Journal of Research in Biology

FicusPublishers

OPEN ACCESS

Original Research paper

An International Online Open Access Publication group

Crotalaria pallida extracts as a putative HIV-protease inhibitors

Authors:
Govindappa M[1], Anil Kumar NV[2] and Gustavo Santoyo[3].

Institution:
[1]Department of Biotechnology, Shridevi Institute of Engineering & Technology, Sira Road, Tumkur-572 106, Karnataka, INDIA,
[2]Department of Chemistry, Manipal Institute of Technology, Manipal University, Manipal-576 104, Karnataka.
[3]Professor Investigador Titular "A", IIQB-UMSNH, Edificio A1 (B5), Ciudad Universitaria, Morelia, Michoacan, Mexico, C.P. 58030.

ABSTRACT:

In the present investigation, we screened the two solvent (methanol and ethanol) extracts of *Crotalaria pallida* different parts (leaf, flower and stem) for phytochemical analysis and screening for HIV protease inhibitors. The two solvent extracts have yielded the presence of major active compounds viz., flavonoids, terpenoids, cardiac glycosides, anthraquinones, coumarins, steroids, tannins, saponins. For HIV protease inhibitor activity, we used pepsin assay as a substitute for screening HIV protease inhibitors in the present investigation. The methanol and ethanol extracts of stem and flower have showed significant inhibition of protease activity as compared to standard (Pepstatin A).

Keywords:
Phytochemicals, Crotalaria pallida, HIV protease.

Corresponding author:
Govindappa M

Email:
dravidateja07@yahoo.co.in

Phone No:
+91-9686114847

Fax:
+91-816-2212629

Web Address:
http://jresearchbiology.com/Documents/RA0069.pdf.

Article Citation:
Govindappa M, Anil Kumar NV and Gustavo Santoyo.
Crotalaria pallida extracts as a putative HIV-protease inhibitors.
Journal of research in Biology (2011) 4: 285-291

Dates:
Received: 29 Jul 2011 /**Accepted:** 11 Aug 2011 /**Published:** 30 Aug 2011

Journal of Research in Biology

INTRODUCTION

A number of anti-HIV drugs used in conventional AIDS therapy are available in the market, unfortunately the administration of these compounds clinically to the AIDS patients exhibited serious side effects (Scinazi et al., 1992). As the data shows, rapid spread of AIDS epidemic and appearance of HIV strain resistance to the currently available drugs suggests that an effective and durable chemotherapy is required inheritably for its treatment. The use of innovative combinations of drugs having diverse mechanisms of anti-HIV activity (Tantillo et al., 1994; Balzarini et al., 1996; Lipsky, 1996) and continuous need for alternative inhibitors are also needed to be developed progressively (Singh et al., 2010). New anti-HIV agents with such activities may be identified through a variety of approaches, one of is through the being screening of natural products.

Plant products have attracted attention as possible anti-HIV drugs, targeted on the specific steps of the viral life cycle, such as viral attachment and entry as well as on essential enzymes and proteins that play a role during viral genome transcription are identified (Matsuse et al., 1999). The approved medications currently in use are mainly restricted to target the two viral enzymes, Protease and Reverse Transcriptase (RT). These inhibitors are very expensive and this has led to a global demand for broader, safer and also cheaper medications (Notka et al., 2004).

HIV protease has been suggested as a potential target for AIDS therapy for a long time (Kramer, 1986; Singh et al., 2010), later it was shown that a frame-shift mutations in the protease region of the pol-gene prevented cleavage of the gag poly protein precursor, which is essential for the mutation of the HIV particles. Blockage of HIV protease leads to the formation of immature non infectious virions (Khol, 1988). The HIV aspartic protease (HIV-PR) 1 is a key enzyme in the virus life cycle and was earlier perceived as a promising therapeutic target and its inhibition has become successfully used in the treatment of AIDS.

