



HIV-1 reverse transcriptase inhibition by *Vitex negundo* L. leaf extract and quantification of flavonoids in relation to anti-HIV activity

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Abstract

This study aimed to determine the activity of ethanolic leaf extract of *Vitex negundo* L. against HIV-1 Reverse Transcriptase (RT) and to identify and quantify the flavonoids present. The effects of ethanolic (85%) leaf extract of *Vitex negundo* L. on RT activity *in vitro* were evaluated with recombinant HIV-1 enzyme, using a non-radioactive HIV-RT colorimetric ELISA kit. In addition, identification and quantification of flavonoids such as Rutin, Luteolin, Myricetin, Quercetin, Kaempferol, Isorhamnetin and Quercetagenin were analysed using HPLC. The plant *Vitex negundo* L. ethanolic leaf extract exhibited the most notable activity of 92.8% against HIV-1 RT at 200 µg/ml concentration. Phytochemical analysis revealed the presence of steroids, triterpenes, alkaloids, flavonoids, anthraquinone glycosides and amino acids. Among 7 flavonoids tested, 6 were identified in the decreasing order of quantity as Kaempferol, Myricetin, Quercetin, Quercetagenin, Isorhamnetin and Luteolin. The study revealed that the plant *Vitex negundo* L. leaf possess anti-RT substances and probably the flavonoids act as anti-virus agents.

Keywords: *Vitex negundo*, flavonoids, HIV-1 reverse transcriptase, phytochemical, anti-HIV activity.

Özet

Vitex negundo L. yaprak özütüyle HIV-1 ters transkriptaz inhibisyonu ve anti-HIV aktivitesiyle ilişkili flavonoidlerin kantifikasyonu üzerine bir çalışma

Bu çalışmada HIV-1 ters transkriptaza karşı *Vitex negundo* L. etanolik yaprak özütünün aktivitesini tespit etmek ve flavonoidlerin varlığını ölçmek amaçlanmıştır. *Vitex negundo* L. etanolik (%85) yaprak özütünün *in vitro* RT aktivitesi üzerinde etkileri, rekombinant HIV-1 enzimi ile radyoaktif olmayan HIV-RT kolorimetrik ELISA kiti kullanarak ölçülmüştür. Ayrıca, Rutin, Luteolin, Myricetin, Quercetin, Kaempferol, Isorhamnetin ve Quercetagenin gibi flavonoidlerin tanımlanması ve kantifikasyonu HPLC kullanılarak analiz edilmiştir. *Vitex negundo* etanolik yaprak özütü 200 µg/ml konsantrasyonda HIV-1 RT'e karşı %92,8 aktivite göstermiştir. Fitokimyasal analizler steroidlerin, triterpenlerin, alkaloidlerin, flavonoidlerin, antrokinon glikoitlerin ve aminoasitlerin varlığını açığa

çıkartmaktadır. Test edilen 7 flavonoidden altısı, azalan miktarlarına göre Kaempferol, Myricetin, Quercetin, Quercetagenin, Isorhamnetin ve Luteolin olacak şekilde belirlenmiştir. Bu çalışma, *Vitex negundo* bitki yaprağının anti-ters transkriptaz özelliği bulunduğunu ve flavonoidlerin retrovirüslere karşı muhtemelen antivirüs ajan olarak rol oynadıkları ortaya çıkarmaktadır.

Anahtar kelimeler: *Vitex negundo*, flavonoidler, HIV-1 ters transkriptaz, fitokimyasal, anti-HIV aktivitesi.

Introduction

Acquired immunodeficiency syndrome (AIDS), caused by the human immunodeficiency virus (HIV), results in life-threatening opportunistic infections and malignancies. HIV leads to the destruction and functional impairment of the immune system, subsequently destroying the body's ability to fight against infections (Kanazawa and Matija, 2001). Moreover, the standard antiviral therapies are too expensive for a common man. In order to manage this condition alternative treatments are explored.

