvenating drugs with multiple applications for longevity, antioxidant activity and immunomodulation. *W. somnifera* (Ashwagandha), *O. sanctum* (Tulasi),

Table 3 — Combinatorial Effect of Extracts on Reverse Transcriptase of Non-ART Isolate

S. No.	Combinations of Extracts	Reverse Transcriptase Inhibition (%) [Mean ± SD]
1	<i>O. sanctum</i> + <i>W. somnifera</i>	52.41 ± 1.46
2	<i>O. sanctum</i> + <i>T. cordifolia</i>	56.42 ± 2.07
3	<i>O. sanctum</i> + <i>A. officinalis</i>	57.44 ± 3.51
4	<i>O. sanctum</i> + <i>R. mucronata</i>	53.78 ± 0.93
5	<i>O. sanctum</i> + <i>Shilajit</i>	57.13 ± 2.09
6	<i>W. somnifera</i> + <i>T. cordifolia</i>	21.32 ± 4.83
7	<i>W. somnifera</i> + <i>A. officinalis</i>	60.24 ± 1.86
8	<i>W. somnifera</i> + <i>R. mucronata</i>	60.55 ± 1.70
9	<i>W. somnifera</i> + <i>Shilajit</i>	45.31 ± 2.22
10	<i>T. cordifolia</i> + <i>A. officinalis</i>	59.40 ± 1.74
11	<i>T. cordifolia</i> + <i>R. mucronata</i>	59.71 ± 4.76
12	<i>T. cordifolia</i> + <i>Shilajit</i>	65.49 ± 3.05
13	<i>A. officinalis</i> + <i>R. mucronata</i>	62.43 ± 2.06
14	<i>A. officinalis</i> + <i>Shilajit</i>	65.84 ± 1.03
15	<i>R. mucronata</i> + <i>Shilajit</i>	65.54 ± 3.27
16	Mixture (<i>O. sanctum</i> + <i>W. somnifera</i> + <i>T. cordifolia</i> + <i>A. officinalis</i> + <i>R. mucronata</i> + <i>Shilajit</i>)	65.61 ± 0.28

Note: Significant ($P < 0.05$) inhibitory activity.

T. cordifolia (Gulvel), *Asparagus racemosus* Willd. (Shatavari), *Tribulus terrestris* Linn. (Gokshura), *Piper longum* Linn. (Pippali), *Terminalia chebula* Retz. (Haritaki) and *Shilajit* to name a few, are classified as *Rasayana* in Ayurveda. They strengthen the host's general resistance by stimulating the immune function, a concept similar to 'prohost therapy' of modern medicine^{22,23}. Besides, various traditional and medicinal uses of Mangrove plants have also been reported²⁴.

In the present study, 17 extracts prepared from the selected plants, *O. sanctum*, *W. somnifera*, *T. cordifolia*, *A. officinalis*, *R. mucronata* and *Shilajit*, were tested for their effect on HIV-reverse transcriptase. Further, only aqueous extracts were evaluated for their combinatorial effect.

The Card test performed to confirm, the sero-status of the HIV positive individuals, revealed development of a single pink band corresponding to the HIV-1. This indicated that the infection was due to HIV-1 only.

It has been observed that, HIV is cultivable from PBMCs of most seropositive persons. The sensitivity of HIV-1 detection by co-cultivation of subject's PBMCs with PHA-stimulated cells from sero negative donor is more than 90% in clinically asymptomatic

patient and close to 100% in clinically symptomatic patients²⁵. Hence, virus has been grown by classical co-cultivation method. Further, the detection of HIV in culture fluid by the reverse transcriptase assay is considered to be the most sensitive and specific assay for the presence of virus in infected lymphocytes or in body fluids^{26,27}.

HIV-reverse transcriptase is one of the vital enzymes for the replication of the virus, as it performs 3 important functions²⁸. This makes it a suitable target for chemotherapeutic intervention. EL-Mekkawy *et al*²⁹ evaluated extracts of 41 medicinal plants used in Egyptian folk medicine for HIV-1 reverse transcriptase inhibitory effects and identified putranjivain A from *P. emblica* as a potent inhibitory compound. Similarly, Rukunga *et al*³⁰ screened extracts from 22 medicinal plants used in preparing traditional remedies in Kenya for activity against HIV-1 reverse transcriptase.

