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Short Communication

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A Screening strategy for selection of Anti-HIV-1 Integrase and anti-HIV-1 Protease Inhibitors from extracts of Indian Medicinal plants

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Abstract

Ethanolic and water extracts from six species of Indian medicinal plants mainly distributed in the region of Western Ghats, India such as *Morinda citrifolia* (leaf), *Garcinia indica* (leaf), *Garcinia cambogia* (leaf), *Salacia oblonga* (leaf), *Coccinium fenestratum* (stem bark) and *Calophyllum inophyllum* (bark) were tested for their inhibitory activities against two prime enzymes of HIV which are HIV-1 protease (HIV-PR) and HIV-1 integrase (HIV-IN). The results revealed that the ethanolic and water extract of the bark extract of *Calophyllum inophyllum* exhibited potent anti-HIV-IN activity with IC_{50} values of 9.8 and 5.6 µg/ml, respectively. Whereas those for anti-HIV-1 PR effect were found to be 63.8 and 16.3 µg/ml, respectively. This result strongly supports the basis for the use of *C. inophyllum* for AIDS treatment by local traditional practitioners of Ayurveda and Unani system of Indian medicine and it is the first report on HIV-1 Protease and HIV-1 Integrase enzyme inhibition by this plant extract.

Keywords: AIDS, HIV-1 protease, HIV-1 integrase

Introduction

In recent years, all other forms of immunodeficiency syndrome have been overshadowed by an epidemic of severe immunodeficiency caused by a retrovirus called Human immunodeficiency virus type 1 or HIV-1. HIV-1 encodes three enzymes, protease (PR), reverse transcriptase (RT) and integrase (IN). HIV-1 PR is responsible for processing of viral proteins into functional enzymes and structural proteins. HIV-1 RT is a multifunctional enzyme that transcripts viral RNA into viral DNA, whereas HIV-1 IN is responsible for the integration of double stranded DNA transcribed from viral RNA into the host chromosome [1]. With the growing drug resistant strains and side effects coupled with the increasing failure of synthetic drugs it is of interest to screen for HIV-1 PR and HIV-1 IN inhibitors from natural sources.

Six medicinal plants mainly distributed in the region of Western Ghats, India were screened for HIV-1 PR and HIV-1 IN inhibitory activity. They are *Morinda citrifolia* L. (leaf), *Garcinia indica* (Dupetit-Thouars) Choisy (leaf), *Garcinia cambogia* (Gaertn.) Desr. (leaf), *Salacia oblonga* L. (leaf), *Coccinium fenestratum* **(**Gaertn.) Colebr. (stem bark) and *Calophyllum inophyllum* L. (bark). These plants were selected for screening owing to their vast medicinal and ethnopharmacological importance. *Morinda citrifolia,* is commonly known as Great Morinda, Indian mulberry. Its fruit (noni) has been used in tropical regions both as food and folk medicine. The recent use of noni as a dietary supplement has increased greatly and is reported to have a

broad range of therapeutic effects, including antibacterial, antiviral, antifungal, antitumor, antihelminthetic, analgesic, hypotensive, anti-inflammatory and immune enhancing effects [2]. *Garcinia indica* (kokam) is an Indian spice, the fruit rind of which is used in cooking, cosmetics and has several medicinal properties [3]. Also, some Tanzanian *Garcinia* species have been shown to possess Anti-HIV-1 Protease inhibitory activity [4]. *Garcinia cambogia* is known to be a potential antiobesity agent [5], and has shown antiulcer activity [6]. *Salacia oblonga* is known to possess wide spectrum activity against several human pathogenic bacteria [7]. *Coscinium fenestratum* is an endangered medicinal plant whose stem is known to possess neurotoxic activity [8] and antidiabetic activity [9]. Previous reports are strongly indicative of Anti HIV-1 Reverse Transcriptase activity of *Calophyllum inophyllum*. The leaves of this tree collected from Malaysia have shown inhibition against HIV-1 Reverse Transcriptase [10]. The seeds of *Calophyllum cerasiferum* and *Calophyllum inophyllum* have shown to contain coumarins which are potent HIV-1 Reverse Transcriptase inhibitors [11]. The screening was carried out for the search of HIV-1 PR and HIV-1 IN inhibitors from natural sources which could lead to the discovery of novel molecules. This report is the first to describe the Anti-HIV-1 Protease and Anti HIV-1 Integrase activity of the bark extract of *Calophyllum inophyllum.*

Materials and Methods

Preparation of plant extracts

Twenty grams of each dried plant were extracted two times with water and ethanol separately under reflux for 3 h. The solvents were removed under reduced pressure to obtain the respective dry extracts and then dissolved in 50% Dimethyl sulfoxide (DMSO) to form stock solutions of 10 mg/ml in order to carry out the bioassay.

