Anti HIV-1 activity, anti bacterial activity and phytochemical analysis of leaf extracts from *Cleistanthus collinus* (Roxb.) Benth. ex Hook.f.

Remya Mohanraja, Shoaib Haidara, Malcolm Nobrea & Anantan Rb

aDepartment of Medical Biotechnology, MGM Institute of Health Sciences, Sector-18, Kamothe, Navi Mumbai-410 209, Maharashtra, India; bFood Chemistry Division, National Institute of Nutrition, Hyderabad-500 007, Andhra Pradesh, India

E-mail: remyam@gmail.com

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This study is an attempt to identify new chemical entities with anti HIV1 activity. *In vitro* anti-HIV activity of *Cleistanthus collinus* extracts was evaluated using the p24 antigen assay. A dose-dependent inhibition of the p24 antigen expression was observed and the extract was found to be efficient against HIV-1. Antibacterial activity was tested against four different bacteria employing the agar well diffusion method and the highest activity was exhibited by the chloroform and methanolic extracts of *C. collinus*. HPLC analysis of the crude extract revealed the presence of phytochemicals like Gallic acid, Chlorogenic acid, 3,4 Dihydroxy B-acid, Diadizin, p-Coumaric acid, Epi-GC Gallate, Ellagic acid, Luteoline, Hesperitin and Quercetin. This report on the anti HIV 1 activity of *C. collinus* and the results obtained from the present study suggest that the extracts could serve as a source for providing potential lead compound for drug discovery against HIV-1.

**Keywords:** *Cleistanthus collinus*, Anti- HIV 1, p24 antigen expression, HPLC, Antibacterial activity, Phytochemicals, Lymphocye culture


The production of drugs for HIV-1 poses great challenges due to the ability of the virus to mutate itself against any kind of inhibitor. In addition, the toxicity of currently available anti-HIV drugs is a hindrance to maintaining patients' adherence to antiretroviral therapy. The inevitable emergence of multi-drug resistant mutants in response to antiretroviral therapies only makes the challenge harder. The rates of success of HAART (highly active antiretroviral therapy) are predicted to decrease gradually with the increase in the emergence of drug-resistant strains, making the continuous development of novel anti-HIV agents a necessity.

Screening anti-HIV agents from natural products may be an effective way for drug discovery. The plant kingdom harbors a rich source of active ingredients that present the advantage of being combined with many other substances giving the plant as a whole, safety, efficiency and much superiority as compared to that of its isolated and pure active components. A variety of natural products, such as ribosome inactivating proteins, alkaloids, flavonoids, lignans, and so on, have been found to inhibit unique enzymes and proteins crucial to the life cycle of HIV, including the reverse transcription process, virus entry, the integrase or protease.

*Cleistanthus collinus* (Roxb.) Benth. Hook f. (Family: Euphorbiaceae) is a tropical plant growing on dry hills in various parts of India. The plant is a rich source of many bioactive compounds. All parts of the plant are astringent, and the alcoholic extracts of leaves, roots and fruits of this plant, are traditionally used as washing agent for clearing septic wound and cure fungal diseases. The stem bark is used in skin diseases, as an antiseptic and in the treatment of hoof sores of cattle. Leaves exhibit antifungal activity.

With this background and abundant source of unique active components harbored in plants, this study was taken up to determine the anti HIV-1...
activity and phytochemical constituents of the crude leaf extracts of C. collinus.

Methodology

Plant material
Leaves of the selected species, C. collinus were collected from Nammakal, Tamil Nadu, India. The plant material was identified by Dr Priti V, Agricultural University, Bangalore, India. To avoid loss, some of the plants were grown in the departmental garden. Leaves were washed and dried under shade. Dried leaf samples were ground into a uniform powder using a blender and stored in polythene bags at room temperature.

Preparation of extracts

Aqueous extraction
Ten gram of dried plant powder was left in distilled water for 6 h at slow heat. After 12 h it was filtered through Whatman No. 1 filter paper and centrifuged at 5000 g for 15 min. The supernatant was collected and concentrated to make the final volume, one-fifth of the original volume. The extract was then autoclaved at 121 °C and 15 lbs pressure, and stored at 4 °C.

Solvent extraction
Ten gram of the dried plant material was extracted with 100 mL of methanol and was kept on a magnetic stirrer (Remi) for 24 h. Thereafter, it was filtered and centrifuged at 5000 g for 15 min. The supernatant was collected and the solvent was evaporated to make the final volume one-fifth of the original volume. It was stored at 4 °C in airtight bottles for further studies. The same procedure was repeated for obtaining ethyl acetate, and chloroform extracts.

