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In vitro anti-HIV properties of coumarins from *Crotalaria pallida* aiton

M.Govindappa^{1*}, T.Umashankar¹, Y.L.Ramachandra², Charles Chee-Jen Chen³

¹Natural Product Laboratory, Department of Biotechnology, Shridevi Institute of Engineering & Technology, Sira Road, Tumkur-572 106, Karnataka, (INDIA)

²Department of PG Studies and Research in Biotechnology & Bioinformatics, Kuvempu University, Jnana Sahyadri, Shankaraghatta-577 451, Shimoga, Karnataka, (INDIA)

³Department of Biotechnology, Southern Taiwan University, 1 Nan-Tai Str., Young-Kang City Tainan 71043, (TAIWAN)

E-mail : dravidateja07@gmail.com

ABSTRACT

Four different parts (flower, stem, root and leaves) of *Crotalaria pallida* were subjected to isolation of coumarins using microwave assisted extraction method. Better results were obtained in two heating cycles of 5 min, 100°C. Four different identification tests was proved the presence of coumarins based on colour development. Each extracts were analyzed with HPLC procedure and chromatographic analysis proved the coumarin presence. The flower extracts having coumarins strongly inhibited the HIV replicating enzymes (α -glucosidase, β -glucosidase, α -glucuronidase, reverse transcriptase and protease). The results suggested that MAE is good for extraction of coumarins, HPLC and other tests confirmed the presence of coumarin and these extracts also strongly inhibited the HIV replicating enzymes. The *Crotalaria pallida* coumarin can be used as drug to control the HIV replication in AIDS management.

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KEYWORDS

Crotalaria pallida;
Coumarins;
Microwave assisted
extraction;
HIV replication enzymes.

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^[29]. 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 di-

verse mechanisms of anti-HIV activity^[3,20,33] and continuous need for alternative inhibitors are also needed to be developed progressively. 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^[22].

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^[24].

HIV protease has been suggested as a potential target for AIDS therapy for a long time^[18,32], 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^[17]. 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.

Glycohydrolase enzymes are found in the Golgi apparatus of eukaryotic cells. These are responsible for glycosylation of proteins. Inhibition of the glycohydrolase enzymes has been found to decrease the infection caused by HIV virion, as the HIV glycoproteins are highly glycosylated^[7]. Reverse transcriptase is an enzyme that reads the sequence of HI viral RNA nucleic acids that have entered the host cell and transcribes the sequence into complementary DNA. Without reverse transcriptase, the viral genome cannot be incorporated in to the host cell; as a result a virus cannot reproduce. Reverse transcriptase is therefore the principal target enzymes of antiretroviral drugs are used to treat HIV infected patients^[9,35]. Reverse transcriptase is convert RNA to DNA for replication, HIV protease is mutates in help in mutation in protease region of pal-gene, a-glucosidase is converts glycogen to glucose and b-glucoronidase catalyses the breakdown of complex carbohydrates. These four important enzymes are present in HI virus leads for replication, mutation, breaking glycogen and carbohydrates in HIV patient.

Crotalaria pallida is an annual, erect herb, up to 150 cm tall, tap root, white or brown and system grooved, solid and glabrous. Novel antimicrobial peptides from *C. pallida* have shown strong antimicrobial activity^[25] and lectin was identified^[28]. Other species of *Crotalaria* have shown various folk and ayurvedic medicines for blood purifier, anemia, psoriasis^[6], cancer activity^[19]. And few species of *Crotalaria* have shown

presence of coumarin^[2,27]. The *Crotalaria pallida* extracts have shown the presence of various bioactive phytochemicals and these compounds also showed significant inhibition of protease activity^[13].

Coumarin from different plants are possess anti-cancer^[10], anti-HIV^[37] and antidiabetic^[12]. The aim of the work is the development of a rapid, reliable and reproducible method of extraction of coumarin and O-coumaric acid from different parts of *C. Pallida* by using microwave assisted extraction method (MAE). Martino et al.^[21] have reported that for coumarin extraction is reliable and more reproducible from MAE from *Melilotus officinalis*. Waksmundzka-Hajnos and Brachet et al. have reported that the application of MAE to the extraction of secondary metabolites from the plants.

