

Anti-HIV-1 Activity of Ellagic acid Isolated from *Terminalia paniculata*

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ABSTRACT

Objective: The present investigation evaluates the potential Anti-HIV-1 and antioxidant activity of the methanol extract of the leaves of *Terminalia paniculata*. This search for inhibitors of Anti-HIV-1 Integrase and Protease from medicinal plants of Western Ghats, India led to the isolation of Ellagic acid from the methanol extract of *T. paniculata* leaves. **Methodology:** The *in vitro* antioxidant activity was determined by different standard methods such as 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), metal chelation, superoxide radical scavenging and lipid peroxidation assays. The *in vitro* anti-HIV-1 enzyme inhibition assays were carried out by standard protocols using Multiplate integration assay. The structure of Ellagic acid was elucidated on the basis of NMR, LC-MS and FTIR studies. **Results:** Ellagic acid has shown excellent *in vitro* anti-HIV-1 activity in the enzyme inhibition assays carried out with an IC₅₀ value of 8.7 µm and 70.4 µm against HIV-1 Integrase and HIV-1 Protease enzymes respectively. For the antioxidant assays carried out Ellagic acid has shown scavenging potential of 92.2 ± 0.03% of DPPH radical, 88.1 ± 0.41% chelation of metal ions, 68.02 ± 0.12% scavenging of superoxide anion radical and 85.52 ± 0.06% inhibition of lipid peroxidation all at a concentration of 40 ppm. **Conclusion:** The isolated bioactive molecule, Ellagic acid has shown highly efficient *in vitro* anti-HIV and antioxidant activity. The present study happens to be the first report on the HIV-1 inhibition ability of *T. paniculata*.

Key words: Anti-HIV, *Terminalia paniculata*, Antioxidant, Ellagic acid, HIV-1 Integrase, HIV-1 Protease.

INTRODUCTION

Extracts of natural materials have always proved to be a vital source in the characterization and developing lead molecules having various biological activities. By thorough screening of extracts of natural products it becomes possible to discover a multitude of structurally diverse drugs and enzyme inhibitors. This kind of diversity cannot be achieved by screening of synthetic entities. Till date, a large number of molecules derived from natural products have been discovered and added to the list of anti-HIV agents.¹ Medicinal plants have proven to be a rich source of potential and new natural bioactive molecule having high therapeutic value. One of the first inhibitor of the enzyme integrase was a phenethyl derivative of caffeic acid (CAPE), which is a plant derived product. This molecule was reported along with Doxorubicin.²

In the life cycle of the deadly Human Immunodeficiency virus, there are several crucial steps which are targeted for therapeutic intervention. HIV-1 Protease is the enzyme which is essential to cleave the newly synthesized polyprotein components at specific sites in order to create mature protein components. These mature protein units in turn aid in forming an infectious HIV virion.³ Without the action of HIV-1 Protease, it is impossible for the HIV virions to attain infectivity.^{4,5} HIV-1 Integrase is the key viral enzyme which is responsible for catalyzing the integration of DNA. *In vitro* assays for 3'-end processing and strand transfer have proven to be invaluable in determining the mechanisms which control integration.^{6,7} The *in vitro* anti-HIV assays have been extremely helpful and have facilitated the discovery and characterization of new HIV-1 inhibitors with proven therapeutic value.

The discovery of more number of inhibitors of HIV would definitely go a long way in improving the existing treatment paradigm for people suffering from HIV. Working towards this, we have successfully isolated and characterized a

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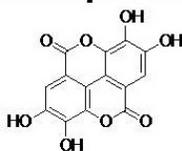
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***Terminalia paniculata*, found in biodiversity hotspot of India – Western Ghats**



Methanol extract of the leaves of *T.paniculata* – Potent Anti – HIV – 1 and Antioxidant activity

Isolation and characterization of bioactive molecule Ellagic acid for the first time from *T.paniculata*



Chemical Formula: $C_{14}H_6O_8$
Exact Mass: 302.01
Elemental Analysis: C, 55.64; H, 2.00; O, 42.36

Graphical Abstract

bioactive molecule namely Gallogen, also known as Ellagic acid from the leaves of *Terminalia paniculata*.