Vitex negundo L., a member of Verbenaceae family, an important medicinal plant is found throughout India. Though almost all plant parts are used, the extract from leaves and the roots is the most important in the field of medicine and is sold as drugs. The leaf extract is used in Ayurvedic and Unani systems of medicine for treatment of various ailments (Kapur *et al.*, 1994). It also has mosquito repellent activity (Hebbalkar *et al.*, 1992), anti-arthritic effect on rats (Tamhankar *et al.*, 1994), analgesic activity on mice (Gupta *et al.*, 1999), hepatoprotective activity (Kapur *et al.*, 1994), anti-inflammatory and anti-allergic activity (Chawla *et al.*, 1992; Jana *et al.*, 1999). Besides being used as a traditional medicine, its antiviral property, especially against HIV, has not yet been explored much.

Flavonoids have been proven to display a wide range of biochemical and pharmacological actions such as anti-carcinogenic, anti-viral, anti-microbial, anti-thrombotic, anti-inflammatory, and anti-mutagenic activities. In addition, flavonoids can act as free radical scavengers and terminate the radical chains reaction that occurs during the oxidation of triglycerides in food system (Turkoglu *et al.*, 2007). Moreover flavonoid compounds represent an important natural source of anti-retrovirals for AIDS therapy due to their significant anti-HIV-1 activity and low toxicity.

One of the possible approaches is the screening of plants based on their ethnomedicinal data for inhibition (Vlietinck *et al.*, 1998). Current strategies for anti-HIV chemotherapy involve inhibition of virus adsorption, virus-cell fusion, reverse transcription, integration, translation, proteolytic cleavage, glycosylation, assembly, or release (Moore and Stevenson, 2000; Miller and Hazuda, 2001). Reverse transcriptase is an enzyme that reads the sequence of HIV RNA that has entered the host cell and transcribes the sequence into complementary DNA. Without reverse transcriptase, the viral genome cannot be incorporated into the host cell and as a result a virus will not replicate. Reverse transcriptase is therefore the principal target enzyme of antiretroviral drugs such as Nevarapine and Delavirpine that are used to treat HIV infected patients (De Clercq, 2007; Woradulayapinji *et al.*, 2005). Therefore this study has been designed to explore the possible anti-HIV activity by RT enzyme inhibition assay and to quantify the flavonoids from the leaves of *Vitex negundo*.

Materials and Methods

Plant material and extraction

The leaves of *Vitex negundo* L. were collected from Kollu hills adjoining downstream areas of Namakkal district, Tamil Nadu, India and authenticated (PARC/2010/587) by Dr. Jayaraman, Plant Anatomy Research Centre, National Institute of Herbal Science, Chennai, India. The plant samples were washed, shade-dried, powdered and extracted in 85% ethanol and filtered. The extracts were then concentrated to dryness under reduced pressure and the residue was freshly dissolved in appropriate buffer on each day

of experiment for the assays. Depending on the assay, extract that could not dissolve in appropriate buffer were dissolved in DMSO and later diluted to different concentration needed for a particular assay.

HIV-1 RT assay

The effect of the plant extract on RT activity *in vitro* was evaluated with recombinant HIV-1 enzyme, using a non-radioactive HIV-1 RT colorimetric ELISA kit (Roche) (Ayisi, 2003; Harnett *et al.*, 2005). The concentration of extract used was 200µg/ml. The extracts, which reduced activity by at least 50%, were considered as active

(Woradulayapinji *et al.*, 2005). Azidothymidine (AZT) was used as a positive control at 100 µg/ml. The control (1) only contained the buffer and reaction mixture (no enzyme and extracts were added). For the control (2) the enzyme and reaction mixture were added for the reaction to take place. The absorbance was read on a microtitre plate reader at 405 nm with a reference wavelength of 490 nm. The mean of the triplicate absorbance were analysed using the formula:

$$\text{Percentage of Inhibition} = 100 - \left(\frac{\text{Mean Sample absorbance} \times 100}{\text{Mean Control-2 absorbance}} \right)$$

Preliminary phytochemical analysis

The ethanolic leaf extract was subjected to preliminary phytochemical screening as per the procedures of Harborne, 1998 and Kokate, 2003.

