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Research Article

PA-9: A Flavonoid Extracted from *Plectranthus amboinicus* Inhibits HIV-1 Protease

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ABSTRACT

221 extracts from various parts of 33 Indian medicinal plants were tested to identify their protease inhibitor potential. The aqueous extract of *Aporosa lindleyana*, methanol and aqueous extracts of *Baliospermum montanum*, chloroform extract of *Gymnema sylvestre*, aqueous extract of *Hedyotis corymbosa* and aqueous extract of *Plectranthus amboinicus* showed significant inhibition of α-Chymotrypsin, Leucine Amino peptidase and Papain.The aqueous extracts of *Plectranthus amboinicus* exhibited significant inhibition of HIV -1 specific Protease. A biology guided fractionation methodology devised in this study pinpointed the flavonoid bioactive fraction PA-9 to exhibit inhibition of HIV-1 protease at 100µg/ml.

Keywords: flavonoid, *Plectranthus amboinicus*

INTRODUCTION

HIV pandemic has led the research community into an urgent pursuit to understand the mechanism of viral spread and devise methods for its control. HIV-reverse transcriptase, protease and integrase enzymes have proven to be good targets for antiretroviral therapy[1].Well known synthetic chemical compounds, as well as, natural compounds derived from aquatic and terrestrial flora and fauna have been identified with anti HIV and immunostimulating activity[2-5]. The effect of various cytokines like IFN, IL4 and TNF on HIV life cycle have been well documented[6,7]. Compounds derived from sponge *Hippospongia sp, Petrosia sp, and Verongia sp* have shown to have RT Inhibitory potentials[8]. Studies on kaempferol and its derivatives extracted from the methanol extract *Securigera securidaca* has shown anti HIV properties[9]. In a study, digallic acid has been reported to inhibit HIV RT[10]. MAP 30 from *Momordica charantia* and GAP 31 from and *Gelonium multiflorum* are well known plant proteins exhibiting inhibition of HIV-1 infection *ex-vivo* in T-lymphocytes and monocytes[11]. Recently, a flavonoid, myricetin[12] has been reported to inhibit HIV infection in an *in-vitro* model. In the present study 221 extracts from different parts of Indian medicinal plants were prepared based on documented antimicrobial and immunomodulating properties[13,14]. The extracts were tested for potential to inhibit α-Chymotrypsin, Leucine Aminopeptidase , Papain and HIV-1 protease.

MATERIAL AND METHOD

Preparation of extracts

The parts of plants of interest were collected, washed, air dried in shade and powdered. The extraction of bioactive components was carried out both sequentially and separately. In sequential extraction the solvents were added as per ascending order of polarity. The extracts were resuspended in either dimethyl sulphoxide (DMSO), phosphate buffered saline (PBS, pH 7.2) or individual solvents as per requirement of the assay. The sequence of extraction is given in Fig 1.

Protease inhibition

Protease inhibitor potential of plant extracts were assayed using previously described protocols with modification[15]. Briefly, Known concentrations of the extract to be assayed was pre incubated with the respective enzymes -α-chymotrypsin ex-porcine pancreas, leucine amino peptidase cytosolic, ex-porcine kidney , and papain ex-papaya latex (Sigma chemical Co., St. Louis, MO, US) at 37°C for 30 minutes in Tris-HCL buffer (pH7.6).Subsequently the respective substrates, N-succinyl-phenylalanine- 4-nitroanilide, L-leucine-4-nitroanilide and Benzoyl-D L – arginine-4 nitroanilide (Sigma chemical Co., St. Louis, MO, US) were added to respective wells and read immediately at 504nm.The OD was followed up for 30 minutes. All reactions were normalized using solvent control.

Percentage Inhibition

$$\% \text{ Inhibition} = \frac{OD_{\text{extract control}} - OD_{\text{positive control}}}{OD_{\text{negative control}}^{*} - OD_{\text{positive control}}}$$

*OD of negative control= 2 SD + Mean of Buffer control, Substrate control and Enzyme control.