Of late, there has been a tremendous rise in interest towards isolating natural antioxidants. This is owing to the mounting evidence everyday on how these antioxidants can go a long way in protecting the human body against the action of free radicals thereby retarding the progression of several chronic diseases.⁸ Phenolic compounds, nitrogen compounds, carotenoids belong to the family of natural antioxidants.⁹

Terminalia paniculata is a medicinal tree native to the Western Ghats, India. Other species of *Terminalia* have been widely researched and have shown several therapeutic properties. But, *T.paniculata* still remains to be unexplored on a larger scale for its possible therapeutic activity and this was the reason to choose this medicinal tree for the present study.

T.paniculata has shown to have hepatoprotective activity¹⁰ and anti-diabetic activity.¹¹ Isolation of Ellagic acid having potent anti-HIV-1 activity coupled with strong antioxidant activity from *T.paniculata* shows a new promising source of natural anti-HIV agent.

MATERIALS AND METHODS

Plant Material

The fresh leaves of *T. paniculata* were collected from Mijar, Moodabidri, Dakshina Kannada District, Karnataka.

Herbaria of the plant was deposited in Herbarium Collection Centre at the Department of Studies in Microbiology, University of Mysore, Mysore. The accession number being *T. paniculata* (MGMB/003/2011-12). The leaf material was air dried and ground into a fine powder. 100 g of the leaf powder was then extracted with 500 ml of methanol. It was then filtered, squeezed and evaporated under reduced pressure to obtain the MeOH extract.

Extraction and Isolation

Briefly, the leaf powder of *T. paniculata* was extracted with MeOH at room temperature and the solvent was evaporated under reduced pressure to obtain the MeOH extract (570.12 mg). A part of the MeOH extract (250 mg) was then used to carry out column chromatography on silica gel by eluting with Hexane / Ethyl acetate (9:1 : 0.9, 9:0.5 : 0.95, 9 : 1, 8.95 : 1.05) to yield four fractions (Fraction F1–Fraction F4). Based on the comparison of R_f value, fractions F1 to F4 were pooled to obtain fraction F5A. Fraction F5A was again subjected to column chromatography using Hexane and ethyl acetate as elution solvents. This in turn gave four sub-fractions F5a, F5b, F5c, F5d. Sub-fraction F5d was purified by preparative TLC with Hexane Ethyl acetate (2 : 1) to obtain the purified compound Ellagic acid (8.4 mg).

Ellagic acid

IR–3570, 3065, 1690, 1615, 1580, 1446, 1395, 1195, 1053, 922, 822, 750.

ESI-MS (negative mode) m/z 301.1 [M-H]; ^1H NMR (CDCl_3) as tetra acetate δ 7.87 (2H, S, aromatic); δ 2.35 (6H, S, CH_3CO_2); δ 2.30 (6H, S, CH_3CO_2).

UV-255 nm

Copies of original spectra (NMR, LC-MS and FTIR) are available from the corresponding author.

Enzymes and Chemicals

Recombinant HIV-1 Protease, substrate peptides and acetyl pepstatin were purchased from Sigma Chemical Co., St. Louis, USA. Recombinant HIV-1 Integrase was expressed in *Escherichia coli*, purified and stored at -80°C until use.

Assay of HIV-1 Protease Inhibitory activity

This assay was carried out according to the protocol reported by Min *et al.*¹² with minor modifications. The recombinant HIV-1 Protease enzyme solution was diluted with a buffer consisting of 50 mM sodium acetate, 1 mM ethylene diamine disodium and 2 mM 2 – Mercaptoethanol. The above mentioned reagents were mixed with glycerol in the ratio 3:1. The sequence of substrate peptide was Arg-Val-Nle-(pNO₂-Phe) Glu-Ala-NH₂. This substrate peptide was diluted with 50 mM sodium acetate buffer. 2 μl of the plant extract and 4 μl of HIV-1 Protease solution were then added to the solution containing 2 μl of 50 mM buffer solution and 2 μl of substrate solution. The reaction mixture was incubated for a period of 1 hour at 37°C . A control reaction was carried out under similar conditions but without the plant extract. The reaction was brought to a halt by heating the reaction mixture at a temperature of 90°C for 1 minute. Subsequently, 20 μl of sterile water was added. An aliquot of 10 ml was then analyzed by HPLC (RP-18 column, 4.6 X 150 mm ID, Supelco 516 C-18, USA). 10 μl of the reaction mixture was injected to the column and gradiently eluted with acetonitrile (10–40%) and 0.2% trifluoroacetic acid in water. The elution profile was carried out at 280 nm. The inhibitory activity of HIV-1 Protease was calculated as follows:

$$\% \text{ inhibition} = (A_{\text{control}} - A_{\text{sample}}) \times 100 / A_{\text{control}}$$