Quantitative analysis of flavonoids using HPLC

The procedure as described by Lawrence Evans (2007) was used for the determination of flavonoids in the plant extracts. The flavonoid standard used in the study includes Rutin, Luteolin, Myricetin, Quercetin, Kaempferol, Isorhamnetin and Quercetagenin (Sigma Chemicals, USA) and were prepared at 1 mg/ml in methanol. A total of 1g of plant extract was extracted with 78 ml of extraction solvent (methanol, water and hydrochloric acid; 50:20:8). Extract was then refluxed at 90°C for 2 h. Then extract was cooled and latter 20 ml of methanol was added and sonicated for 30 minutes. All solutions were filtered through a 0.45µM cellulose acetate membrane filter (Paul, USA) before being injected into the HPLC. Aliquots of the filtrate (20µl) were injected on to an HPLC (Lachrom L-7000) column using C18 (Merck) (25 X 0.4 cm, 5µm) separately and eluted with mobile phase solvent mixture comprising Water: Methanol: Phosphoric acid (100:100:1, v:v:v) with a flow rate at 1.5 ml/min. The UV detection was

carried out at 270 nm. The chromatograms were recorded and the areas measured for the major peak to quantify the flavonoids in the tested plant sample.

Results

The results shown in Table 1 indicates the inhibition percentage of ethanolic leaf extract of *Vitex negundo* L. against the reverse transcriptase (RT) enzyme. The most notable activity of about 92.8% was detected against RT at 200µg/ml. The phytochemical analysis of the plant extract revealed the presence of steroids, triterpenes, alkaloids, flavanoids, antroquinone glycosides and aminoacids. In this study, flavonoids content of the ethanolic extract of *Vitex negundo* L. leaves were evaluated. The HPLC chromatogram of the flavonoid standard used and the chromatogram of the tested plant extract is shown in Figure 1 & 2. Results revealed that the extract consisted of different amount of various flavonoid types. As shown in Figure 1, the retention times of Rutin, Quercetin, Kaempferol Luteolin, Isorhamnetin, Myricetin, and Quercetagenin were at 19.40, 24.80 29.70, 33.80, 38.70, 41.26 and 43.8 min, respectively.

Table 1. Effect of ethanolic leaf extract of *Vitex negundo* L. on the activity of recombinant HIV-1 reverse transcriptase

Extract/Control	Mean absorbance \pm SD	Percentage of Inhibition
Control 1	0.003 \pm 0.01	100
Control 2	1.32 \pm 0.01	0
AZT	0.193 \pm 0.00	85.37
<i>V. negundo</i> L.	0.094 \pm 0.01	92.8

Control 1- buffer and reaction mixture (no enzyme and extract); Control 2- enzyme and reaction mixture (no extract); AZT – Azidothymidine (Positive control). The plant extract showing percentage of inhibition greater than 50% has been considered as positive in inhibiting recombinant HIV-1 reverse transcriptase enzyme.

