



Short Communication

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

Chaitra Narayan L¹, Ravishankar Rai V^{1*}, Supinya Tewtrakul²

*Corresponding author:

Ravishankar Rai V

¹Department of Microbiology,
University of Mysore, Mysore,
Karnataka, India

²Department of Pharmacognosy
and Pharmaceutical Botany,
Faculty of Pharmaceutical
Sciences, Prince of Songkla
University, Kanchanawit street,
Hat-Yai, Songkhla 90112, Thailand

Abstract

Ethanollic 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 ethanollic and water extract of the bark extract of *Calophyllum inophyllum* exhibited potent anti-HIV-IN activity with IC₅₀ 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 transcribes 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, antihelminthic, 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-NH₂, 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-NO₂-Phe-bearing hydrolysate were 4.709 and 2.733 min, respectively. The inhibitory activity of HIV-1 PR was calculated as follows :% inhibition = $(A_{\text{control}} - A_{\text{sample}}) \times 100 / A_{\text{control}}$; 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-ACCCTTTTAGTCAGTGTGGAAAATCTCTAGCAGT-3'(LTR-D1) and 3'-GAAAATCAGTCACACCTTTTAGAGATCGTCA-5' (LTR-D2), respectively. Those of the target substrate DNA (digoxigenin-labelled target DNA, TS-1) and its 3'-labelled complement were 5'-TGACCAAGGGCTAATTCACCT-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 MnCl₂, 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 sterilized 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 MgCl₂ 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.

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

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 IC₅₀ values were calculated using the Microsoft Excel program.

Results and Discussion

Ethanollic- 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 IC₅₀ value of 5.6 µg/ml, followed by its EtOH extract with an IC₅₀ 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: IC₅₀ 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 |
| <i>Morinda citrifolia</i> | Rubiaceae | Water | >100 | >100 |
| <i>Morinda citrifolia</i> | | Ethanol | >100 | >100 |
| <i>Garcinia indica</i> | Clusiaceae | Water | 87.8 | >100 |
| <i>Garcinia indica</i> | | Ethanol | 86.2 | 69.5 |
| <i>Garcinia cambogia</i> | Guttiferae | Water | >100 | 67.7 |
| <i>Garcinia cambogia</i> | | Ethanol | >100 | 70.4 |
| <i>Salacia oblonga</i> | Hippocrateaceae | Water | 37.0 | 63.8 |
| <i>Salacia oblonga</i> | | Ethanol | 51.5 | 65.3 |
| <i>Coscinium fenestratum</i> | Menispermaceae | Water | >100 | >100 |
| <i>Coscinium fenestratum</i> | | Ethanol | >100 | >100 |
| <i>Calophyllum inophyllum</i> | Clusiaceae | Water | 5.6 | 16.3 |
| <i>Calophyllum inophyllum</i> | | 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 IC₅₀ ± S.D., n=4 for HIV-1 IN inhibitory activity ; those of HIV-1 PR activity are IC₅₀ ± S.D., n= 4