Where, A is the relative peak of the product hydrolysate. Acetyl pepstatin was used as a positive control.

Assay of HIV-1 Integrase inhibitory activity

Oligonucleotide substrates

Oligonucleotides of long terminal repeat donor DNA (LTR-D) and target substrate (TS) DNA were purchased from QIA-

GEN operon, USA. The sequence of biotinylated LTR donor DNA being 5'-biotin-ACCCTTTTAGTCAGTGTGG-AAAATCTCTAGCAGT-3' (LTR-D1) and the sequence of its unlabelled complement being 3'-GAAAATCAG-TCACACCTTTTAGAGATCGTCA-5' (LTR-D2). The sequence of digoxigenin labeled target DNA is 5'-TGACCAAGGGCTAATTCAGT-digoxigenin and that of its 3' - labeled complement was digoxigenin - ACTGGTTCCCGATTAAGTGA-5' (TS-2).

Multiplate Integration Assay

This assay has been carried out according to the method described by Tewtrakul *et al.*,¹³ A mixture consisting of 12 μl Integrase buffer, 75 mM MnCl_2 , 5 mM dithiothritol, 25% glycerol and 500 $\mu\text{g}/\text{ml}$ bovine serum albumin along with 1 μl of 5 pmol/ml digoxigenin - labeled target DNA and 32 μl of sterilized water were added into each well of a 96 - well microtiter plate. To this reaction mixture, 6 μl of plant extract and 9 μl of 1/5 dilution of Integrase enzyme was added. Following this, the microtiter plate was incubated at a temperature of 37°C for 80 minutes. After this, wells were washed with Phosphate Buffered Saline four times. Following this, 100 μl of 500 mU/ml alkaline phosphatase labeled anti-digoxigenin antibody was added and again incubated for 1 hour at 37°C . To this, 150 μl of Alkaline phosphatase buffer was added to each of the well and incubated for 1 hour at 37°C . The absorbance of the plate was measured at 405 nm using a microplate reader. A control reaction was carried out consisting of a reaction mixture of 60% DMSO and Integrase enzyme. Sumarin, which is a polyanionic inhibitor of integrase enzyme was used as a positive control. % inhibition against HIV-1 Integrase was calculated as follows:

$$\% \text{ inhibition against HIV-1 Integrase} = (OD_{\text{sample}} - OD_{\text{control}}) \times 100$$

Where, OD = absorbance determined from wells of microtiter plate.

Free radical scavenging activity

DPPH radical scavenging activity

1,1 - diphenyl-2-picryl hydrazyl (DPPH) is a stable free radical which is extensively used to determine the free radical scavenging ability of various compounds. It is known to have maximum absorbance at 515 nm. The absorbance decreases when antioxidants donate protons to DPPH. The DPPH radical scavenging activity was assayed according to the protocol of Sultanova *et al.*¹⁴ The reaction mixture consisted of 300 μM DPPH solution in methanol

dissolved in different concentrations of the plant extract. The absorbance was detected at 515 nm after incubation for 20 minutes at room temperature. The radical scavenging activity was calculated as a % of the reduction in radicals. The assay was carried out in triplicates. Ascorbic acid was used as reference compound. The % of radicals scavenged was calculated using the formula:

$$\% \text{ radicals scavenged} = [(A \text{ of control} - A \text{ of sample}) / A \text{ of control}] \times 100$$