HIV 1– Protease inhibition

The cleavage of peptide substrate Acetyl-Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2 by HIV -1 protease was tested as per previously described protocol with modifications (16). Briefly, the reaction mix containing 10mM Sodium acetate (pH 5.0), known concentration of test extract, HIV-1

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Table 1: MIC and percentage activity of extracts found to inhibit protease

S. No	Name of the Plant	α-Chymotrypsin	Leucine Aminopeptidase	Papain
1	*Aporosa lindleyana*	M: 400µ g/ml 57%	Aq:400µ g/ml 52% M: 400µ g/ml 60%	Aq: 400µg/ml 57% M: 400µ g/ml 57%
2	*Baliospermum montanum*	M: 400µ g/ml 52% Aq: 400 µ g/ml 60%	M: 400µ g/ml 50% Aq: 400 µ g/ml 57%	Aq: 400µg/ml 67% M: 400µ g/ml 62%
3	*Gymnema sylvestre*	C: 200µ g/ml 72%	C: 200µ g/ml 57%	C: 200µ g/ml 52%
4	*Hedyotis corymbosa*	Aq: 200µ g/ml 53%	Aq: 200 µ g/ml 59%	Aq: 200µg/ml 62%
6	*Plectranthus amboinicus*	Aq: 400µ g/ml 60%	Aq: 400 µ g/ml 52%	Aq: 400µg/ml 52%

M = Methanol, Aq = Aqueous, C = chloroform

Table 2: Percentage inhibition and Minimum Inhibitory concentration of extracts showing HIV –1 Protease inhibition

S. No	Plant extract used	Percentage Inhibition	MIC (µ g/ ml)
1	Acetyl Pepstatin	58%	30µg/ml
2	*Aporosa lindleyana (Aq)*	Negative	-
3	*Aporosa lindleyana (M)*	Negative	-
4	*Baliospermum montanum* (Aq)	Negative	-
5	*Baliospermum montanum* (M)	Negative	-
6	*Hedyotis corymbosa* (Aq)	Negative	-
7	*Plectranthus amboinicus* (Aq)	63%	100µg/ml
8	*Gymnema sylvestre* (C)	Negative	-

M = Methanol, Aq = Aqueous, C= chloroform

Table 3: Phytochemical analysis of bioactive fraction of PA-9

Compound tested for	Name of the test	Inference
Alkaloids	1. Mayer's test 2. Wagner's test 3. Dragendroff's test	Negative
Triterpenoids	1.Libermann-Buchard 2. Thionyl chloride Test	Negative
Flavonoids	1.Lead acetate test 2.Test using sodium hydroxide	Both Positive
Phenol	Ferric chloride test	Negative
Saponin	Foam test	Negative
Sugar	Fehling's test	Negative
Anthroquinone	Test with benzene	Negative
Amino acid	Nin hydrin test	Negative
Sterols	1. Salkowski test 2. Liebermann-Buchard	Negative

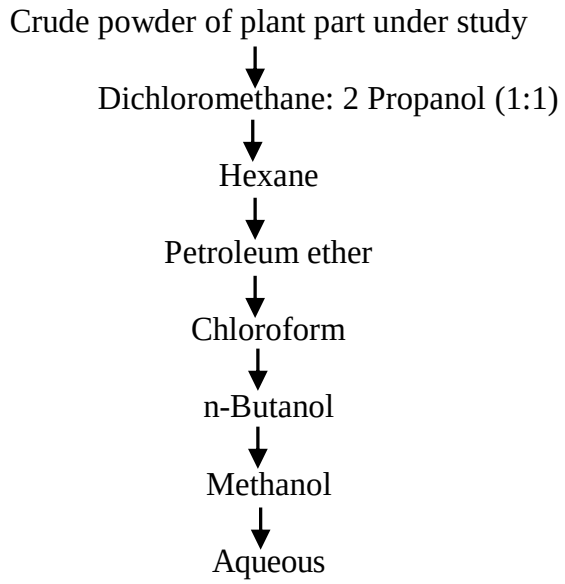

Figure 1: Sequential extraction of plant material in solvents based on polarity

Substrate (0.4µ g/ml) and 2 U HIV-1 Protease (Sigma) were incubated at 37°C for 2 hrs. TFA was added and the hydrolysate was subjected to HPLC analysis (Shimadzu Asia Pacific PTE Ltd, Singapore).The mobile phase constituted of acetonitrile: 0.1% trifluoro acetic acid. Acetyl pepstatin was used as the inhibitory control.

Interpretation of percentage inhibition

$$\% \text{ Inhibition} = \frac{\text{Product peak area of control} - \text{Product peak area of sample}}{\text{Product peak area of control}}$$

The concentration of extract showing 50% inhibition was taken as its IC_{50} value.

Fractionation of plant extract

Silica gel column chromatography

The aqueous extract was treated with n-Butanol and water in the ratio of 1:1, evaporated, washed with petroleum ether and extracted with water. The resulting extract was filter sterilized and the sterile filtrate was added onto a silica gel column. Methanol: water (30:70) was used as mobile phase. The fractions were collected and screened for HIV-1 protease inhibitor potential. The bioactive fractions were further sub- fractionated using LC18 column using methanol: water as mobile phase and the sub fractions further tested for protease inhibitor activity.