Anti HIV-1 Activity

Isolation and culture of lymphocytes
Isolation and culture of lymphocytes was performed by a modification of the method described by Kulkarni et al.9. 5 mL of blood was collected from a HIV positive individual (confirmed by sandwich ELISA using HIV-Check (Xcyton, India), and also from an uninfected individual based on the procedure described by Hahn et al.10 in EDTA vacutainers after obtaining informed consent. It was double diluted with RPMI tissue culture media, layered on 3 mL Ficoll-hypaque (Sigma, USA), and centrifuged at 1500 rpm for 15 min. The lymphocyte ring obtained was collected in a separate NUNC sterile tube containing 13 mL RPMI. The contents of the tube were mixed well and centrifuged at 1000 rpm for 5min. The supernatant was discarded and the pellet was dissolved in 1 mL RPMIC (RPMI containing 20 % FCS). The cell viability was checked using Trypan blue method. Phytohaemagglutinin (PHA) was added at a concentration of 1 µL per 1 million cells. 500 µL of the culture was added to each well of a 24 well NUNC plate. The culture was incubated at 37 °C in a 5 % CO2 incubator for 24 h.

Addition of plant extract
After 24 h 1 µL of IL-2 (1:100 in RPMIC) was added to each well along with 1 µL of different dilutions (2, 3, 4 & 5 mg/mL) of the aqueous extract of C. collinus. One of the wells in which the standard drug AZT was added was used as the positive control. The plates were incubated at 37 °C in a 5 % CO2 incubator for 72 h. Different dilutions of the extracts were also added to wells with healthy lymphocytes and incubated at 37 °C in a 5 % CO2 incubator for 72 h. Cytotoxicity assay was carried out using Trypan blue dye11.

Measurement of p24 antigen expression
The effect of the crude extracts of C. collinus on HIV-1 replication in vitro was tested by viral core protein p24 expression using a commercial kit (Vironistika HIV-1 Antigen Micro Elisa system, Germany) as described previously by Gary et al.12. HIV infected cell cultures containing the HIV-1 antigen was incubated in Micro Elisa strips whose wells were coated with antibodies (murine monoclonal) to HIV-1 p24 core antigen. Following incubation, the specimen was aspirated and the wells were washed with phosphate buffer. Subsequently, anti-HIV-1 conjugate (Antibody to HIV-1 (human) coupled to horse radish peroxidase) was added. The labeled antibodies bind to the previously formed solid phase antibody/antigen complexes. Following wash and incubation with TMB (tetramethylbenzidine) substrate, a blue colour was developed which turned yellow and the reaction was stopped with sulfuric acid. Optical density (OD) was measured using the ELISA reader. Percentage of p24 antigen inhibition was calculated by comparing OD of the treated wells with the control well. The results were presented as the average and standard error of triplicate experiments. The statistical significance was checked at p < 0.5.

Antibacterial activity
A modification of the well diffusion assay protocol given by Juliani et al.13, was used to screen the extract for antimicrobial activity against Salmonella typhi.
(MTCC 734), Salmonella paratyphi A (MTCC 3220), Vibrio cholera (MTCC 3904) and Klebsiella pneumoniae (MTCC 109). Nutrient agar plates were swabbed with the respective broth culture of the organisms and kept for 15 min for absorption to take place. Wells were made in agar plates using the broad end of a sterile Pasteur pipette (5 mm diameter). Then, 10 μL of the crude extract in methanol, ethyl acetate, chloroform and hot water, at concentrations 2, 3, 4 and 5 mg/mL were added to each well. A mixture of penicillin and streptomycin (1:1) was used as positive control. The various solvents with which dilutions were made, were used as negative controls. Plates were incubated at 37 ºC for 24 h and the diameters of the inhibition zones were measured in millimeter after the incubation period.

Phytochemical analysis by HPLC

Standards and chemicals
HPLC grade methanol, other chemicals (acetone, ethanol, benzene, petroleum ether) and authentic polyphenol standards were purchased from Himedia Laboratories (Mumbai, India).

Sample preparation and HPLC Analysis
Powdered leaf was used for HPLC analysis. The samples were then heated under reflux for 1 h with 6 mL of 25 % hydrochloric acid and 20 mL MeOH, the hydrolysate was diluted with methanol to 100 mL and filtered. (PTFE Syringe filter, Whatman, UK). 1 mL of this solution was injected for HPLC analysis. Analysis was performed after three separate extraction of each sample and each extract was diluted and injected in triplicate. Polyphenols in the samples was identified by comparison of their retention times with the standard compounds.