Metal Chelation assay

The chelating potential of the plant extract for metal ions such as ferrous ions (Fe^{2+}) was tested by adding 100 μl of different concentrations of the plant extract to 135 μl of distilled water. To this, 2 mM FeCl_2 , 5 mM ferrozine was also added. The reaction mixture was incubated for 10 minutes and absorbance measured at 562 nm. EDTA was used as control. The % chelation of the plant extract for Fe^{2+} was calculated as:

$$\% \text{ chelation} = (A_0 - A_1) / A_0 \times 100$$

Where A_0 is the absorbance of the control and A_1 is the detected absorbance in presence of the plant extract.¹⁵

Superoxide radical scavenging assay

To check the ability of the plant extract to scavenge superoxide anion radicals, an *in vitro* non – enzymatic phenazine methosulphate-nicotinamide adenine dinucleotide (PMS –NADH) system was generated. This system consisted of a reaction mixture of PMS, NADH and oxygen. It was assayed by the reduction of nitroblue tetrazolium (NBT). The absorbance was measured at 560 nm. BHA was used as control.^{16,17}

The % scavenging of superoxide anion radical was calculated as follows:

$$\% \text{ inhibition} = \text{Control} - \text{Sample} / \text{Control} \times 100$$

Lipid peroxidation assay

Freshly excised goat liver was processed to obtain 10% homogenate in cold phosphate buffered saline using a homogenizer. It was then filtered to obtain a clear homogenate. The degree of lipid peroxidation was assayed by the TBARS.¹⁸ The standard method of Ohkawa *et al.*,¹⁹ with minor modifications was followed. Different concentrations of the plant extract (10–40 ppm) in water were added to the liver homogenate. The reaction was initiated by the addition of 100 μl of 15

mM FeSO_4 solution to 3 ml of liver tissue homogenate. After 30 minutes, 100 μl of this reaction mixture was transferred into a test tube containing 1.5 ml TBA in 50% acetic acid. After this, the reaction mixture was heated for 30 seconds on a boiling water bath. A pink colored complex was formed, the absorbance of which was measured at 535 nm. % of inhibition of lipid peroxidation was calculated as follows:

$$\% \text{ inhibition} = \text{Control} - \text{Sample} / \text{Control} \times 100$$

Phytochemicals

Total flavonoid content

The content of total flavonoid was determined according to the protocol of Sudha *et al.*²⁰ The absorbance was measured against the blank at 510 nm. Quercitin was used as reference standard. The results were expressed as mg of Quercitin equivalent per gram of dry weight.

Total flavonol content

The content of total flavonol was assessed as described by Miliauskas *et al.*²¹ Absorbance was measured at 510 nm using a UV–Visible Spectrophotometer. The total flavonol content was expressed as mg of Quercitin equivalent per gram of plant extract.

Total phenolic content

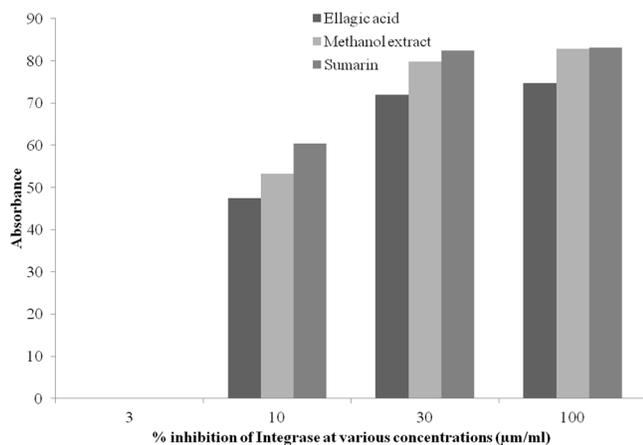
The total phenolic content was determined by using Folin–Ciocalteu reagent. In the procedure followed, 200 μl of the plant extract was mixed with 1.5 ml of Folin–Ciocalteu reagent. After 5 minutes, 1 ml of saturated Na_2CO_3 was added to the mixture. The mixture was then incubated for 90 minutes at room temperature in dark. The absorbance was measured at 725 nm against the blank.²² The content of total phenolics was expressed as mg of Gallic acid equivalent (GAE) per gram of dry weight.

