Anti HIV-1 and antimicrobial activity of the leaf extracts of *Calotropis procera*

Remya Mohanraj, Jyotirmoy Rakshit, Malcolm Nobre
Department of Medical Biotechnology, MGM University of Health Sciences, Kamothe, Navi Mumbai, Maharashtra, India

Calotropis procera R.Br. (Asclepiadiaceae) is an important medicinal plant whose leaves and roots have multiple uses. The plant is a rich source of many bioactive compounds which are of medicinal importance. The study was undertaken to investigate the anti HIV-1 and antibacterial activities of the crude leaf extracts of *C. procera*. Hot water extract was used for evaluating the efficacy against the growth of HIV. Inhibition of p24 antigen's expression was used as the method for the study. Antibacterial activity was tested against four different bacteria using agar well diffusion method. Zones of inhibition were measured with different concentrations of the extracts and some of them gave better values than the antibiotics used. The results were presented as the average and standard error of triplicate experiments. The statistical significance was checked at $P<0.5$. A dose-dependent inhibition of the p24 antigen expression was observed and the extract was found to be efficient against HIV-1. Ethyl acetate extract was effective against all the bacteria tested. The results observed support ancient and traditional medicinal values of *C. procera*.

Key words: Anti HIV-1 activity, antibacterial activity, *Calotropis*

INTRODUCTION

Nature has been a source of medicinal agents for centuries. The importance of herbs in the management of human ailments cannot be overemphasised. It is clear that the plant kingdom harbours an inexhaustible source of active ingredients valuable in the management of many intractable diseases. Furthermore, the active components of herbal remedies have the advantage of being combined with many other substances that appear to be inactive. However, these complementary components give the plant as a whole, safety and efficiency, much superior to that of its isolated and pure active components.[1]

Antibiotic resistance has become a global concern.[2] There has been an increasing incidence of multiple resistances in human pathogenic microorganisms in recent years, largely due to indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases. This has forced scientists to search for new antimicrobial substances from various sources like the medicinal plants. Search for new antibacterial agents should be continued by screening many plant families. Recent work revealed the potential of several herbs as sources of drugs.[3] The screening of plant extracts and plant products for antimicrobial activity has shown that higher plants represent a potential source of novel antibiotic prototypes.[4] Numerous studies have identified compounds within herbal plants, which are effective antibiotics.[5] Traditional healing systems around the world that utilise herbal remedies are an important source for the discovery of new antibiotics;[6] some traditional remedies have already produced compounds that are effective against antibiotic-resistant strains of bacteria.[7] The results of this study indicate the need for further research into traditional health systems.[8] It also facilitates pharmacological studies leading to synthesis of a more potent drug with reduced toxicity.[9]

The production of drugs for HIV-1 is quite complicated because of the capability of the virus to mutate itself against any kind of inhibitor. Also, the toxicity of currently available anti-HIV drugs makes it difficult to maintain patients' adherence to antiretroviral therapy.[10] The inevitable emergence of drug-resistant mutants, especially multi-drug resistant mutants, in response to antiretroviral therapies makes things worse.[11] 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. Therefore, continuous development of novel anti-HIV agents is necessary. 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.[12] Screening anti-HIV agents from natural products may be a more effective way for drug discovery.

Address for correspondence: Dr. Remya Mohanraj, Department of Medical Biotechnology, MGM University of Health Sciences, Kamothe, Navi Mumbai, Maharashtra, India. E-mail: remyam@gmail.com
Received: 22-11-2009; **Accepted:** 03-07-2010; DOI: 10.4103/0973-8258.74132

Of the large number of plant species reported in the ethnobotanical interest, two species of *Calotropis*, *Calotropis procera* and *Calotropis gigantean*, in India, hold a pride of place largely because of their other uses and economic values. *C. procera* has bitter, healing, laxative and anthelmintic properties and it relieves strangury, cures ulcers and acts as an expectorant. The leaves are used to relieve stomach pain, flower is a tonic, appetiser, stomachic, cures piles, asthma and wounds. Its milky juice is a blistering agent. Its flowers are useful in cholera.[13] In Indian traditional medicine, the latex of this plant has been used for the treatment of skin diseases, rheumatism, and aches.[14] It has been reported to exhibit potent anti-inflammatory, analgesic, and weak antipyretic activities when administered orally.[15-18] With this background and abundant source of unique active components harboured in plants, this study was taken up to determine the anti HIV-1 and antibacterial properties of the crude leaf extracts of *C. procera*.