Literature survey shows that there are great potential natural products acting as HIV protease inhibitors for eg; limonin and nomilin from *Citrus* sp. (Battinelli, 2003), maslinic acid from *Geum japonicum* (Xu, 1996), oxygenated triterpenoids (Ganoderic acid-α) from *Ganoderma lucidum* (Mekkawy, 1998), ursolic acid and uvaol from *Crataegus pinatitids* (Min, 1999), coumarins from *Calophyllum brasiliense* (Ricardo et al., 2004) and other natural products (Chinsembu and Hedimbi, 2010).

Crotalaria pallida is a terrestrial, annual, erect herb, up to 150 cm tall. Taproot white or brown and stem grooved, solid, glabrous. Stipules present. Leaves trifoliolate, alternate spiral, stalked, leaflets elliptic or obovate, more than 2 cm long/ wide, hairy on upper surface, margin entire, apex obtuse or rounded, base acute, pinnately veined. Flowers bisexual, grouped together in a terminal raceme, stalked, petals 5, yellow. Fruit a rounded pod. A novel antimicrobial peptide has been exhibited as a strong antimicrobial agent against human pathogenic micro-organisms (Pellegrini et al., 2009) and lectin (Rego et al., 2002).

In the folk and Ayurvedic medicines, *C. juncea* is used as blood purifier, abortificient, astringent, demulcent, emetic, purgative and in the treatment of anaemia, impetigo, menorrhagia and psoriasis (Chauhan and Singh, 2010). *Crotalaria* species possesses anticancer properties (Kumar et al., 2008). Presence of coumarin has reported from Crotalaria *madhurensis* (Bhakshu et al., 2008) and *Crotalaria ramosissima* (Rao and Narukulla, 2007).

The literature survey indicates that there are no reports available from India regarding the phytochemicals and their anti-HIV properties of *C. pallida*. In the present study was aimed to examine the phytochemical analysis of methanol and ethanol extract of stem, leaves and flower of *C. pallida* and was screened for anti-HIV properties using standard methods. The findings from this work may add to the overall value of the medicinal potential of the plant.

MATERIALS AND METHODS

Collection of plant material

The plant was collected in November 2009 from the college campus, Shridevi Institute of Engineering & Technology, Sira Road, Tumkur, Karnataka, India. The plant was identified by their vernacular names and later it was compared with the herbarium of Department of Studies in Botany, Manasa Gangothri, University of Mysore, Mysore and Government Ayurvedic College, Mysore, India.

Extract Preparation

Each individual plant parts were used in the extraction followed by methodology as described by Martino et al. (2006). Microwave assisted extraction was performed using a closed-vessel system (GMS 17M 07 WHGX SOLO Microwave). Two grams of powdered different plant parts were subjected to extraction in a vessel with of solvent

(ethanol and methanol) up to the volume of 2ml for two cycles of 5 min individually. The resulting extracts were filtered through Whatman filter No.2. Clarified supernatant was kept in refrigeration for further studies (phytochemical analysis, coumarin identification and HIV protease inhibition).

Phytochemical analysis

Phytochemical analysis was carried out for saponins, flavonoids, terpenoids, steroids, phenol, alkaloids (Obdoni and Ochuko, 2001), tannins (Kaur and Arora, 2009) and coumarin spectrophotometrically as described by Yao et al. (2008). Wagner's and Heger's reagents were used for alkaloid foam test for saponins, Mg-HCl and Zn -HCl for flavonoids, Keller-Killani test for cardiac glycosides, Salkonoski test for terpenoids, acetic anhydride and sulphuric acid for steroids, chloride and gelatin for tannins, ferric chloride for phenol, hexane and diluted ammonia for anthraquinones test. All these experiments were carried out for each solvent extracts separately.

Enzyme pepsin inhibition assay

Pepsin has a quite close resemblance in proteolytic activity with HIV-1 protease one key enzyme of HIV-1 life cycle as both of them belong to same aspartate enzyme family (Maria et al., 2004). This enzyme was used as substitute of HIV-1 protease to check out anti-HIV activity of plant extracts in the present investigation (Singh et al., 2010).