Statistics

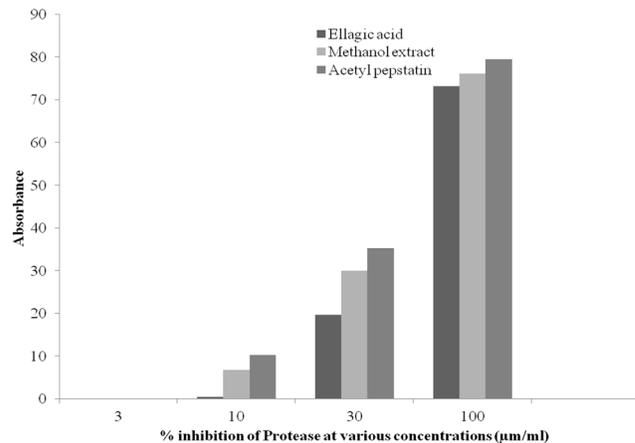
Statistical analysis for the results of HIV-1 Protease inhibitory activity was expressed as mean \pm S.D. of three determinants. Whereas, the results of HIV-1 integrase inhibitory activity were expressed as mean \pm S.D. of four determinants. For the antioxidant assays, the statistical comparison among the groups were performed with one way ANOVA followed by Tukey's HSD as Post Hoc Test (Whenever F value was significant) using statistical presentation system software version.¹⁶

Table 1: Antioxidant activity (%) of methanol extract and bioactive compound at 40 ppm. Each value is expressed as mean \pm S.D. (n=3)

Antioxidant activity	Methanol extract	Ellagic acid	Reference standard
DPPH	92.2 \pm 0.03	88.3 \pm 0.36	89.2 \pm 0.01
Metal Chelation	97.1 \pm 0.12	88.1 \pm 0.41	91.2 \pm 0.06
Superoxide radical	78.1 \pm 0.09	68.02 \pm 0.12	91.03 \pm 0.03
Lipid peroxidation	90.04 \pm 0.01	85.52 \pm 0.06	88.55 \pm 0.17

**Figure 1: HIV-1 Integrase inhibition activity of Ellagic acid**

Each value is expressed as mean \pm S.D. (n=3).

**Figure 2: HIV-1 Protease inhibition activity of Ellagic acid**

Each value is expressed as mean \pm S.D. (n=3).

RESULTS AND DISCUSSION

From the methanol extract of the leaves of *T. paniculata*, a polyphenol compound Ellagic acid was isolated. Ellagic acid isolated from *Tristanopsis* species has shown to possess antiplasmodial activity,²³ antioxidant activity.²⁴ Derivatives of Ellagic acid isolated from *Terminalia chebula* Retz has shown to downregulate the expression of quorum sensing genes to attenuate *Pseudomonas aeruginosa*.²⁵ Ellagic acid is a polyphenolic compound. It is a dimeric derivative of Gallic acid. In the combined form it exists with its precursor hexahydroxydiphenic acid or in the bound form as Ellagitannins. Ellagic acid has been known to possess antimutagenic, antiviral, skin whitening and antioxidative properties.²⁶ It is of high importance to note that Ellagic acid has been approved for use as a food additive in Japan for its functioning as an antioxidative agent.²⁷ It can hence be said that Ellagic acid is completely safe for human consumption. Ellagic acid is effective against several carcinogens such as nitrosamines, azoxymethane, mycotoxins and polycyclic aromatic hydrocarbons.

Table 1 shows the antioxidant activities of the methanol extract versus Ellagic acid and the reference compound used for the study. Ellagic acid isolated from *T. paniculata* leaves was a potent DPPH radical scavenger. The radical quenching ability of the methanol extract against DPPH

radical at 40 ppm was found to be 92.2 \pm 0.03% (Figure 3) whereas that of Ellagic acid was 88.3 \pm 0.36%.

Metal ion chelating activity of an antioxidant molecule prevents the generation of oxy radicals and its consequent oxidative damage. Chelation of metal ion decreases the concentration of the catalyzing transition metal in lipid peroxidation.²⁸ The metal ion chelating potential (%) of the methanol extract, Ellagic acid and EDTA at 40 ppm were 97.1 \pm 0.12, 88.1 \pm 0.41 and 91.2 \pm 0.06 respectively (Figure 4).