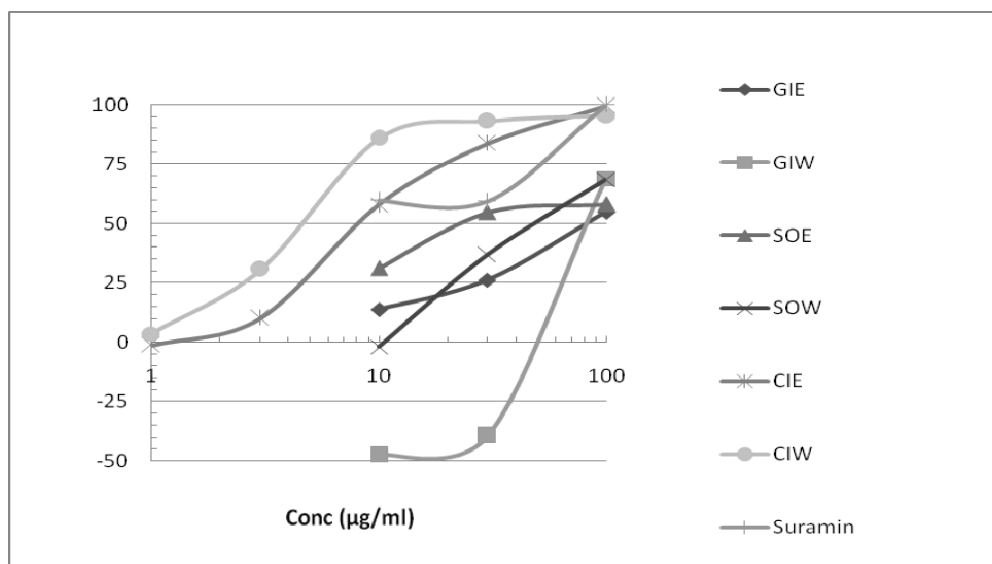


Fig 1 (a)

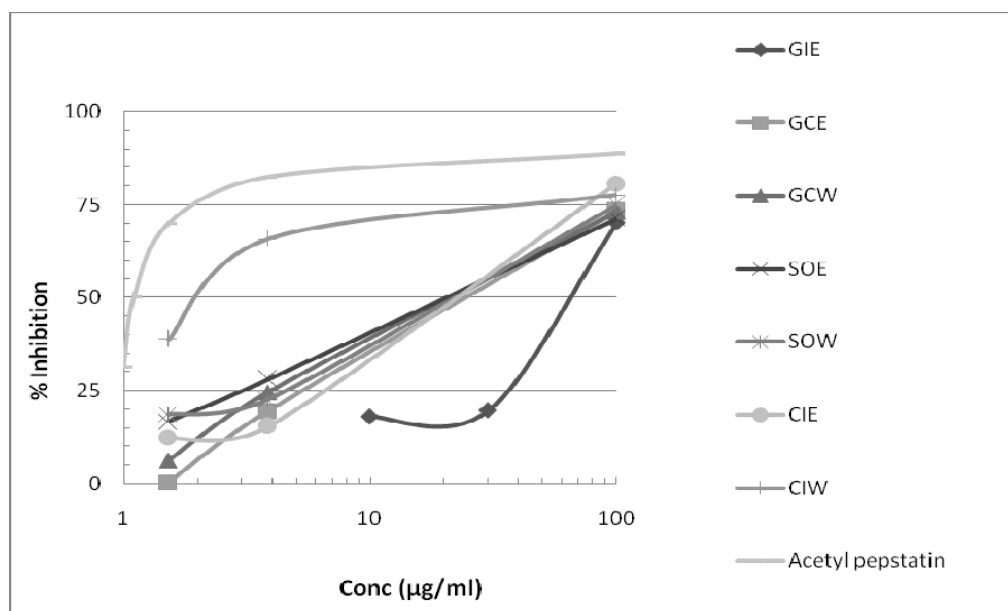


Fig 1 (b)

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.

Acknowledgements

The authors wish to express their sincere thanks to the National Research University, Prince of Songkla University, Thailand for financial support; to the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand, for providing laboratory facilities and to Dr. Robert Craigie, National Institute of Health (NIH), Bethesda, Maryland, USA, for providing an HIV-1 IN enzyme. We also express our sincere thanks to Institution of Excellence, University of Mysore, Mysore, India for providing the fellowship to one of the authors. There is no conflict of interest of any regard.