MATERIALS AND METHODS

Plant Material

Leaf of the selected species, viz. *C. procera*, was collected from Navi Mumbai, Maharashtra, India. They were identified by Dr. Priti V, Agricultural University, Bangalore, India, and a voucher specimen of the same has been deposited in the institutional herbarium. 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 grams of the dried plant powder was left in distilled water for 6 hours at slow heat (35°C). After 12 hours, it was filtered through Whatman No.1 filter paper and centrifuged (Remi, India) at 5000 *g* for 15 minutes. 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 grams of the dried plant material was extracted with 100 ml of methanol and was kept on a magnetic stirrer (Remi) for 24 hours. Thereafter, it was filtered and centrifuged at 5000 *g* for 15 minutes. 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, methanol and chloroform extracts.

Antibacterial activity

A modification of the well diffusion assay protocol[19] was used to screen the extract for antimicrobial activity against *Salmonella typhi* (MTCC 734), *Salmonella paratyphi* A (MTCC 3220), *Vibrio cholerae* (MTCC 3904) and *Klebsiella pneumoniae* (MTCC 109). Nutrient agar plates were swabbed with the respective broth culture of the organisms and kept for 15 minutes 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 in which dilutions were made, were used as negative controls. Plates were incubated at 37°C for 24 hours and the diameters of the inhibition zones were measured in millimeter after the incubation period.

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*.[20] In brief, 5 ml of blood was collected from a HIV positive individual [confirmed by sandwich enzyme-linked immunosorbent assay (ELISA) using the commercial kit HIV-Check (Xcyton, India)], and also from HIV uninfected individual based on the procedure described by Hahn *et al*.,[21] in ethylenediaminetetraacetic acid (EDTA) vacutainers, after taking their 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 minutes. The lymphocyte ring obtained was collected in a separated NUNC sterile tube containing 13 ml RPMI. The contents of the tube were mixed well and centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and the pellet was dissolved in 1 ml RPMIC (RPMI containing 20% FCS). T he cell viability was checked using Trypan blue method.[22] Phytohaemagglutinin (PHA) was added at a concentration of 1 µl per 1 million cells. Then, 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% CO_2 incubator for 24 hours.

Addition of different dilutions of crude extract

After 24 hours, 1 µl of IL-2 (1:100 dilutions in RPMIC) was added to each well. Different dilutions (2, 3, 4 and 5 mg/ml) of the hot water extract of *C. procera* were made and 1 µl of each dilution was added to each well. One of the wells in which no extract was added was used as the control. The plates were incubated at 37°C in a 5% CO_2 incubator for 72 hours. Different dilutions of the extracts were also added to wells with healthy lymphocytes and incubated at 37°C in a 5% CO_2 incubator for 72 hours. Cytotoxicity assay using Trypan blue dye exclusion assay[22] was carried out in the cells from the above culture.

Measurement of p24 antigen expression

The effect of the crude extracts of *C. procera* on HIV-1 replication *in vitro* was tested by viral core protein p-24

expression using a commercial kit (Vironistika HIV-1 Antigen Micro Elisa system, Germany) as described previously by Gary *et al*.[23] HIV infected cell cultures containing the HIV-1 antigen were 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 labelled antibodies bind to the solid phase antibody/antigen complexes previously formed. Following wash and incubation with tetramethylbenzidine (TMB) substrate, a blue colour was developed which turned yellow and the reaction was stopped with sulphuric acid. Optical density was measured using the ELISA reader. Percentage of p24 antigen inhibition was calculated by comparing optical density (OD) of the treated wells with the control well. The experiments were repeated thrice.

RESULTS

It was observed that the crude aqueous extract from the leaves of *C. procera* showed potent anti HIV-1 activity. 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].

The inhibitory effects of the solvent extracts of *C. procera* leaf against four different pathogenic bacterial strains were examined. Among all the solvent extracts, the ethyl acetate extract showed the best inhibitory activity. The effects of different extracts on the bacteria tested are shown in Table 2. The maximum zone of inhibition was obtained against *Sa. typhi* and *Sa. paratyphi* A with 5 mg/ml of ethyl acetate extract. The zone of inhibition in all the cases increased with an increase in concentration.