Superoxide is the most important radical produced *in vivo* in the mitochondria. During electron transfer chain, it regularly leaks out of mitochondria. The superoxide anion scavenging potential (%) of methanol extract, Ellagic acid and BHA at 40 ppm were found to be 78.1 \pm 0.09, 68.02 \pm 0.12 and 91.03 \pm 0.03 respectively (Figure 5). Though, superoxide anion is a weak oxidant, it is capable of giving rise to potentially dangerous hydroxyl radicals and singlet oxygen. Both of these are known to trigger oxidative stress. Hence, the scavenging of superoxide radical by antioxidants has physiological implications.^{29,30}

The oxidation of lipids or lipid peroxidation is a crucial step in the pathogenesis of several diseases as has been evident by several studies in recent years. Lipid peroxidation is a

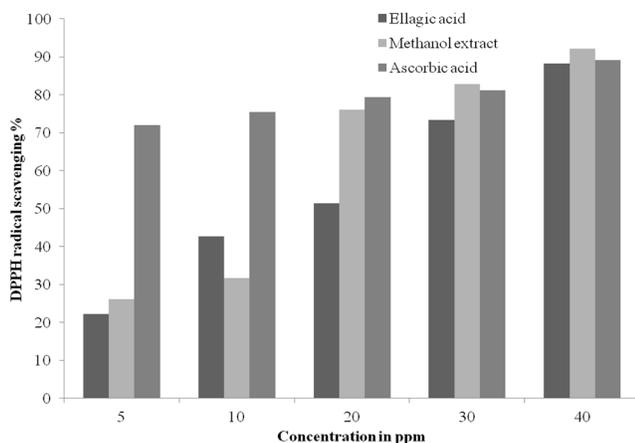


Figure 3: DPPH radical scavenging activity of Ellagic acid

Each value is expressed as mean \pm S.D. (n=3).

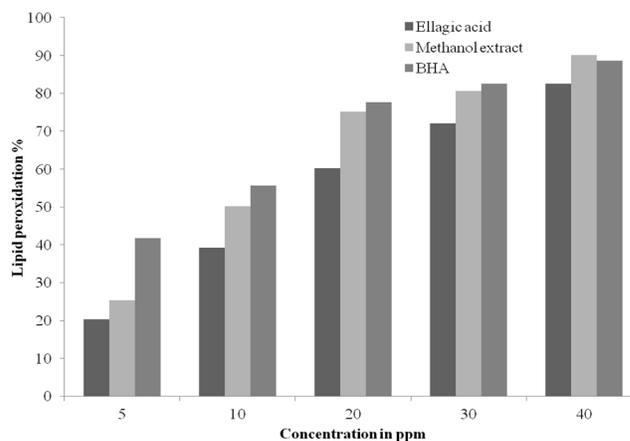


Figure 4: Metal ion chelating activity of Ellagic acid

Each value is expressed as mean \pm S.D. (n=3).

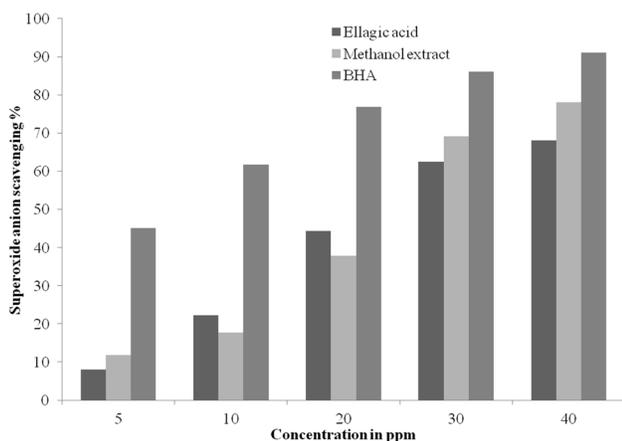


Figure 5: Superoxide anion scavenging activity of Ellagic acid

Each value is expressed as mean \pm S.D. (n=3).

naturally occurring process happening in small amounts in the body. It mainly occurs by the effect of several reactive oxygen species. These reactive oxygen species act by readily attacking the polyunsaturated fatty acids in the fatty acid membrane. This initiates a self-propagating chain reaction. The destruction of membrane lipids coupled with the end products of such lipid peroxidation reactions are especially dangerous for the viability of cells and tissues (Figure 6).