Enzymes and chemicals

Recombinant HIV-1 PR, substrate peptides and acetyl pepstatin, were purchased from Sigma Chemical Co., St. Louis, USA.

Recombinant HIV-1 IN was expressed in *Escherichia coli,* purified according to the method described by Jenkins [12] and stored at -80 °C until use.

Assay of HIV-1 protease inhibitory activity

This assay was modified from the previously reported method [13]. In brief, the recombinant HIV-1 PR solution was diluted with a buffer composed of a solution containing 50 mM of sodium acetate (pH 5.0), 1 mM ethylenediamine disodium (EDTA.2Na) and 2 mM 2-mercaptoethanol (2-ME) and mixed with glycerol in the ratio 3:1. The substrate peptides, Arg-Val-Nle-NH_2, was diluted with a buffer solution of 50 mM sodium acetate (pH 5.0). Two microliters of plant extract and four microliters of HIV-1 PR solution (0.025 mg/ml) were added to a solution containing 2 µl of substrate solution (2 mg/ml), and the reaction mixture of 10 µl was incubated at 37°C for 1 h. A control reaction was performed under the same condition but without the plant extract. The reaction was stopped by heating the reaction mixture at 90°C for 1 min. Subsequently, 20 µl of sterile water was added and an aliquot of 10 µl was analyzed by HPLC using RP-18 column (4.6 mm X 150 mm i.d., Supelco 516 C-18-DB 5 µm, USA). Ten microliters of the reaction mixture was injected to the column and gradiently eluted with acetonitrile (15-40%) and 0.2% trifluoroacetic acid (TFA) in water, at a flow rate of 1.0 ml/min. The elution profile was monitored at 280 nm. The retention times of the substrate and p-NO_2-Phe-bearing hydrolysate were 4.709 and 2.733 min, respectively. The inhibitory activity of HIV-1 PR was calculated as follows :% inhibition = $(A_{control} - A_{sample})$ X $100/A_{control}$; whereas A is the relative peak of the product hydrolysate. Acetyl pepstatin was used as a positive control.

Assay of HIV-IN inhibitory activity

Oligonucleotide substrates

Oligonucleotides of long terminal repeat donor DNA (LTR-D) and target substrate (TS) DNA were purchased from QIAGEN Operon, USA and stored at -25°C before use. The sequence of biotinylated LTR donor DNA and its unlabelled complement were 5'-biotin-ACCCTTTTAGTCAGTGTGGAAAATCTCTA GCAGT-3'(LTR-D1) and 3'-GAAAATCAGTCACACCTTTTAGAGATCGT CA-5' (LTR-D2), respectively. Those of the target substrate DNA (digoxigenin-labelled target DNA, TS-1) and its 3'-labelled complement were 5'-TGACCAAGGGCTAATTCACT-digoxigenin and digoxigenin-ACTGGTTCCCGATTAAGTGA-5' (TS-2), respectively.

Multiplate integration assay (MIA)

The integration reaction was evaluated according to the method previously described [14]. Briefly, a mixture (45 µl) composed of 12 µl of IN buffer [containing 150 mM 3-(*N*-morpholino)propane sulfonic acid, pH 7.2 (MOPS), 75 mM $MnCl_2$, 5 mM dithiothritol (DTT), 25% glycerol and 500 µg/ml bovine serum albumin], 1 µl of 5 pmol/ml digoxigenin-labelled target DNA and 32 µl of sterilzed water were added into each well of a 96-well plate. Subsequently, 6 µl of sample solution and 9 µl of 1/5 dilution of integrase enzyme was added to the plate and incubated at 37 °C for 80 min. After wells were washed with PBS four times, 100 µl of 500 mU/ml alkaline phosphatase (AP) labelled anti-digoxigenin antibody were added and incubated at 37°C for 1 h. The plate was washed again with washing buffer containing 0.05% Tween 20 in PBS four times and with PBS four times. Then, AP buffer (150 µl) containing 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM $MgCl_2$ and 10 mM *p*-nitrophenyl phosphate was added to each well and incubated at 37°C for 1 h. Finally, the plate was measured with a microplate reader at a wavelength of 405 nm. A control composed of a reaction mixture, 50% DMSO and an integrase enzyme, while a blank is buffer-E containing 20 mM MOPS (pH 7.2), 400 mM potassium glutamate, 1 mM ethylenediaminetetraacetate disodium salt (EDTA·2Na), 0.1% Nonidet-P40 (NP-40), 20% glycerol, 1 mM DTT and 4 M urea without the integrase enzyme. Suramin, a polyanionic HIV-1 IN inhibitor was used as a positive control.