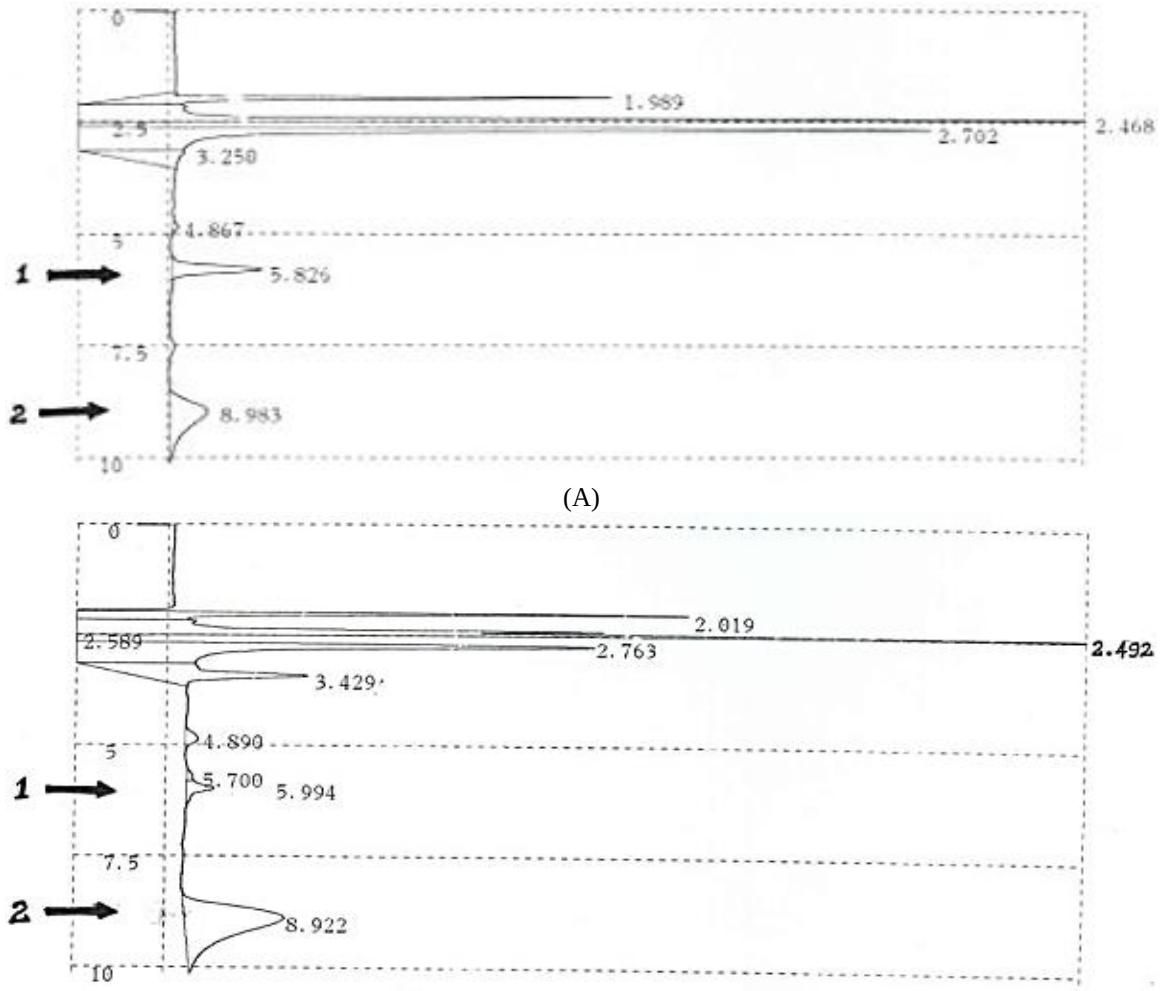

Figure 2: HIV-1 Protease inhibition by PA-9 (A) HIV-1protease activity on peptide substrate with lysed hydrolysate and unlysed substrate peaks at retention time 5.8 and 8.9 minutes respectively (B) Reduction in HIV-1 protease activity by plant extract.

Phytochemical identification

The extracts were prepared for phytochemical identification by refluxing with 2N HCl in methanol, concentrated and saponified with 5% KOH in ethanol, dried and resuspended in water followed by chloroform extraction. These extracts were used for identification of flavonoids, phenol, alkaloids, triterpenoids, saponin, tannins, sugars, anthroquinone, amino Acids and sterol using standard tests[17,18].

RESULTS

A total of 221 extracts were prepared from parts of 33Indian medicinal plants using seven solvents and tested for protease inhibitor potential.The aqueous extract of *Aporosa lindleyana*, methanol and aqueous extracts of *Baliospermum montanum*, chloroform extract of *Gymnema sylvestre*, aqueous extract of *Hedyotis corymbosa* and aqueous extract of *Plectranthus amboinicus* showed significant inhibition of the protease tested. The inhibition of the three proteases is depicted in Table 1.