DISCUSSION

The urgent need for new anti-HIV/AIDS drugs is a global concern. In addition to obvious economical and commercial hurdles, HIV/AIDS patients are faced with multifarious difficulties associated with the currently approved anti-HIV drugs.

Table 1: Effect of the leaf extracts of *Calotropis procera* on the inhibition of p24 antigen expression

Concentration of leaf extract	Percent (%) inhibition (mean±S.E)	*P* values
2 mg/ml	8±1.3	0.046
3 mg/ml	18.17±1.2	0.038
4 mg/ml	28.84±1.2	0.042
5 mg/ml	60±1.3	0.044
Control	NIL	–

n=3

Table 2: Antibacterial activity of the leaf extracts of C. procera

Concentration of extract (mg/ml)	Zone of inhibition (mm) observed against the pathogenic bacteria under study															
	Sa. typhi				*Sa. paratyphi A*				*K. pneumoniae*				*V. chloreae*			
	Ethyl acetate extract	Hot water extract	Chloro-form extract	Metha nol extract	Ethyl acetate extract	Hot water extract	Chloro form extract	Methanol extract	Ethyl acetate extract	Hot water extract	Chloro-form extract	Methanol extract	Ethyl acetate extract	Hot water extract	Chloroform extract	Methanol extract
5	22±1.3*	17±1.5*	12±1.8*	11±1.5*	22±1.1*	17±1.6*	11±2.2*	10±1.4*	0	0	0	17±1.6*	9±1.5*	10±1.6*	13±1.4*	12±1.1*
4	20±1.6*	0	11±1.1*	10±2.0*	20±1.3*	0	12±2.0*	11±1.7*	0	0	0	15±2.1*	7±1.4*	9±1.1*	12±1.5*	10±1.4*
3	19±1.8*	0	10±1.2*	0	19±1.2*	0	10±1.8*	0	0	0	0	15±2.0*	0	7±1.3*	10±1.2*	09±1.6*
2	17±1.2*	0	0	0	15±1.9*	0	0	0	0	0	0	13±1.8*	0	7±2.0*	7±1.3*	0
Antibiotic	24±1.4*	24±1.4*	24±1.3*	24±1.4*	21±1.5*	21±1.3*	21±1.5*	21±1.4*	24±1.3*	24±1.5*	24±1.7*	24±1.7*	22±1.5*	22±1.6*	22±1.4*	22±1.6*
Solvent	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Result is an average of three replicates±SE; *$P<0.5$

At present, due to continuous emergence of drug resistance and side effects of currently available drugs, more and more efforts have been made on searching for more effective anti-HIV drugs. The narrow spectrum of activity has limited the therapeutic usefulness of the various reverse transcriptase and protease inhibitors that are currently available on the market. This has given rise to the necessity to look for new anti-retrovirals with better efficacy, safety and affordability. As has always been the case in the search for cures, natural sources offer great promise. Medicinal plants are chemically complex and diverse. Botanical compounds provide a wide spectrum of biological and pharmacological properties.[24]

Perusal of the literature has revealed that most of these promising naturally derived anti-HIV compounds are flavonoids, terpenoids, alkaloids, polysaccharides or proteins.[12]

Triterpenoids, anthocyanins, a yellow bitter acid, resins and the cardiacglycosides, calotropogenin,[25] calotropin,[26] uscharin, calotoxin and calactin,[27] are found in *C. procera.* Also, phytochemical screening of the crude extract revealed the presence of saponin, tannins, sequiterpene and alkaloids.[28] This leads to a conclusion that the presence of these compounds could have lead to the anti HIV-1 activity of *C. procera.* The U.S. Food and Drug Administration (FDA) has approved a number of anti-HIV drugs for clinical use.[29] 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.[30,31] Activity shown by the extracts of *C. procera* 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.[32] They can be used in the treatment of infectious diseases caused by microbes.