We followed the method of Aoyagi (1978) and Singh et al. (2010), for this assay, 50 µg pepsin, 800 µg haemoglobin and different parts extracts were taken in 500 µl of reaction mixture. The mixture was allowed to incubate at 37^0C, after 20 min, 700 µl of 5% TCA was added to stop the reaction. It was then centrifuged at 14, 000 g for 5 min and the supernatant was collected. Optical Density (OD) was recorded spectrophotometrically at 280 nm. Separate blanks were used or both positive and negative control as well as for sample. For positive control enzyme and substrate were taken and followed the above procedure and for negative control pepstatin A was taken as a well known inhibitor of both pepsin and HIV-protease. Each sample was taken in triplicate, so this assay gives reproducible results.

RESULTS

Phytochemical analysis

The phytochemical screening showed that the two different solvent ethanol and methanol extracts of *C. pallida*, the alkaloids, flavonoids, terpenoids, saponins, phenols, cardiac glycosides, steroids, coumarin and tannins were present in all the solvent extracts. The methanol extract yielded strongly all the phytochemicals compared to ethanol (**Table 1**). In ethanol leaf extracts, it

Table 1. Phytochemical analysis of methanol and ethanol extracts of *Crotalaria pallida*

Sample	Phytochemicals								
	Flavonoids	Terpenoids	Phenol	Cardiac glycosides	Tannin	Steroids	Saponins	Coumarins	Anthraquinones
Ethanol extract									
Leaf	-	+	-	+	+	+	+	+	-
Flower	+	+	-	+	+	+	+	+	-
Stem	+	+	-	+	+	+	+	+	-
Methanol extract									
Leaf	-	+	+	+	+	+	+	+	-
Flower	+	+	+	+	+	+	+	+	-
Stem	+	+	-	+	+	+	+	+	-

Repeated the each experiments thrice, +: presence, -: absent

showed the absence of flavonoids, the ethanol stem extract showed absence of phenol and ethanol flower and stem extracts showed absence of tannin. The ethanol stem extract exhibited the absence of steroids. Methanol leaf extracts showed the absence of flavonoids and methanol stem extract exhibited the absence of phenol.

The ethanolic flower and stem extracts exhibited highest amount of coumarins in tested methods where as methanol flower and stem extracts exhibited highest amount of coumarins.

The ethanol and methanol stem and flower extracts exhibited strong inhibition of pepsin enzyme. Strong inhibition was noticed in methanol stem extract followed by ethanol stem extract, methanol flower extract and ethanol flower extract while other extracts with negligible toxic effect (**Table 2**).

Table 2. Effect of *Crotalaria pallida* constituents on HIV protease inhibition

Sample	Absorbance at 280 nm
Control (without any extract/ inhibitor)	0.4212 ± 0.0085^{a}
Control with Pepstatin A	0.0016 ± 0.0085^{g}
Control with methanol leaf extract	0.2430 ± 0.0085^{c}
Control with methanol flower extract	0.0046 ± 0.0085^{d}
Control with methanol stem extract	0.0029 ± 0.0085^{f}
Control with ethanol leaf extract	0.3568 ± 0.0085^{b}
Control with ethanol flower extract	0.0051 ± 0.0085^{d}
Control with ethanol stem extract	0.0036 ± 0.0085^{e}

Repeated the each experiments thrice, +: presence, -: absent, According to Duncan's Multiple Range Test (DMRT), values followed by different subscripts are significantly different at P<0.05, SE-standard error of the mean.

DISCUSSION

AIDS-related diseases remain one of the leading causes of death globally. The declines in new infections and AIDS-deaths may be attributed to the scale-up of anti-retroviral therapy (ART) programmes, especially in the developing world. Presently, no reliable and friendly treatment can be claimed to combat this disease. The current anti-HIV drugs have focused on reverse transcriptase and protease inhibitors have experienced drug resistance by HIV strains. This imposes the demand for the development of new drugs particularly of plant origin owing to their success as sources of anti -HIV drugs. The use of plants for management of different diseases has become a common practice since olden days in developing countries. Especially in India, most of the people are depending on traditional medicines for their primary health care including the management of HIV/AIDS.