The methanol extract was evaluated for the concentration of total phenolics, total flavonoids and flavonols. The content of total phenolics was found to be 59.5 mg GAE/g extract. The flavonoid and flavonol content was found to be 27.5 mg and 13.2 mg GAE/g extract respectively.

The results of the present investigation clearly indicate that the isolated compound is most potent against HIV-1 Integrase with an IC_{50} value of 8.7 μ m/ml and against HIV-1 Protease

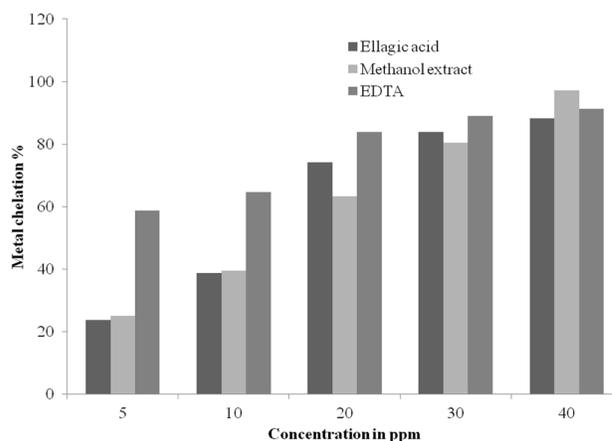


Figure 6; Lipid peroxidation inhibition activity of Ellagic acid

Each value is expressed as mean \pm S.D. (n=3).

with an IC_{50} value of 70.4 μ m/ml respectively (Figure 1 and Figure 2). In our previous study, the anti-HIV-1 activity of several Indian medicinal plants has been reported,³¹ but the active fractions were not characterized. In this regard, the present study was designed to isolate the most potent active component from the numerous plants which we screened for anti-HIV-1 activity.

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CONFLICT OF INTEREST

There is no conflict of interest of any regard.

ABBREVIATIONS

NMR: Nuclear Magnetic Resonance
 LC-MS: Liquid Chromatography Mass Spectroscopy

FTIR: Fourier Transform Infrared Spectroscopy
 EDTA: Ethylene Diamine Tetra Acetic acid
 NBT: Nitroblue Tetrazolium

Highlights of the paper

- Potential bioactive molecule Ellagic acid isolated for the first time from *Terminalia paniculata*.
- First report of In vitro Anti-HIV-1 activity from *T. paniculata*. IC₅₀
- The isolated molecule as well as all the solvent extracts of *T. paniculata* have shown excellent antioxidant activity in the *In vitro* models tested.

About Authors



Prof Ravishankar Rai V: Has received his M. Sc and Ph. D from University of Mysore, India.

Currently Dr. Rai is working as Professor and Chairman at Department of Studies in Microbiology, University of Mysore, Mysore, India. He has been awarded fellowship of UNECSO Biotechnology Action Council, Paris (1996), Indo- Israel Cultural Exchange Fellowship (1998) Biotechnology Oversea fellowship, Government of India (2008) Indo-Hungarian Exchange fellowship (2011). He has been invited by Academia Sinica, Taiwan as Visiting Fellow (2010). Indian National Science Academy Bilateral Exchange Program fellowship has been awarded to Dr Rai to visit Turkey during

2015. Recently Dr Rai has edited a book on Biotechnology: Concepts and Applications published by Alpha Science International, Oxford, UK. and he edited three more books for CRC Press and Wiley Publication. Dr. Rai has received funding for research from various agencies including DBT, DST, UGC, DRDO, ICMR and NMPB, New Delhi His interests include: Food Biotechnology, Bacterial Quorum Sensing, Microbiological corrosion, Bioprospecting of Medicinal plants, Forest diseases and Management. Presently he is the coordinator for innovative Program on Food Quality and Safety, funded by University Grants Commission and DST PURSE, New Delhi.



Chaitra Narayan: Is a doctoral student at the Department of Studies in Microbiology, University of Mysore, India. She has been working on Bioactive molecule characterization and therapeutic activities of Indian medicinal plants with special emphasis on In vitro Anti-HIV-1 activity of medicinal plants.

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