HPLC based HIV-1 protease inhibition

The extracts showing significant protease inhibition in previous assay were tested for HIV-1 protease inhibition potential. Table 2 details the activity shown by extracts tested for HIV-1 protease inhibition. The aqueous extract of *Plectranthus amboinicus* showed 63% inhibition of HIV –1 protease at a concentration of 100µ g/ml. Acetyl pepstatin was taken as positive control and had a 58% inhibition at 30µg/ml. Figure 2a-b depicts representative HPLC pattern of peptide substrate during different test conditions.

Biology guided fractionation of bioactive plant extracts

Fractions obtained using column chromatography of aqueous extract of *Plectranthus amboinicus* were air dried and tested for HIV–1 protease inhibitor potential at a concentration of 100µg/ml. Out of the 15 fractions tested; fraction-9 (PA-9) exhibited 56% HIV protease inhibition. (Table3). PA-9 obtained by partial fractionation of aqueous extract of *Plectranthus amboinicus* was further fractionated using High performance liquid chromatography (HPLC). Six sub- fractions were obtained using methanol: water as mobile phase. Among these Sub-fractions, sub fraction- II showed the highest HIV-1 protease inhibition. Phytochemical testing of column fraction 9 of *Plectranthus amboinicus* (PA-9).

Flavonoids were the major components of PA- 9 fraction of *Plectranthus amboinicus.*Table 3 depicts the result of the phytochemical tests carried out on PA-9

DISCUSSION

Though several plants are indexed with possible antimicrobial, immunomodulatory and blood purifying properties in literature, scientific analysis has proved that not all of them possess the properties listed or claimed[19,20]. This study was carried out using well-designed scientific protocols for validating the protease inhibitor properties of Indian medicinal plants, as natural products, with anti HIV properties have been identified from marine and terrestrial sources. In a study by Matsuse *et al*[16], water extract of *Erythroxylum citrifolium*, *Waltheria indica* and methanolic extract of *Xylopia frutescens* showed HIV-1 Protease Inhibition at IC_{50} of 43,48 and 46 µ g/ml respectively. In another study, column fractions mangostin and γmangostin isolated from ethanolic extract of fresh fruit peel of *Garcinia mangostana*[21] were found to inhibit HIV -1 Protease at IC_{50} of 5.12 ± 0.41 µ M and 4.18 ± 0.32µ M respectively. Mitsuya *et al.*[22] screened some low molecular weight compounds for HIV-1 protease inhibitory activity and found a compound which could inhibit viral maturation in Molt-4 cells. Nakashima *et al.*[23] have used indirect immunoflourescence and laser flow cytometric analysis to identify Gemin D, NobotaninB ,Camelliin B and Trapanin B to inhibit virus adsorptionat a concentration of 4.0,0.9,1.0 and 1.3µ g/ml respectively. *Plectranthus barbatus* has been documented with HIV enzyme inhibitory as well as anti-inflammatory potentials24). In a recent study Nutan et al[25] have shown the HIV protease inhibitor activity of ellagic acid & gallic acid from *Lagerstroemia speciosa* L. In our study out of 221 extracts screened for their protease inhibitor potentials,the methanol extract of *Aporosa lindleyana*, the aqueous / methanol extracts of *Baliospermum montanum*, the chloroform extract of *Gymnema sylvestre*, the aqueous extract of *Hedyotis corymbosa* and the aqueous extract of *Plectranthus amboinicus* showed 50- 60% inhibition of general protease at 400µ g/ml.Although the chloroform extract of *Gymnema sylvestre* had significant inhibitory activity, the solvent control consisting of chloroform alone showed 30 % inhibition and hence turned out insignificant. On testing the seven extracts from these four plants for HIV-1 specific protease inhibition, the aqueous extract of *Plectranthus amboinicus* showed 63% inhibition of HIV–1 protease at a concentration of 100µ g/ml, which is significantly lower that than the amount needed to inhibit the other proteases tested. A methodology of biology guided fractionation has been evolved in the study by combining chemical fractionation along with stage wise bio-assay protocol to pinpoint the bioactive fraction of the identified medicinal plant.The biology guided fractionation of the aqueous fraction of *Plectranthus amboinicus* points towards a flavonoid fraction PA-9 to have significant HIV protease inhibitor potential.This is the first study to confirm the inhibition of HIV protease by *Plectranthus amboinicus*. Virus growth inhibitory studies using PA-9 shall be the confirmatory procedure to prove the HIV specific inhibitory activity of this compound.

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