To the best of our knowledge from literature survey, no reports on MAE of coumarin from the plant material *Crotalaria pallida* from determination of the content of coumarin and O-coumaric acid, HPLC procedure suitable for the determination and identification of coumarin in the suitable and *in vitro* anti-HIV studies by inhibition of four important enzymes are responsible for viral replication.

MATERIALS AND METHODS

Plant material

Plants of *Crotalaria pallida* Aiton were collected in April, 2011 in natural population at flowering stage from Shridevi Institute of Engineering & Technology, Tumkur, India campus.

The freshly cut plants were sorted out, dried in the drying room with active ventilation at room temperature (24-26^o C) until constant weight. All the different parts were ground with a blade mill to obtain a homogenous drug powder. The drug material was stored in closed jars and preserved in dark conditions in the refrigerator. The humidity value of the sample did not change during the storage.

Chemicals

All solvents from Spectrum Reagents and Chemicals Pvt. Ltd, Cochin, Kerala, India, pepsin, a-glucosidase, b-glucoronidase, reverse transcriptase from Sigma-Aldrich, Bangalore, India.

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Extraction

Each individual plant parts were used in the extraction followed by methodology as described by Martino et al.,^[21]. Microwave assisted extraction was performed using a closed vessel system (GMS 17M 07 WHGX SOLO Microwave) and setting the microwave power at 100 W. 0.2 g of the powdered drug were placed into the extraction vessel in addition with solvent up to volume of irradiation after extraction time had elapsed, the vessels were allowed to cool temperature before opening. Here we used, two different temperatures, one or two cycles for 5 min for all the methanol extracts.

Identification of coumarin in the extracts

Test 1

3 ml of methanol extract was evaporated to dryness in a vessel and the residue was dissolved in hot distilled water. It was then cooled and divided into two test portions, one was reference, second was the test. To the second test tube, 0.5 ml of 10 NH₄OH was added. The occurrence of intense/fluorescence under UV light is a positive test for the presence of coumarins and derivatives. The experiment was carried out for all the experiments in three replicates^[16].

Test 2

5 ml of the extract was evaporated to dryness and the residue was dissolved in 2 ml of distilled water. The aqueous solution was divided into two equal parts in test tubes. One part was the reference. To the other test tube, 0.5 ml of 10% ammonia solution was added and the test tubes were observed under UV light indicated. The occurrence of a bluish green florescence under UV light indicated the presence of coumarin derivatives^[16].

Test 3

To the concentrated alcoholic extract of drug few drops of alcoholic FeCl₃ solution was added. Formation of deep green colour, which turned yellow on addition of conc. HNO₃, indicates presence of coumarins.

Test 4

The alcoholic extract of drug was mixed with 1N NaOH solution (one ml each). Development of blue-green fluorescence indicates presence of coumarins.

HPLC analysis

Coumarin and *o*-cumarinic acid were quantified by high performance liquid chromatography–ultraviolet diode array detection (HPLC–UV/DAD). A Jasco system (Japan) consisting of a PU-1580 pump and a MD-1510 detector ($\lambda = 275$ nm) was used. Sample solutions were injected using a Spectra System auto-sampler AS3000. Experimental data were acquired and processed by Borwin PDA and Borwin Chromatograph Software. Chromatographic separations were carried out using a LiChrosorb RP-18 column (250mm×4.6 mm, 7_μm Merck, Germany) and a security guard RP-18 column (LiChrocart® 4-4 Purospher®, 5_μm). The mobile phase consisted of water with orthophosphoric acid 1:10,000 (solvent A), methanol (solvent B) and acetonitrile (solvent C). The starting mixture (80% A, 5% B and 15% C) was modified as follows: in 20 min the mobile phase composition became 65% A, 20% B, 15% C and was kept constant for 10 min; in the following 10 min the mixture composition came back to the initial eluting system. The flow rate was set at 0.7 ml/min.

