Crotalaria pallida extracts as a putative HIV-protease inhibitors

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.

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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 inherently 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-a) 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, abortifcient, 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°C, 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>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Anthraquinones</th>
<th>Coumarins</th>
<th>Saponins</th>
<th>Flavonoids</th>
<th>Terpenoids</th>
<th>Phenol</th>
<th>Cardiac glycosides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol extract</td>
<td>Leaf</td>
<td>Flower</td>
<td>Stem</td>
<td>Leaf</td>
<td>Flower</td>
<td>Stem</td>
<td>+</td>
</tr>
<tr>
<td>Methanol extract</td>
<td>Leaf</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

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

Repeated each experiment 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

<table>
<thead>
<tr>
<th>Sample</th>
<th>Absorbance at 280 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (without any extract/inhibitor)</td>
<td>0.4212±0.0085</td>
</tr>
<tr>
<td>Control with Pepstatin A</td>
<td>0.0016±0.0085</td>
</tr>
<tr>
<td>Control with methanol leaf extract</td>
<td>0.2430±0.0085</td>
</tr>
<tr>
<td>Control with methanol flower extract</td>
<td>0.0046±0.0085</td>
</tr>
<tr>
<td>Control with methanol stem extract</td>
<td>0.0029±0.0085</td>
</tr>
<tr>
<td>Control with ethanol leaf extract</td>
<td>0.3568±0.0085</td>
</tr>
<tr>
<td>Control with ethanol flower extract</td>
<td>0.0051±0.0085</td>
</tr>
<tr>
<td>Control with ethanol stem extract</td>
<td>0.0036±0.0085</td>
</tr>
</tbody>
</table>

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 glycosides (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.

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