Department of Pharmaceutical Biology, Faculty of Pharmacy, University of Ljubljana, Slovenia

Screening of selected plant extracts for *in vitro* inhibitory activity on HIV-1 reverse transcriptase (HIV-1 RT)

A. Mlinarič, S. Kreft, A. Umek and B. Štrukelj

Methanolic-aqueous extracts of 70 plants were investigated for their ability to inhibit HIV-1 reverse transcriptase activity *in vitro*. Two thirds of the extracts screened showed more than 50% inhibition. Two extracts inhibited the enzyme completely while four exhibited more than 90% inhibition. Tannins as nonspecific HIV-1 RT inhibitors were detected and removed from the extracts. The IC_{50} values of the most potent extracts after the removal of tannins for the HIV-1 RT inhibition are as follows: *Sambucus racemosa* 0.017 mg/ml and *Geranium phaeum* 0.067 mg/ml. Daunomycine was chosen as a standard substance in the non-radioactive immuno assay used for screening. As a result from the future isolation and characterization of these compounds, new leading structures are expectable.

1. Introduction

Enormous efforts have been dedicated to research on compounds that could be applied as therapeutic agents using by the patients infected with the human immunodeficiency virus, the causative agent of AIDS. Three HIV enzymes are essential to the life cycle of the virus: reverse transcriptase, protease and integrase.

HIV-1 reverse transcriptase is one of the possible targets to inhibit the reproduction of the HIV. It causes a transcription of viral RNA into a DNA that is integrated in the host cell and carries information for the formation of new viral particles. Many inhibitors have been and still are discovered while only a small number is used in therapy [1]. These agents are predominantly nucleoside inhibitors.

New lead compounds have to be found due to the high ability of HIV to develop resistance against therapeutic agents. One of the possible methods is screening of different substances, synthetic and natural, on the HIV-1 RT inhibitory activity. Many natural compounds that exhibit strong HIV-1 RT inhibitory activity have been described so far [2–9].

In order to simplify the search, screening strategies and commercially available screening assays have been developed. Besides frequently used radioactive methods also an ELISA based non-radioactive assay was developed. It has proven to be quick, safe and reliable for the screening purposes of our study. From the rich and diverse natural pool of the Slovenian flora, selected plants were used for the screening on HIV-1 RT inhibitory activity.

2. Investigations and results

Table 1 demonstrates that methanolic-aqueous extracts of 70 different plant species belonging to 60 genera were used in our survey. In some cases different species of the same genus were used in order to find possible similarities in their activity and also some known HIV-1 RT inhibitory plants (according to the bibliography searched) were included in our study. 49 extracts expressed more than 50% inhibition while only 4 extracts caused inhibition higher than 90%. The ten most active extracts were tested on the presence of tannins which were removed, if present. The IC_{50} for the three most potent extracts was calculated while for other extracts only % of inhibition is displayed. Extracts of *Sambucus racemosa* and *Geranium phaeum* were the most active.

The results of this study could be a starting point for the isolation of new active principles with potential new structures and properties.

3. Discussion

There are many published papers discussing plants and HIV-1 RT inhibition. We decided to test selected species growing in Slovenia that were not included in screenings yet and also some known HIV-1 RT inhibitory species. This was also an opportunity to test commercially available non-radioactive reverse transcriptase assays. Almost every extract exhibited certain inhibitory activity and to our opinion it is a consequence of present polyphenolic compounds. Therefore only the most potent extracts after detection and removal of tannins were chosen for future isolation and characterization. Daunomycin that is considered as a relatively strong RT inhibitor [10] was chosen as a comparative substance and extracts that are more active (on the w/w ratio) than daunomycin were searched for. The IC_{50} of daunomycin measured in our study was 0.380 mg/ml which is higher than reported [10] but still in the same range.

Species from different genera and families were chosen and also some species that belong to the same family or genus were compared in order to see if there is any intrafamiliar or intrageneal similarity. This was confirmed in some cases, but more research has to be done (three members of Apiaceae family, two species of *Dentaria*, two species of *Ranunculus*, two species of *Euphorbia* for example).

It was interesting to notice that in some cases activation was observed as well *(Allium sativum*, *Citrus paradisi*, *Helleborus niger*, *Vinca minor*, *Viscum album)*. In *Allium sativum* this percentage is remarkable (−56.4%). One reason could be activation of the enzyme reaction, however, the exact mechanism remains to be investigated.

Tannins and other polyphenols may be responsible or at least contribute to the enzyme inhibition. That was obvious after removal of tannins for the following species: *Lysimachia vulgaris*, *Glycyrrhiza glabra*, *Juglans regia*. On the other hand, even after removal, some species (as evident from the Table 2), still possessed inhibitory activity in the range over 70%. This leads to the conclusion that other inhibitory compounds than tannins could be present.