All mixtures obtained by microwave assisted extraction were centrifuged for 5 min at 3000 rpm (Centrifugette 4206 ALC, Milano, Italy), and before injection each sample was filtered with 0.45_μmGHP membrane, in order to remove the solid residue. The injection volume was 5_μl. Each determination was carried out in triplicate. Quantitative determination of the considered phytochemicals in the extracts was performed using external standards by means of six points calibration curve. Recovery was investigated by adding about 3.0 mg, 1.5 mg and 8.0 mg of coumarin, and *o*-coumaric acid respectively, to 1 g of drug powder. The obtained sample was then accurately mixed, extracted and analysed. 91% of coumarin, and 89% of *o*-coumaric acid were recovered.

In vitro anti-HIV activity

Glycohydrolase enzyme assay

Glycohydrolase enzymes are found in the eukaryotic host cells Golgi apparatus and are responsible for glycosylation of proteins. Inhibition of the glycohydrolase enzymes has been found to decrease the infectivity of the HIV virion, as the HIV envelop proteins are highly

glycosylated during the life cycle of the virus. α -Glucosidase has been found to be partly responsible for the glycosylation of HIV gp120^[7].

To measure the inhibition of the glycohydrolase enzymes, α -glucosidase and β -glucuronidase were used with their corresponding substrates p-nitrophenyl- α -D-glucopyranose and p-nitrophenyl- β -D-glucuronide in a 96 well microlitre plate in a colorimetric enzyme based assay^[7] the substrates and enzymes were dissolved in their appropriate 50 mM buffers (2-morpholinoethanesulphonic acid monohydrate-NaOH, pH 6.5, for α -glucosidase and β -glucuronidase and sodium acetate, pH 5.6, for β -glucosidase). The final enzyme was 200 μ l and contained 2 mM substrate, \sim 0.25 μ g enzyme and the different parts methanol MAE extracts at 0.2 mg/ml separately. The reaction was allowed to proceed for 15 min at 25°C before termination with 60 μ l 2M glycine-NaOH, pH 10 and measured the absorbance at 412 nm for samples.

HIV reverse transcriptase (RT) assay

The effect of methanol MAE extracts on reverse transcription was tested using a non-radioactive HIV-RT colourimetric ELISA kit. The protocol outlined in the kit was followed using 2 ng of enzyme in a well and incubated the reaction mixture for 2h at 37°C. Extracts were tested at 0.2 mg/ml^[14].

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^[23]. This enzyme was used as substitute of HIV-1 protease to check out anti-HIV activity of plant extracts in the present investigation^[32].

We followed the method of Aoyagi and Singh et al.^[32], for this assay, 50 μ g pepsin, 800 μ g haemoglobin and different parts extracts were taken in 500 μ l of reaction mixture. The mixture was allowed to incubate at 37°C, after 20 min, 700 μ l 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 lopinavir

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 AND DISCUSSION

We used microwave assisted extraction procedure in order to isolate coumarin and o-coumaric acid from *C. pallida* by using the ratio sample weight/solvent constant with a value of 0.05 g/ml. Three replicates were maintained for all the parts extraction.

The extractions were performed employing as extraction solvents 50% (v/v) aqueous MtOH at two different temperature (50 and 100) in one and two cycle. The best results were obtained employing 80% aqueous methanol. Being the amount of the two phytochemicals are different significantly.

Based on reports of Martino et al.^[21] identified microwave assisted extraction is suitable to isolate or yielded more coumarin by using closed vessel system after trying other methods and they also reported that the other two methods are unsatisfactory. From our findings, coumarin and o-coumaric acid yields were obtained in 5 min of two cycles at 100°C and the percent of coumarins is varies on parts. The flower extract yielded more coumarin compared to stem, root and leaves (TABLE 1). From MAE, the short extraction time is required and allowed a good extraction of coumarin without any degradation problem. Similar results were obtained from *Melilotus officinalis* using ethanol solvent^[21].