$$\% \text{ Inhibition against HIV IN} = \frac{OD_{control} - OD_{sample}}{OD_{control}} \times 100$$

where OD = absorbance detected from each well

Statistics

For statistical analysis, the results of anti-HIV-1 PR activity were expressed as mean ± S.D. of three determinations, while anti-HIV-1 IN activity were mean ± S.D. of four determinations. The IC_{50} values were calculated using the Microsoft Excel program.

Results and Discussion

Ethanolic- and water extracts from six species of Indian medicinal plants including *Morinda citrifolia* (leaf), *Garcinia indica* (leaf), *Garcinia cambogia* (leaf), *Salacia oblonga* (leaf), *Coccinium fenestratum* (stem bark) and *Calophyllum inophyllum* (bark) were investigated against HIV-1 PR and HIV-1 IN activities (Table 1). The result indicated that the aqueous extract of *Calophyllum inophyllum* possessed the most potent inhibitory activity against HIV-1 IN with an IC_{50} value of 5.6 µg/ml, followed by its EtOH extract with an IC_{50} value of 9.8 µg/ml.

Calophyllum inophyllum has been reported to possess anti-HIV-1 RT activity. The active compounds responsible for this activity are calanolide A and B [10]. Reports show a wide range in chemical composition within trees growing in different geographical location. The use of multivariate statistical analyses (PCA) shows geographical distribution of inophyllums and indicate those rich in HIV-1 active (+)-inophyllums. Reports suggests the presence of interesting chemotypes which could be used as plant source for anti-HIV-1 drugs [16].

Table 1: IC_{50} values of aqueous and ethanolic extracts of six Indian medicinal plants against HIV-1 IN and HIV-1 PR activity

Botanical Name	Family	Extract	IC_{50} (µg/ml) ± S.D.	
			HIV-1 IN	HIV-1 PR
Morinda citrifolia	Rubiaceae	Water	>100	>100
Morinda citrifolia		Ethanol	>100	>100
Garcinia indica	Clusiaceae	Water	87.8	>100
Garcinia indica		Ethanol	86.2	69.5
Garcinia cambogia	Guttiferae	Water	>100	67.7
Garcinia cambogia		Ethanol	>100	70.4
Salacia oblonga	Hippocrateaceae	Water	37.0	63.8
Salacia oblonga		Ethanol	51.5	65.3
Coscinium fenestratum	Menispermacae	Water	>100	>100
Coscinium fenestratum		Ethanol	>100	>100
Calophyllum inophyllum	Clusiaceae	Water	5.6	16.3
Calophyllum inophyllum		Ethanol	9.8	63.8
Sumarin(positive control for HIV-1 IN)			2.2±0.2	
Acetyl pepstatin (positive Control for HIV-1 PR)			3.4±0.1	

The results are IC_{50} ± S.D., n=4 for HIV-1 IN inhibitory activity ; those of HIV-1 PR activity are IC_{50} ± S.D., n= 4