In the present study, effect of 17 extracts on reverse transcriptase of two different clinical isolates, non-ART and ART was evaluated by HIV reverse transcriptase inhibition assay. As all the 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, 5 mg/ml. As illustrated in Table 2, the selected plants and *Shilajit* showed significant ($P < 0.05$) inhibitory activity on non-ART as well as ART isolate. Furthermore, 11 of 17 extracts showed more than or equal to 50% ($\geq 50\%$) inhibition of non-ART isolate, whereas, only 9 of 17 extracts showed $\geq 50\%$ inhibition of ART isolate. In general, *Shilajit* extracts showed $\geq 50\%$ inhibition of both the clinical isolates.

The development of resistant viruses is the major obstacle for sustained viral suppression and long-term clinical benefit of antiretroviral therapy. During early phase of HIV therapy, insufficient drug potency resulting from monotherapy was the major cause of drug failure and resistance. However, the current therapy, "Highly active antiretroviral therapy" (HAART) refers to use of combination of antiretroviral agents for treatment of HIV infection³¹.

Consequently, in the present study, different combinations of aqueous extracts of selected plants and the herbomineral were evaluated for their combinatorial effect on reverse transcriptase of non-ART isolate using reverse transcriptase inhibition assay. According to Table-3, all the 16 combinations showed significant ($P < 0.05$) inhibition of RNA

Dependant DNA Polymerase (RDDP) function of HIV-reverse transcriptase.

Many compounds of plant origin such as alkaloids, carbohydrates, coumarins, flavonoids, tannins, terpenoids to name a few, have been reported to inhibit HIV-reverse transcriptase. Galloylquinates and galloylshikimates (Tannin) isolated from *Castanopsis hystrix* A. DC. and caffeoylquinates (Tannin) isolated from *Lonicera japonica* Thunb. showed HIV-reverse transcriptase inhibitory activity³². Nakashima *et al*³³ reported a new HIV-reverse transcriptase inhibitor extracted and purified from red alga *Schizymenia pacifica*. The chromatographic behavior and chemical properties of this sea algal extract suggest that it is a sulfated polysaccharide. An Alkaloid, Michellamine B isolated from leaves of *Ancistrocladus korupensis* D.W. Thomas. & Gereau acts both at an early stage of the HIV life cycle by inhibiting reverse transcriptase as well as at later stages by inhibiting cellular fusion and syncytium formation³⁴, whereas, coumarins, calanolide A, calanolide B and its dihydroderivative isolated from different species of the genus *Callophyllum* have shown inhibition of the HIV replication in laboratory as well as clinical isolates. Among these, Calanolide A is currently in phase II clinical trial as non nucleoside reverse transcriptase inhibitor (NNRTI)³⁵.

In the present study, the phytochemical analysis of *O. sanctum* showed presence of resins, alkaloids, tannin and steroidal terpenes. *W. somnifera* revealed presence of alkaloids, saponin and flavonoids, whereas, *T. cordifolia* showed presence of resins, alkaloids and saponin. *A. officinalis* showed presence of resins and tannin. In general, presence of carbohydrates, steroidal ring and cardiac glycosides was noted in all the plants. However, anthraquinone was not detected in any of the test material. Moreover, *Shilajit* revealed presence of tannins, saponins, carbohydrates and cardiac glycosides¹⁴, whereas, literature review of *R. mucronata* divulged presence of tannin¹⁰.

In our earlier study we found that, most of these plants and *Shilajit* exerted their anti-HIV activity via multiple mechanisms of action; viz., interference with the gp120/CD4 interaction and inhibition of recombinant HIV-reverse transcriptase^{14,15}. In the present study, they also showed potent inhibitory activity against reverse transcriptase of 2 clinical isolates which may be due the phytochemical(s) present.

Conclusion

Thus, the present study does seem to justify the traditional use of plants and *Shilajit* for the treatment of infectious diseases of viral origin. They further indicate that these might be of value as sources or leads for novel antiviral compounds which can be useful in the continuing fight against HIV.

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