TABLE 1 : Effect of microwave assisted extractions on yield of coumarin and O-coumaric acid from different parts of *Crotalaria pallida*

Extracts	Temperature (°C)	Cycles X minutes	Coumarin ^a (mg/g)±SD	o-coumaric acid ^a (mg/g)±SD
Flower	50	1 x 5	3.141±0.031	1.226±0.068
		2 x 5	3.622±0.074	1.663±0.071
	100	1 x 5	3.576±0.053	1.142±0.076
		2 x 5	3.881±0.082	1.224±0.058
Stem	50	1 x 5	2.885±0.057	1.164±0.047
		2 x 5	3.224±0.054	1.347±0.023
	100	1 x 5	3.011±0.087	1.024±0.059
		2 x 5	3.443±0.044	1.161±0.051
Root	50	1 x 5	2.541±0.058	1.004±0.063
		2 x 5	2.658±0.061	1.221±0.043

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Extracts	Temperature (°C)	Cycles X minutes	Coumarin ^a (mg/g)±SD	o-coumaric acid ^a (mg/g)±SD
Leaf	100	1 x 5	2.779±0.074	1.313±0.038
		2 x 5	2.882±0.068	1.461±0.052
	50	1 x 5	2.116±0.063	1.012±0.021
		2 x 5	2.312±0.023	1.232±0.053
	100	1 x 5	2.086±0.040	1.119±0.027
		2 x 5	2.168±0.026	1.227±0.057

^a Determined by HPLC, 50% (v/v) aqueous methanol, SD: standard error

All MAE extractions were subjected to know the presence of coumarin using different methods of Jagessar and Cox^[16]. In all the four different identification methods confirmed the presence of coumarin and coumarin related compounds (TABLE 2). This results of findings giving strong evidence of coumarin presence. Presence of coumarin in plant extracts was screened^[16] using different plant. This confirms MEA method is yielded more coumarin.

TABLE 2 : Identification of coumarin(s) from different parts of extracts in different experiments

Extracts	1	2	3	4
Flower	+	+	+	+
Stem	+	+	+	+
Root	+	+	+	+
Leaf	+	+	+	+
o-coumaric acid	+	+	+	+
Coumarin	+	+	+	+

1: test 1, 2:test 2, 3:test 3, 4:test 4, +:presence

All the flower and stem methanol extracts of *Crotalaria pallida* showed significant inhibition of RT was observed. No significant activity was observed with root and leaf (Figure 1). The flower (83%) and stem (80%) MAE methanol extracts showed highest RT inhibitory activity compare to root (64%) and leaf (64%) whereas standard zidovudine 300 has shown 81%. Almost all extracts inhibited the RT at varied range. This report is confirmed with the earlier reports^[14]. Yu et al.^[36] have reviewed the coumarins importance in inhibition of viral replication and cell cycle. Dharmaratne et al.^[11] have clearly identified the coumain were found to be inactive in both the HIV-1 RT and whole virus systems.

Many plant extracts were able to inhibit the three glycohydrolase enzymes already reported^[7,14]. More inhibition of three enzymes was observed from flower

(74% for a-glucosidase, 63% for b-glucosidase and 76% for a-glucuronidase) followed by stem whereas leaf and root extracts also showed more inhibitory activity (TABLE 3). Hanett et al. and Collins et al.^[7] have reported that plant extracts showed the inhibitory activity of these enzymes. The inhibitory effect is due to presence of coumarin in the extract. Our MAE extracts of *Crotalaria pallida* having more coumarin are possibly inhibited these enzyme activities effectively.

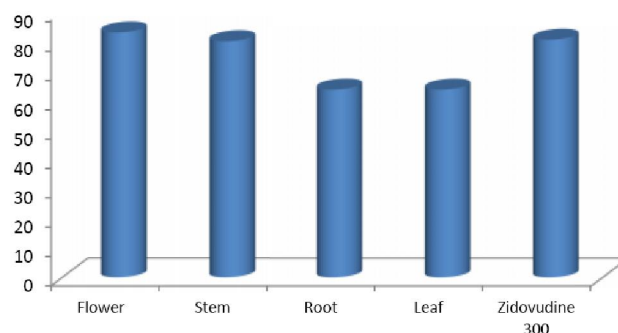


Figure 1 : Effect of plant extracts (200 µg/ml) on the activity of recombinant HIV reverse transcriptase