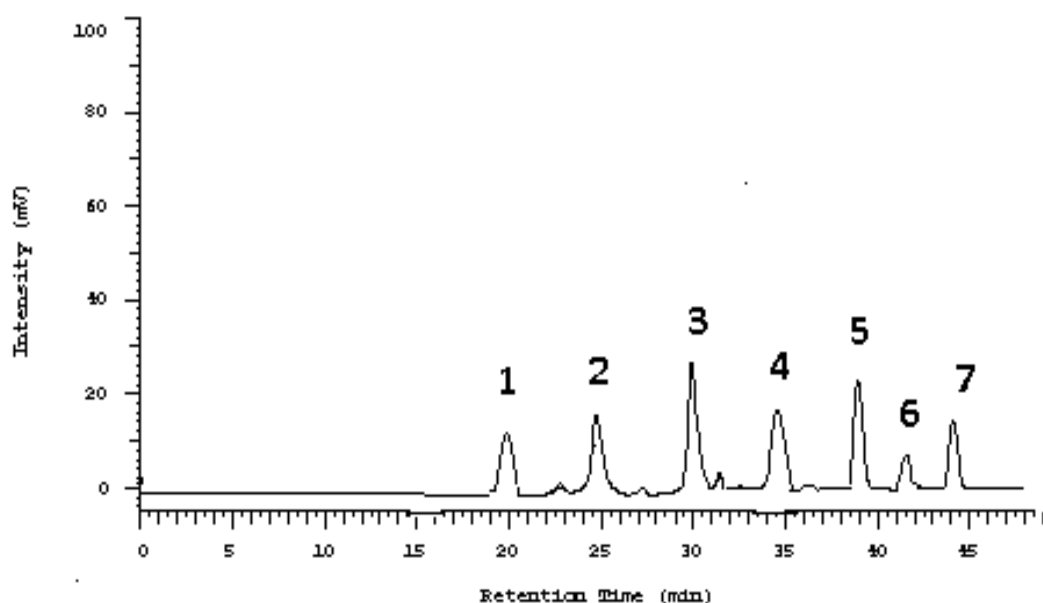
**Figure 1.** HPLC Chromatogram of the flavonoid standards used in the study. 1. Rutin 2. Quercetin, 3. Kaempferol, 4. Luteolin, 5. Isorhamnetin, 6. Myricetin, 7. Quercetagetin.

Figure 2 exhibits the presence of Quercetin, Kaempferol, Luteolin, Isorhamnetin, Myricetin and Quercetagetin in *Vitex negundo* leaf extract as per the retention time. The flavonoid Rutin was not identified in the chromatogram of the plant sample showing its absence in the plant extract. Amount of tested flavonoid compounds in the extract were calculated by measuring the area obtained for the peaks and in the order as Kaempferol (20.61 mg/g) > Myricetin (18.75 mg/g) > Quercetin (14.73 mg/g) > Quercetagetin (12.13 mg/g) > Isorhamnetin (11.01 mg/g) > Luteolin (6.40 mg/g).

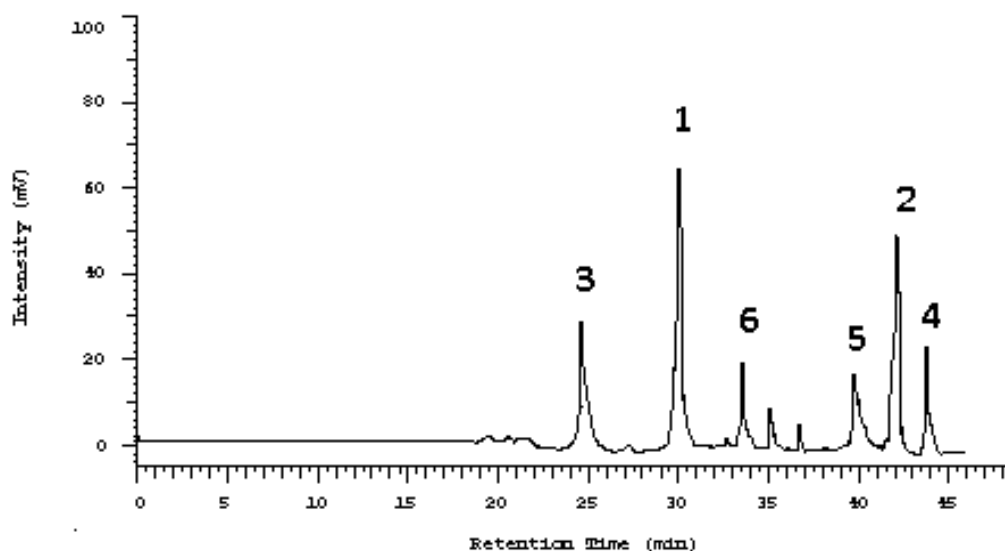


Figure 2. HPLC chromatogram of *Vitex negundo* leaves. Probable flavonoids quantity as per area and Retention time compared with the standard HPLC chromatogram: 1. Kaempferol ; 2. Myricetin ; 3. Quercetin ; 4. Quercetagenin ; 5. Isorhamnetin ; 6. Luteolin.