Our results have showed protease inhibitor activity and phytochemical screening showed the presence of many active compounds. The various plant active molecules exhibited as anti-HIV properties, such as flavonoids (Mantas et al., 2000), terpenoids (Sun et al., 2003), cardiac glyucosides (Prinsloo et al., 2010), tannin (Sakagami et al., 1999), steroids (You et al., 2003), saponins (Konoshima et al., 1995), coumarins (Zhou et al., 2000) and athraquinones are under study (Schinazi et al., 1990).

The structure of the dimeric enzyme of HIV-1-PR superficially resembles that of other aspartic proteases such as pepsin (Hutchins et al., 1991; Wlodawer and Erickson, 1993). However, whereas pepsin exists as a 326-residue monomer, with two differing domains forming a cleft containing the active site, HIV-1-PR forms a similar groove in the interface between its two 99-residue subunits. As in pepsin, HIV-1-PR has two highly mobile arms of about 10 residues each, which surround and anchor the substrate in the region of the active site. The flaps themselves are not necessary for enzyme activity, although the absence of flaps reduces enzymatic activity (Chatfield and Brooks, 1995; Silva *et al*., 1996).

The main aim of our present investigation is the screening of *Crotalaria pallida* phytochemical extracts for HIV protease inhibition. The plant has potential antimicrobial compounds, two solvent extracts of *Crotalaria pallida* showed a very significant inhibition of pepsin enzymatic activity. The previous reports suggested that there are close structural and functional similarities between pepsin and HIV protease. The plant extracts have showed inhibitory activity of pepsin enzyme, may be these extracts inhibit the activity of HIV protease. Our results, the methanol and ethanol extracts of stem and flower have proven potential inhibition of the pepsin enzyme activity due to the different phytochemical constituents, may be our extracts have strong inhibitory activity of HIV protease. Many similar works has been done with plant

extracts (Cos et al., 2004; Debouck, 1992; Aiken and Chen, 2005; Polya, 2003). The current study can append one more alternative HIV protease inhibitor to solve the problem especially arresting the HIV replication. But, it needs further characterization of active molecules in the extracts, purification and mode of action on HIV replication is needed.

ACKNOWLEDGEMENTS

We thank **Dr MR Hulinaykar**, Managing Trustee, Sri Shridevi Charitable Trust (R.) and **Dr MA Venkatesh**, Principal, SIET, Tumkur, India for encouragement, **Dr P Sharanappa**, DOS in Biosciences, Hemagangothri, University of Mysore, Hassan, India for assistance in identifying plant.

REFERENCES

Aiken C and Chen CH. 2005. Betulinic acid derivatives as HIV-1 antivirals. *Trends in Molecular Medicine* 11(1):31-36.

Aoyagi T, Umezawa H, Takita T and Shiba T. 1978. In Bioactive peptides produced by micro-organisms. Eds. Halsted Press. 129-151.

Bhakshu LM, Ratnam KV and Venkataraju RR. 2008. Medicinal properties and antimicrobial activity of *Crotalaria madurensis* var. *kurnoolica*. *Ethanobotanical Letters*. 12:758-762.

Balzarini J, Pelemans H, Karlsson A, De Clerq E and Kleim JP. 1996. Concomitant combination therapy for HIV infection preferable over sequential therapy with 3TC and non-nucleoside reverse transcriptase inhibitors. *Proceedings of Natural Academic Science USA*. 93:13152-13157.

Battinelli L, Mengoni F, Lichtner M, Mazzanti G, Saija A, Mastroianni CM and Vullo V. 2003. Effect of limonin and nomilin on HIV-1 replication on infected human mononuclear cells. *Planta Medica* 69:910-913.

DC and Brooks BR. 1995. HIV-1 Protease Cleavage Mechanism Elucidated with Molecular Dynamics Simulation. *J. Am. Chem. Soc*., 117:5561 -5572.

Chauhan HS and Singh SK. 2010. Antimicrobial activity of seed and flower parts of *Crotalaria juncea* Linn. *American-Eurasian Journal of Scientific Research* 5(3):212-215.

Chinsembu KC and Hedimbi M. 2010. Ethanomedical plants and other natural products with anti-HIV active compounds and their putative modes of action. *International Journal of Biotechnology and Molecular Biology Research* 1 (6):74-91.