TABLE 3 : Inhibition of glycohydrolases (percent) in the presence of plant extracts at 200 µg/ml concentration

Extracts	Percent inhibition		
	α-glucosidase	β-glucosidase	α-glucuronidase
Flower	74	63	76
Stem	75	59	75
Root	62	52	60
Leaf	64	47	63
Zidovudine 300	81	80	81

The methanol flower and stem extracts exhibited strong inhibition of pepsin protease enzyme. Strong inhibition was noticed in methanol flower and stem extract. The root and leaf extracts have showed negligible toxic effect (TABLE 4).

TABLE 4 : Effect of *Crotalaria pallida* coumarin constituents on HIV protease inhibition

Extracts	Absorbance
Flower	0.2630+
Stem	0.2449+
Root	0.0039+
Leaf	0.1126+
Control with lopinavir	0.0016+
Control (without any extract/inhibitor)	0.4212+

Our results have showed protease inhibitor activity and phytochemical screening showed the presence of

many active compounds due to presence of coumarins^[37].

The structure of the dimeric enzyme of HIV-1-PR superficially resembles that of other aspartic proteases such as pepsin^[15,34]. 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^[5,30].

The *Crotalaria pallida* coumarin extracts have pepsin enzymatic activity that correlates HIV protease inhibitor 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 of stem and flower have proven potential inhibition of the pepsin enzyme activity due to coumarin constituents, may be our extracts have strong inhibitory activity of HIV protease. Many similar works has been done with plant extracts^[1,8,31]. 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.

Quantification was performed by comparing the chromatographic peak areas for extracted samples using HPLC (Figure 2 and 3). The assignments of the HPLC peaks were confirmed by comparison of retention times with authentic standards as mentioned by Mortino et al.^[21].

In conclusion, this study shows the importance of MAE in extraction of coumarin from *Crotalaria pallida* and showed inhibitory activity against enzymes such as glycohydrolases, HIV-RT and pepsin. The potential mechanism for the clinically seen improvement of AIDS suffers when administered with flower and stem extract of *Crotalaria pallida* retained the inhibition of HIV-RT. Other identification methods confirm the strong presence of coumarin and HPLC data given clear evi-

dence that strong presence of coumarin. Purification of the active ingredient coumarin will allow more conclusive data regarding the mechanism by which improves general health status of HIV positive patients.

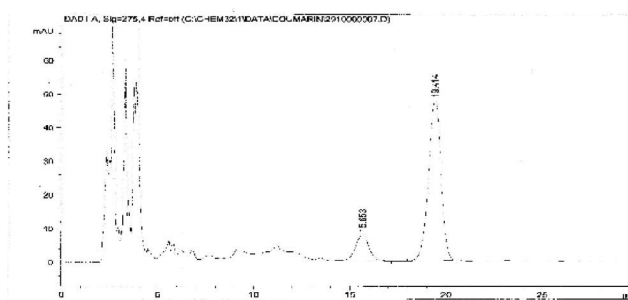


Figure 2 : Chromatographic profile coumarins peaks: (1) coumarin: 15.653; (2) o-coumaric acid:19.414 in flower extract

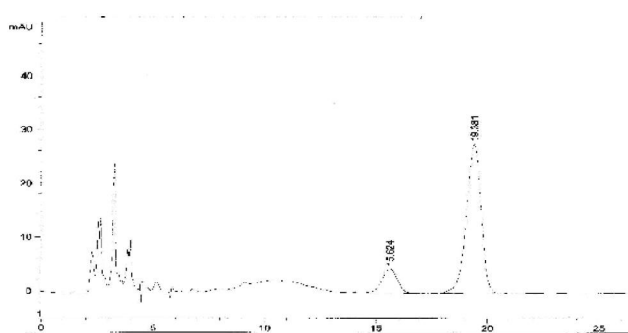


Figure 3 : Chromatographic profile coumarins peaks: (1) coumarin: 15.624; (2) o-coumaric acid:19.381 in stem extract

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