An earlier study had reported that crude methanol extract of *C. procera* showed antibacterial activity against *Staphylococcus aureus, Staphylococcus epidermidis, Plesiomonas shigelloides, Shigella dysenteriae,* and *Vibrio cholerae*. On the other hand, aqueous extract showed antibacterial activity against *St. aureus, St. epidermidis, Staphylococcus saprophyticus, Streptococcus pyogenes P. shigelloides, Sh. dysenteriae, V. cholerae, Shigella Flexner, Shigella sonnei* and *Pseudomonas aeruginosa.* Both the extracts did not show any activities against *Sa. typhi and Shigella boydi*.[33] In contrast, the present study reveals that the crude ethyl acetate extract of *C. procera* showed the best antibacterial activity against *Sa. typhi* and *Sa. paratyphi* A. Also, the choloroform extracts showed a better activity against *V. cholerae* than the methanolic and hotwater extracts. 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 presence of glycoside, calotropine, a powerful bacteriolytic agent in the latex of *C. procera*[34] could be attributed to the potent antimicrobial activities of *C. procera*. The reputation of *C. procera* as a remedy for different microbial diseases traditionally including diarrhoea and dysentery is supported by the antibacterial screening. Also, fluoroquinolone resistance in *Sa. typhi* and *Sa. paratyphi* A and tetracycline resistance in *V. cholerae* are being increasingly reported.[35,36] The efficacy of *C. procera* extract against these pathogens suggests that it could serve as a source for providing potential lead compound for drug discovery.

The traditional healers use primarily water as the solvent but we found in this study that the plant extracts by methanol, ethyl acetate and chloroform provided more consistent antimicrobial activity compared to those extracted by water.

CONCLUSIONS

The results obtained in the present study give credential to the use of *C. procera* in traditional medicine. It is our strong conviction that these results will inspire and motivate even more researchers to look for new leads from plants and other natural sources.

ACKNOWLEDGMENT

We thank MGM University of health Sciences for the laboratory support.

REFERENCES

1. Shariff ZU. Modern herbal therapy for common ailments. Nature Pharamcy Ser 2001;1:9-84.
2. Afolayan AJ. Extracts from the shoots of *Arctotis artotoides* inhibit the growth of bacteria and fungi. Pharm Biol 2003;41:22-5.
3. Iwu MM. Ethnomedicine and drug discovery. In: Iwu MM, Wootton JC, editors. Threapeutic Agents from Ehtnomedicine. Amsterdam: Elsevier Science; 2002. p. 309-20.
4. Westh H, Zinn CS, Rosdahl VT. An international multicenter study of antimicrobial consumption and resistance in *Staphylococcus aureus* isolates from 15 hospitals in 14 countries. Microb Drug Resist 2004;10:169-76.
5. Basile A, Sorbo S, Giordano S, Ricciardi L, Ferrara S, Montesano D, *et al*. Antibacterial and allelopathic activity of extract from *Castanea sativa* leaves. Fitoterapia 2000;71:110-6.
6. Okpekon T, Yolou S, Gleye C, Roblot F, Loiseau P, Bories C, *et al*. Antiparasitic activities of medicinal plants used in Ivory coast. J Ethnopharmacol 2004;90:91-7.