Chromatographic equipment and condition
The chromatographic analyses were performed on a 250 mm × 4.6 mm i.d., C18 (ODS), Shimadzu, Japan with 0.5 % aqueous solution of Orthophosphoric acid and Methanol (HPLC Grade) as mobile phase at a flow rate of 1 mL min⁻¹. The HPLC equipment comprised Hewlett-Packard (HP) 1050 Chem Station Software, an HP model 35900 interface unit, an HP 9000 Series 300 computer, and an HP Desk Jet 500 Printer. A Waters 486 tunable absorbance detector was operated at 254 nm; detector sensitivity was 0.05 AUFS and the column oven temperature was 30°C. Determinations were performed after three separate extractions of each sample, and each extract was injected in triplicate.

Results and discussion
Due to continuous emergence of drug resistance and side-effects of currently available drugs, more and more efforts have been spent on searching for more effective anti-HIV drugs. As has always been the case in the search for cures, natural sources offer great promise14. Herbal medicines provide rational means for the treatment of many diseases that are obstinate and incurable in western systems of medicine. In the past decade, there has been a sustained bioprospective effort to isolate the active leads from plants and other natural products for preventing transmission of HIV and management of AIDS15. It has been reported that the viral load of some HIV patients had gone down to undetectable levels after taking plant mixtures16. Medicinal plants and other natural products including mushrooms are used as primary treatment for HIV-related problems such as skin disorders, nausea, depression, insomnia, and body weakness17.

In the present study, it was observed that the crude aqueous extract from the leaves of C. collinus showed potent anti HIV 1 activity suggesting that it could serve as a source for providing potential lead compound for drug discovery. Also, the extracts were not cytotoxic at the concentrations used for the current study. The percentage of inhibition of the p24 antigen expression was dose dependent and increased with increasing concentration of the extract (Table 1). 50 % p24 antigen inhibition was seen at approximately 20 mg/mL concentration.

Screening of bioactive agents from plants is one of the most intensive areas of natural product research today. Findings from such studies lead to the discovery of pharmacologically active compounds aiding in drug discovery18. Perusal of literature revealed that most of the promising naturally derived anti-HIV compounds are flavonoids, terpenoids, alkaloids, polysaccharides or proteins4. HPLC

<table>
<thead>
<tr>
<th>Concentration of leaf extract</th>
<th>Percent (%) Inhibition (mean ± SE)</th>
<th>p values</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mg/mL</td>
<td>08.00 ± 1.3</td>
<td>0.046</td>
</tr>
<tr>
<td>10 mg/mL</td>
<td>18.17 ± 1.2</td>
<td>0.038</td>
</tr>
<tr>
<td>20 mg/mL</td>
<td>54.63 ± 1.3</td>
<td>0.042</td>
</tr>
<tr>
<td>40 mg/mL</td>
<td>73.80 ± 1.7</td>
<td>0.044</td>
</tr>
<tr>
<td>Control</td>
<td>NIL</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1 — Effect of leaf extracts of C. collinus on the inhibition of p24 antigen expression

Result is an average of 3 replicates ± SE; *p < 0.5
analysis of the leaf powder of *C. collinus* revealed the presence of polyphenols like, Gallic acid, Chlorogenic acid, 3,4Dihydroxy B-acid, Diadizin, p-Coumaric acid, Epi-GC Gallate, Diadizin, Ellagic acid, Luteoline, Hesperitin and Quercetin (Table 2). The chromatogram obtained is shown in Fig. 1. It has already been reported that gallic acid is an inhibitor to HIV integrase\(^{19}\). Also, quercitin is a known antiviral agent\(^{20}\). Moradi *et al.* reported that the antiviral activity of medicinal plants is related to their total phenolic content\(^{21}\). It could, therefore, be concluded that the presence of these compounds could have lead to the anti HIV I activity exhibited by *C. collinus*.

The U.S. Food and Drug Administration (FDA) has approved a number of anti-HIV drugs for clinical use\(^{22}\). However, these medications have limitations such as high cost, peripheral neuropathy and decreased sensitivity due to the rapid emergence of drug-resistant mutant virus strains, and adverse effects like bone marrow suppression and anaemia\(^{23,24}\). Activity shown by the extracts of *C. collinus* against HIV-1 suggests that it could serve as a good source for the effective discovery of anti-HIV agents with decreased toxicity.

The use of antibiotics is thought to influence the prevalence of resistance in bacteria and to be a risk factor for the emergence of antibiotic resistance in human pathogens. That is the reason why screening of bioactive agents from plants is one of the most intensive areas of natural product research today. The presence of antibacterial substances in the higher plants is well established\(^{25}\). They can be used in the treatment of infectious diseases caused by microbes.