References

- [1]. Tewtrakul, S., Nakamura, N., Hattori, M., Fujiwara, T., and Supavita, T. Flavanone and Flavonol Glycosides from the leaves of *Thevetia peruviana* and their HIV-1 Reverse Transcriptase and HIV-1 Integrase Inhibitory Activities. *Chem and Pharmaceutical Bull.* 2002; 50(5) : 630-635.
- [2]. Peter, P.I. Clinical Research of *Morinda citrifolia* L.-Noni. *Noni Clinical Res J.* 2007; 1 (1-2).
- [3]. Mishra, A., Bapat, M.M., Tilak, J.C., and Thomas, P. A., Devasagayam. Antioxidant activity of *Garcinia indica* (kokam) and its syrup. *Curr Sci.* 2006; 91 (1).
- [4]. Magadula, J.J., and Tewtrakul, S. Anti-HIV-1 protease activities of crude extracts of some *Garcinia* species growing in Tanzania. *African J of Biotechnol.* 2010; 9 (12) : 1848-1852.
- [5]. Heymsfield, S.B., Allison, D.B., Vasselli, J.R., Pietrobelli, A., Greenfield, D., Nunez, C. *Garcinia cambogia* (hydroxycitric acid) as a potential antiobesity agent: a randomized controlled trial. *J of Amer Med Ass.* 1998; 280 (18) : 1596-600.
- [6]. Mahendran, P., Vanisree, A.J., Devi, C.S2. The antiulcer activity of *Garcinia cambogia* extract against indomethacin-induced gastric ulcer in rats. *Phyther. Res.* 2002; 16 (1) : 80-83.
- [7]. Rao T.M., Prayaga Murty P. In-vitro antibacterial activity of *Salacia oblonga* Wall. *Recent Res in Sci and Technol*, 2010; 2 (6).
- [8]. Wattanathorn, J., Uabundit, N., Itarat, W., Mucimapura, S., Laopatarakasem, P., Sripanidkulchai, B. Neurotoxicity of *Coscinium fenestratum* stem, a medicinal plant used in traditional medicine. *Food and Chem Toxicol.* 2006; 44(8) : 1327-1333.
- [9]. Shirwaikar, A., Rajendran, K., and Punitha, I.S.R. Antidiabetic activity of alcoholic stem extract of *Coscinium fenestratum* in streptozotocin-nicotinamide induced type 2 diabetic rats. *J of Ethnopharmacol.* 2005; 28 : 369-374.
- [10]. Patil, A.D., Freyer, A.J., Eggleston, D.S., Haltwanger, R.C., Bean, M.F., Taylor, P.B., Caranfa, M.J., Breen, A.L., Bartus, H.R., Johnson, R.K. The inophyllums, novel inhibitors of HIV-1 Reverse Transcriptase isolated from the Malaysian tree, *Calophyllum inophyllum* Linn. *J of Med Chem.* 1993; 36 (26) : 4131- 4138.
- [11]. Claude Spino, Marco Dodier, Subramaniam Sotheeswaran. Anti-HIV coumarins from calophyllum seed oil. *Bioorg and Med Chem Let.* 1998; 8 : 3475 - 3478.

- [12]. Jenkins, T.M., Engelman, A., Ghirlando, R., Craigie, R. A soluble active mutant of HIV-1 integrase. *J of Biol Chem.* 1996; 271 : 7712-7718.
- [13]. Tewtrakul, S., Subhadhirasakul, S., Kummee, S. HIV-1 protease inhibitory effects of medicinal plants used as self medication by AIDS patients. *Songklanakarin J of Sci and Technol.* 2003; 25 : 239-243.
- [14]. Tewtrakul, S., Miyashiro, H., Hattori, M., Yoshinaga, T., Fujiwara, T., Tomimori, T., Kizu, H., Miyaichi, Y. Inhibitory effects of flavonoids on human immunodeficiency virus type-1 integrase. *J of Trad Med.* 2001; 18 (6) : 229-238.
- [15]. Laurea, F., Raharivelomanana, P., Butauda, J., Bianchinia, J., Gaydou, E.M. Screening of anti-HIV-1 inophyllums by HPLC-DAD of *Calophyllum inophyllum* leaf extracts from French Polynesia Islands. *Analytica chimica acta* . 2008; 624 : 147-153.