Discussion

For centuries water extract of fresh mature leaves are used in Ayurveda medicine as anti-inflammatory, analgesic and anti-itching agents internally and externally. However the ethanolic extract of *V. negundo* leaves resulted in the isolation of a new flavones glycoside along with five known compounds which were evaluated for their antimicrobial activities by Sathyamoorthy et al., (2007). However studies on anti-HIV activity of *V. negundo* are few. For example the water extracts of *Vitex negundo* (aerial part) was shown to have HIV-1 RT inhibition ratio (% IR) higher than 90% at a 200 μ g/ml concentration (Woradulayapinij et al., 2005). Similarly the present study also showed that the polar solvent extract of ethanolic leaf extract of *Vitex negundo* L. had 92.8% inhibition of recombinant HIV-1 reverse transcriptase enzyme at 200 μ g/ml. Previous phytochemical studies on *V. negundo* L. had revealed the presence of volatile oil, triterpenes, diterpenes, sesquiterpenes, lignan, flavonoids, flavones glycosides, iridoid glycosides, and steroids as physiologically active compounds (Azhar and Abdul, 2004; Mukherjee et al., 1981).

For centuries, preparations that contain flavonoids as the principal physiologically active constituents have been used by physicians and lay healers in attempts to treat human diseases

(Havsteen, 1983). Flavonoids are the largest classes of naturally-occurring polyphenolic compounds (Geissman and crout, 1969). Evidence has been presented that substances closely related to flavonoids inhibit the fusion of the viral membrane with that of the lysosome (Miller and Lenard, 1981). Therefore the many claims from lay medical practitioners of the prophylactic effects of flavonoids against viral attack have substantial support (Beladi et al., 1977). Although the mechanism of the inhibition remains unclear, it seems that prostaglandins participate in the fusion of cell membranes. Since flavonoids inhibit their formation, a rationale can be constructed for the protective effect of flavonoids against viral diseases (Nagai et al., 1995a, 1995b; Carpenedo et al., 1969). Moreover from the previous reports it is clear that certain naturally occurring flavonoids can inhibit reverse transcriptases of different origins (Spedding et al., 1989) From the above reviews it is clear that the flavonoids have anti-microbial activity, particularly the antiviral. Therefore the present study is focused particularly on flavonoids among other phytochemicals. Moreover the choice

of flavonoid standards used in this study was based on those commonly found in herbs and vegetables which have been studied earlier and evidenced to possess anti-HIV activity.

Schinazi *et al.* (1997) showed that the flavonols such as quercetin, myricetin, and quercetagenin, which was used as standard control in this study, have earlier been reported to inhibit certain viruses in vitro, including the Rauscher murine leukemia virus and the HIV virus. Among the 17 flavonols tested by Schinazi *et al.* (1997) only 3-O-glucosides of kaempferol, quercetin, and myricetin caused significant inhibition of HIV-1 at nontoxic concentrations. At the same time other comparative studies with other flavonoids revealed that the presence of both the double bond between positions 2 and 3 of the flavonoids pyrone ring, and the three hydroxyl groups introduced on positions 5,6 and 7 (ie, baicalein) were a prerequisite for the inhibition of RT-activity. Removal of the 6-hydroxyl group of baicalein required the introduction of three additional hydroxyl groups at position 3,3' and 4' (quercetin) to afford a compound still capable of inhibiting the RT-activity. Quercetagenin which contains the structures of both baicalein and quercetin with an additional hydroxyl group on the 5' position also proved strong inhibitors of RT activity (Ono *et al.*, 1990). Thus the activity of *Vitex negundo* leaf extract against HIV-1 RT in the present study might be due to the presence of above mentioned flavonoids particularly due to the presence of high quantity of Kaempferol, myricetin and quercetin. However this needs to be explored and confirmed. Probably this is the first report from India confirming the possible anti-HIV activity of *Vitex negundo* L.

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