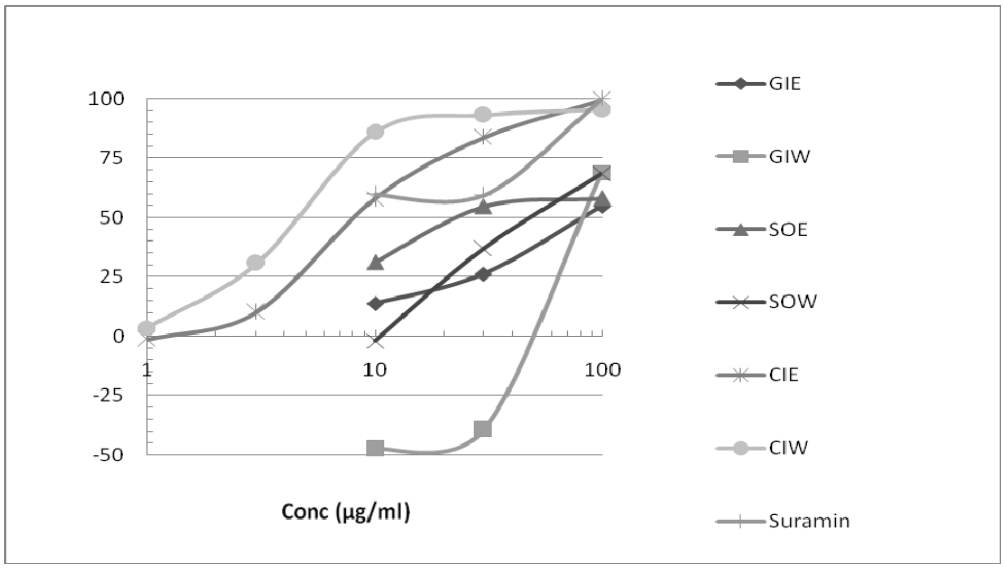

Fig 1 (a)

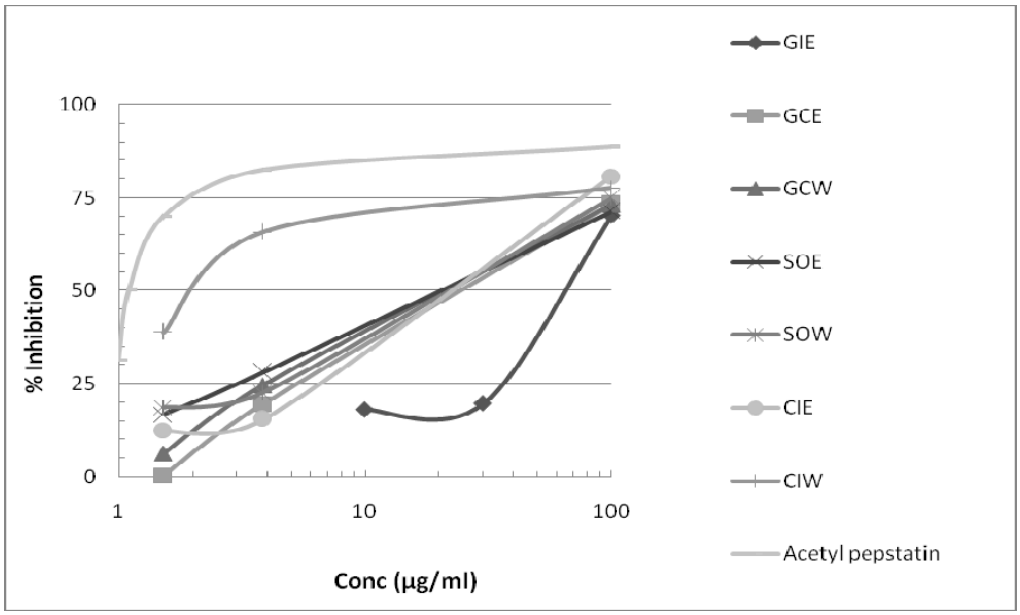

Fig 1 (b)

Fig. 1. Dose-concentration dependence against HIV-1 IN (a) and HIV-1 PR (b) of Indian Medicinal plants. GIE = *Garcinia indica* (EtOH extract), GIW = *Garcinia indica* (water extract), GCE = *Garcinia cambogia* (EtOH extract), GCW = *Garcinia cambogia* (water extract), SOE = *Salacia oblonga* (EtOH extract), SOW = *Salacia oblonga* (water extract), CIE = *Calophyllum inophyllum* (EtOH extract), CIW = *Calophyllum inophyllum* (water extract)

In conclusion, the present study supports the use of *Calophyllum inophyllum* for treatment of AIDS by traditional practitioners and it is the first report for anti-HIV-1 IN and PR activity of this plant. The isolation of active compounds from this extract will be further investigated.

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