Cos P, Maes L, Berghe DV, Hermans N, Pieters L and Vlietinck A. 2004. Plant Substances as Anti-HIV Agents Selected According to Their Putative Mechanism of Action. *J. Nat. Prod.* 67(2):284-293.

Debouck C. 1992. The HIV-1 Protease as a Therapeutic Target for AIDS. *AIDS Research and Human Retroviruses* 8(2):153-164.

Hutchins C, Greer J and Hoover DJ. 1991. Comparative Modeling of Proteins in the design of Novel Renin Inhibitors. *Crit. Rev. Biochem. Mol. Biol*., 26(1):77-127.

Kaur GJ and Arora DS. 2009. Antibacterial and phytochemical screening of *Anethum graveolens, Foeniculum vulgare* and *Trachyspermum ammi*. *BMC Complementary and Alternate Medicine* 9:30.

Khol NE, Emini EA, Schleif WAF, Scolnick EM and Sigal IS. 1988. Active human immunodeficiency virus protease is required for viral infectivity. *Proceedings of National Academy of Sciences, USA* 85:4686-4690.

Konoshima T, Yashida T, Ashiwada Y, Consentino LM and Lee KH. 1995. Anti-AIDS agents, triterpenoids saponins a anti-HIV principles from fruits of *Gledissia japonica* and *Gymnocladus chinensis* and a structure activity correlation. *Journal of Natural Products* 58(9):1372-1377

Kramer RA, Schaber MD, Shalka AM, Ganguly K, Wong-Staal F and Reddy EP. 1986. HTLV-III gag protein is processed in yeast cells by the virus pol protease. *Science* 231:1580-1585.

Kumar S, Praveen F, Goyal S and Chauhan A. 2008. Indegenous herbal coolant for combating heat stress in the Indian Arid Zone. *Indian Journal of Traditional Knowledge* 7(4):679.

Lipsky JJ. 1996. Antiretroviral drugs for AIDS. *Lancet* 348:800-803.

Mantas A, Deretey E, Ferretti FH, Estrada MR and Csizmadia IG. 2000. Structural analysis of flavonoids with anti-HIV activity. *Journal of Molecular Structure : Theochem* 504(1-3):171-179.

Martino E, Ramaiola I, Urbano M, Bracco F and Collina S. 2006. Microwave-assisted extraction of coumarin and related compounds from *Melilotus officinalis* (L.) Pallas as an alternative to Soxhlet and ultrasound-assisted extraction. *Journal of Chromatography* 1125:147-151.

Matsuse IT, Lim Y, Hattori M, Correa M and Gupta MP. 1999. A search for anti-viral properties in Panamanian medicinal plants the effect of HIV and its essential enzymes. *Journal of Ethanopharmacology* 64:15-22.

Mekkawy SE, Meselhy MR and Nakamura N.1998. Anti-HIV-1 and anti-HIV- 1 protease substances from *Ganoderma lucidum*. *Phytochemistry* 49:1651-1657.

Notka F, Meier G and Wagner R. 2004. Concentrated inhibitory activities of *Phyllanthus amarus* on HIV replication *in vitro* and *ex vitro*. *Antiviral Research* 64:93-102.

Maria MY, Justo P, Cristina M, Julio G, Manuel A, Francisco M and Javier V. 2004. Rapeseed protein hydrolysates: a source of HIV protease peptide inhibitors. *Food Chemistry* 87:387-392.

Min BS, Jung HJ and Lee JS. 1999. Inhibitory effect of triterpenes from *Crataegus pinatifida* on HIV-1 protease. *Planta Medica* 65:374-375.

Obdoni BO and Ochuko PO. 2001. Phytochemical studies and comparative efficacy of the crude extract of some homostatic plants in Edo and Delta States of Nigeria. *Glob. J. Pure Appl. Sci.* 8b:203-208.

Pelyrini PB, Farias LR, Saude AC, Costa FT, Bloch C Jr, Silva LP, Oliveira AS, Gomes LE, Sales MP and Franco OL. 2009. A novel antimicrobial peptide from *Crotalaria pallida* seeds with activity against human and phytopathogens. *Current Microbiology* 59(4):400-404.