7. Kone WM, Kamanzi Atindehou K, Terreaux C, Hostettmann K, Traore D, Dosso M. Traditional medicine in North Cote-d'Ivoire screening of 50 medicinal plants for antibacterial activity. J Ethnopharmacol 2004;93:43-9.
8. Romero CD, Chopin SF, Buck G, Martinez E, Garcia M, Bixby L. Antibacterial properties of common herbal remedies of the southwest. J Ethnopharmacol 2005;99:253-7.
9. Ebana RU, Madunagu BE, Ekpe ED, Otung IN. Microbiological exploitation of cardiac glycoside and alkaloids from *Garcinia kola*, *Borreria ocymoides, Kola nitida* and *Citrus aurantifolia*. J Appl Bacteriol 1991;71:398-401.
10. Sliciano JD, Kajdas J, Finzi D, Quinn TC, Chadwick K, Margolick JB, *et al*. Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting CD4 T cells. Nat Med 2003;9:727-8.
11. Grant RM, Hecht FM, Warmerdam M, Liu L, Liegler T, Petropoulos CJ, *et al*. Time trends in primary HIV-1 drug resistance among recently infected persons. J Am Med Assoc 2002;288:181-8.
12. Cos P, Maes L, Vanden Berghe D, Hermans N, Pieters L, Vlietinck A. Plant substances as anti-HIV agents selected according to their putative mechanism of action. J Nat Prod 2004;67:284-93.
13. Bors W, Saran M, Elstner EF. Screening for plant antioxi-dants. In: Linskens HF, Jackson JF, editors. Modern methods of plant analysis. New Series, Vol. 13. Berlin, Heidelberg, New York: Springer-Verlag; 1992. p. 277-95.
14. Anonymous. *Wealth of India: Raw materials III.* New Delhi (India): CSIR Publication and Information Directorate; 1992.
15. Kumar VL, Basu N. Anti-inflammatory activity of the latex of *Calotropis procera*. *J Ethnopharmacol 1994;***44**:123-5.
16. Sangraula H, Dewan S, Kumar VL. Evaluation of anti-inflammatory activity of latex of *Calotropis procera* in different models of inflammation. *Inflammopharmacol 2002*;**9**:257-64.
17. Dewan S, Sangraula H, Kumar VL. Preliminary studies on the analgesic activity of latex of *Calotropis procera*. *J Ethnopharmacol 2000*;**73**:307-11.
18. Dewan S, Kumar S, Kumar VL. Antipyretic effect of latex of *Calotropis procera*. *Indian J Pharmacol 2000;***32**:252.
19. Juliani JR, Biurrun F, Koroch AR, Oliva MM, Demo MS, Trippi VS. Chemical constituents and antimicrobial activity of the essential oil of *Lantana xenica*. Planta Medica 2002;68:762-4.
20. Kulkarni SS, Tripathy S, Paranjap RS, Mani NS, Joshi DR, Patil U, *et al*. Isolation and preliminary characterization of two HIV-2 strains from Pune, India. Indian J Med Res 1999;109:115-22.
21. Hahn BH, Shaw GM, Arya SK, Popovic M, Gallo RC, Wong-Staal F. Molecular cloning and charecterization of HTLV-III virus associated with AIDS. Nature 1984;312:166-9.
22. Mishell BB, Shiigi SM. Selected methods in cellular immunology. San Francisco: W.H. Freeman and Company; 1980.
23. Gary M, Andre C, Natasha O, Noel G, John V. HIV incidence results from two assays employed in the serological testing algorithm for recent HIV seroconversion (STARHS). J Virol Methods 2003;113:79-86.
24. Patwardhan M, Gautam M. Botanical immunodrugs: Scope and opportunities. Drug Discov Today 2005;10:495-502.
25. Haslam E. Natural polyphenols (vegetable tannins) as drugs: Possible modes of action. *J Nat Prod 1996;59:205-15.*
26. Tyler VE. Pyhtomedicines: Back to the future. J Nat Prod 1996;62:1589-92.
27. Anonymous. *Wealth of India: Raw materials.* New Delhi (India): CSIR Publication and Information Directorate; 1959.
28. Sehagal R, Arya S, Kumar VL. Evaluation of cytotoxic potential of latex of Calotropis. Indian J Pharmacol 2005;37:334-5.
29. De Clercq E. Perspectives of non-nucleoside reverse transcriptase inhibitors (NNRTIs) in the therapy of HIV-1 infection. Farmaco 1999;54:26-45.
30. Lee KH, Morris-Natschke SL. Recent advances in the discovery and development of plant-derived natural products and their analogs as anti-HIV agents. Pure Appl Chem 1999;71:1045-51.
31. Vandamme AM, Van Vaerenbergh K, D' Clercq E. Anti-human immunodeficiency virus drug combination strategies. Antivir Chem Chemother 1998;9:187-203.
32. Srinivasan D, Nathan S, Suresh T, Perumalsamy O. Antimicrobial activity of certain Indian medicinal plants used in folkloric medicine. J Ethnopharmacol 2001;74:217-20.
33. Nazma YM, Nasiruddin S, Mubassara S and MA. Antioxidant and antibacterial activities of *Calotropis procera* Linn American-Eurasian. J Agric Environ Sci 2008;4:550-3.
34. Mueen Ahmed KK, Rana AC, Dixit VK. Calotropis species (Ascelpediaceae): A comprehensive review. Pharmacogn Mag 2005;1:48-52.
35. Joshi S, Amarnath S. Fluoroquinolone resistance in Salmonella typhi and S. paratyphi A in Bangalore, India. *Trans R Soc Trop Med Hyg* 2005;101:308-10.
36. Iwanaga M, Insisiengmay S, Higa N, Sisavath L. Tetracycline resistant and polymyxin B sensitive vibrio cholerae O1 E1 tor isolated from the recent epidemics. Jpn J Trop Med Hyg 2000; 28: 15-8.

Source of Support: Nil, **Conflict of Interest:** None declared.