The inhibitory effects of the solvent extracts of *C. collinus* leaf against four different pathogenic bacterial strains were examined in the present study. The effects of different extracts on the bacteria tested are shown in Table 3. The maximum zone of inhibition

### Table 2 — Phytochemical constituents from the leaf extracts of *C. collinus*

<table>
<thead>
<tr>
<th>Name</th>
<th>Retention Time(min)</th>
<th>Lambda Max.</th>
<th>Area(μVsec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid</td>
<td>0.721</td>
<td>261.6</td>
<td>108110</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>2.889</td>
<td>327.5</td>
<td>2805669</td>
</tr>
<tr>
<td>3,4 Dihydroxy B-acid</td>
<td>2.889</td>
<td>327.5</td>
<td>1349200</td>
</tr>
<tr>
<td>Diadizin</td>
<td>2.889</td>
<td>327.5</td>
<td>1349200</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>3.808</td>
<td>326.9</td>
<td>808581</td>
</tr>
<tr>
<td>Epi_GCGallate</td>
<td>3.808</td>
<td>326.9</td>
<td>367894</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>10.068</td>
<td>203.6</td>
<td>506287</td>
</tr>
<tr>
<td>Luteoline</td>
<td>12.315</td>
<td>204.2</td>
<td>759038</td>
</tr>
<tr>
<td>Hesperitin</td>
<td>12.315</td>
<td>204.2</td>
<td>532039</td>
</tr>
<tr>
<td>Quercetin</td>
<td>12.315</td>
<td>204.2</td>
<td>1019114</td>
</tr>
</tbody>
</table>

![Fig 1 — Chromatogram showing Phytochemical constituents from the leaf extracts of *C. collinus*](image)

### Table 3 — Antimicrobial activity of the leaf extracts of *C. collinus*

<table>
<thead>
<tr>
<th>Concentration of Extract</th>
<th>S. typhi Zone of inhibition (mm) observed against the pathogenic bacteria under study</th>
<th>S. paratyphi A Zone of inhibition (mm) observed against the pathogenic bacteria under study</th>
<th>K. pneumonia Zone of inhibition (mm) observed against the pathogenic bacteria under study</th>
<th>V. cholerae Zone of inhibition (mm) observed against the pathogenic bacteria under study</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>13± 18± 19± 11+</td>
<td>10± 11± 11± 10±</td>
<td>E</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>1.3* 1.5* 1.8* 1.5*</td>
<td>1.1* 1.6* 2.2* 1.4*</td>
<td>1.6* 1.5* 1.4* 1.1*</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 mg/mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 mg/mL</td>
<td>09± 13± 17± 09+</td>
<td>08± 09± 09± 07±</td>
<td>0 0 0 0</td>
<td></td>
</tr>
<tr>
<td>40 mg/mL</td>
<td>09± 12± 12± 08±</td>
<td>08± 0 07±</td>
<td>0 0 0 0</td>
<td></td>
</tr>
<tr>
<td>20 mg/mL</td>
<td>11± 11± 08±</td>
<td>07± 0 0</td>
<td>0 0 0 0</td>
<td></td>
</tr>
<tr>
<td>Antibiotic</td>
<td>24± 24± 24±</td>
<td>21± 21± 21±</td>
<td>24± 24± 24±</td>
<td></td>
</tr>
<tr>
<td>Solvent</td>
<td>0 0 0</td>
<td>0 0 0</td>
<td>0 0 0</td>
<td></td>
</tr>
</tbody>
</table>

Result is an average of 3 replicates ± SE; *p < 0.5

E - Ethyl acetate extract; H – Hotwater extract; C- Chloroform extract; M – Methanol extract
against *S. typhi* and *S. paratyphi* was exhibited by choloroform extracts of *C. collinus*. Maximum zone of inhibition against *V. cholera* and *K. pneumoniae* was observed using the methanolic extracts of *C. collinus*. The zone of inhibition in all the cases increased with an increase in concentration.

An earlier study has reported that acetone extracts of *C. collinus*, showed the best activity against, *E. coli*, *K. pneumoniae*, *S. aureus* and *V. cholerae*. In contrast to the earlier findings, the present study reveals that the chloroform and methanol extracts of *C. collinus* are the most effective ones. This could be because of the fact that successive isolation of botanical compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. The reputation of *C. collinus* as a remedy for different microbial diseases traditionally including its use as an antiseptic, is supported by the antibacterial screening.

**Conclusion**

The present study reports for the first time a direct inhibitory effect of extracts of *C. collinus*, on HIV-1 replication. Plant- based traditional medical knowledge systems which have been developed and adapted over many generations may constitute a big advantage in the development of new anti-HIV therapeutics. The present study is an example where traditional medical knowledge led to the identification of an extract, which might potentially be useful in the treatment/management of HIV/AIDS. It is our strong conviction that the results obtained in the present study will inspire and motivate even more researchers to look for new leads from plants and other natural sources.

**References**

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