Polya GM. 2003. Protein and non-protein protease inhibitors from plants. *Studies in Natural Product Chemistry* 29(10):567-641.

Prinsloo G, Meyer JJ, Hussein AA, Munoz E and Sanchez R. 2010. A cardiac glycoside with *in vitro* anti-HIV activity isolated from *Elaeodendron croceum*. *Natural Product Research* 24(8):1743-1746.

Rao MS and Narukulla R. 2007. A new trimethoxychalcone from *Crotalaria ramosissima*. *Fitoterapia* 78(6):446-447.

Rego EJ, De Carvalho DD, Maragoni S, de Oliveira B and Novello JC. 2002. Lectins from seeds of *Crotalaria pallida* (smooth rattlebox). *Phytochemisrty* 60(5):441-446.

Ricardo RC, Elizabet EM, Teresa RA, Badia A, Andre A, Christopher KJ and Mario VT. 2004. Cytotoxic effect of Mammea type coumarins from *Calophyllum brasiliennse*. *Life Sciences* 75(13): 1635-1647.

Sakagami H, Satoh K, Ide Y, Koyama N, Premanathan M, Arakaki R, Nakashima H, Hatano T and Yoshida T. 1999. Induction of apoptosis and anti-HIV activity by tannin and lignin related substances. *Basic Life Sciences* 66: 595-611.

Scinzi R, Mead J and Feorino P. 1992. Insights into HIV chemotherapy. *AIDS Research Human Retroviruses* 8:963.

Schinazi RF, Chu CK, Babu JR, Oswald BJ, Saalmann V, Cannon DL, Erikkson BFH and Nasr M. 1990. Anthraquinones as new class of antiviral agents human immunodeficiency virus. *Antiviral Research* 13(5):265-272.

Silva AM, Cachau RE, Sham HL and Erickson JW. 1996. Inhibition and catalytic mechanism of HIV-1 aspartic protease. *J. Mol. Biol*. 255(2):321-346.

Singh KP, Upadhyay B, Prasad R and Kumar A. 2010. Screening of *Adhatoda vasica* Nees as a putative HIV-protease inhibitor. *Journal of Phytology* 2(4):78-82.

Sun FC, Kashiwada Y, Morris-Natshke SC and Lee KH. 2003. Plant derived terpenoids and

analogues as anti-HIV agents. *Current Tropical Medicinal Chemistry* 3(2):155-169.

Tantillo C, Ding J, Jacobo-Monila A, Nanni RG, Boyer PL, Hughes SH, Pauwels R, Andries K, Janssen PAJ and Arnold E. 1994. Locations of anti-AIDS drug binding sites and resistance mutations in the three-dimensional structure of HIV -1 reverse transcriptase, implications for mechanisms of drug inhibition and resistance. *Journal of Molecular Biology* 243:369-387.

Xu H-X, Zeng F-Q, Wan M and Sim K-Y. 1996. Anti-HIV triterpene acids from *Geum japonicum*. *Journal of Natural Products* 59:643-645.

Yao SQ, Rongpig Y, Hao BW, Zhong-you, Tu YQ and Hu YJ. 2008. Ultraviolet spectrophotometric determination of psoralen furan herbs in the total content of two coumarin. *Pharmacognosy Medicine* 42.

You Z, Mian AM, Agarwal RP and Lee HJ. 2003. Snthesis, anti-V an cytotoxic activity of new AZT conjugates of steroid acids. *Nucleosides, Nucleotides, Nucleic Acids* 22(11):2049-60.

Zhou P, Takaishi Y, Duan H, Chen B, Honda G, Itoh M, Taka Y, Kodzhimotov OK and Lee KH. 2000. Coumarins and biocoumarin from *Ferula sumbul*: anti-HIV activity and inhibition of cytokine release. *Phytochemistry* 53(6):689-697.

Wlodawer A and Erickson JW. 1993. Structure-Based Inhibitors of HIV-1 Protease. *Annu. Rev. Biochem*., 62:543-583.