The study was also an evaluation of the assay for testing plant extracts. The assay was optimized regarding the na-

Table 1: Inhibition of methanolic-aqueous extracts of selected plants on HIV-1 RT

Family	Species	Inhibition (%)
Apiaceae	*Daucus carota*	72.3
Apiaceae	*Pastinaca sativa*	61.7
Apiaceae	*Sanicula europaea*	71.3
Apocynaceae	*Vinca minor*	−4.6
Araliaceae	*Hedera helix*	−0.1
Aristolochiaceae	*Asarum europaeum*	59.1
Asclepiadaceae	*Vincetoxicum hirundinaria*	53.9
Asteraceae	*Achillea millefolium*	82.2
Asteraceae	*Artemisia absinthium*	57.2
Asteraceae	*Calendula officinalis*	67.1
Asteraceae	*Centaurea jacea*	88.5
Asteraceae	*Cirsium oleraceum*	79.1
Asteraceae	*Erigeron anuus*	73.8
Asteraceae	*Eupatorium cannabinum*	76.8
Asteraceae	*Matricaria perforata*	79.5
Asteraceae	*Tanacetum vulgare*	80.8
Boraginaceae	*Pulmonaria officinalis*	76.7
Boraginaceae	*Symphytum officinale*	60.7
Boraginaceae	*Symphytum tuberosum*	61.1
Brassicaceae	*Alliaria petiolata*	34.4
Brassicaceae	*Barbarea vulgaris*	34.2
Brassicaceae	*Capsella bursa-pastoris*	73.3
Brassicaceae	*Dentaria bulbifera*	1.7
Brassicaceae	*Dentaria pentaphyllos*	2.5
Caprifoliaceae	*Lonicera caprifolium*	62.7
Caprifoliaceae	*Sambucus nigra*	42.5
Caprifoliaceae	*Sambucus racemosa*	106.4
Cichoriaceae	*Aposeris foetida*	22.9
Crassulaceae	*Sedum maximum*	5.5
Equisetaceae	*Equisetum arvense*	56.1
Equisetaceae	*Equisetum hienale*	65.3
Euphorbiaceae	*Euphorbia amygdaloides*	81.2
Euphorbiaceae	*Euphorbia cyparissias*	78.9
Fabaceae	*Glycyrrhiza glabra*	88.1
Fabaceae	*Lotus corniculatus*	80.3
Gentianaceae	*Centaurium erythraea*	52.3
Gentianaceae	*Gentiana cruciata*	56.2
Geraniaceae	*Geranium phaeum*	107.4
Geraniaceae	*Pelargonium radum*	66.6
Hypericacae	*Hypericum perforatum*	59.5
Juglandaceae	*Juglans regia*	89.5
Lamiaceae	*Clinopodium vulgare*	72.8
Lamiaceae	*Galeobdolon montanum*	7.2
Lamiaceae	*Lamium maculatum*	5.4
Lamiaceae	*Rosmarinus officinalis*	78.2
Lamiaceae	*Stachys palustris*	76.7
Lamiaceae	*Thymus serpyllum*	82.1
Liliaceae	*Allium sativum*	−54.6
Liliaceae	*Allium ursinum*	25.0
Liliaceae	*Polygonatum multiflorum*	44.2
Loranthaceae	*Viscum album*	−8.4
Orchidaceae	*Orchis morio*	43.9
Papaveraceae	*Chelidonium majus*	87.7
Polygonaceae	*Rumex obtusifolius*	86.3
Polypodiaceae	*Asplenium ruta-muraria*	87.7
Polypodiaceae	*Asplenium trichomanes*	54.0
Polypodiaceae	*Polypodium vulgare*	53.9
Primulaceae	*Lysimachia vulgaris*	98.8
Ranunculaceae	*Helleborus niger*	−5.0
Ranunculaceae	*Ranunculus lanuginosus*	57.2
Ranunculaceae	*Ranunculus repens*	55.7
Rubiaceae	*Cruciata laevipes*	89.6
Rubiaceae	*Galium aparine*	48.0
Rubiaceae	*Galium odoratum*	65.1
Rutaceae	*Citrus paradisi*	9.8
Scrophulariaceae	*Veronica verna*	51.5
Solanaceae	*Atropa belladonna*	25.6
Solanaceae	*Scopolia carniolica*	46.7
Taxaceae	*Taxus baccata*	35.0
Thymelaecaceae	*Daphne mezereum*	86.9

Table 2: The ten most active extracts

Family	Species	Inhibitory activity	IC_{50}
Geraniaceae	*Geranium phaeum*	107.4	0.021 mg/ml
		90.4*	0.067 mg/ml
Caprifoliaceae	*Sambucus racemosa*	106.4	0.017 mg/ml
Primulaceae	*Lysimachia vulgaris*	98.8	
		53.2*	
Fabaceae	*Glycyrrhiza glabra*	92.1	
		77.2*	
Rubiaceae	*Cruciata laevipes*	89.6	
Juglandaceae	*Juglans regia*	89.5	
		75.4*	
Asteraceae	*Centaurea jacea*	88.5	
Papaveraceae	*Chelidonium majus*	87.7	
Polypodiaceae	*Asplenium ruta-muraria*	87.7	
Thymelaceae	*Daphne mezereum*	86.9	

(% of inhibition before and after removal of tannins where present; IC_{50} values of the most potent extracts)
*: after removal of tannins

ture of our samples. Extracts were dissolved in 10% DMSO (v/v) aqueous solution [10] instead of lysis buffer recommended by the manufacturer and additional control samples were added to quantify the inhibition caused by the solvent. The assay was found to be very sensitive and the results were accurate and reproducible.

It is difficult to discuss the possible mechanism of enzyme inhibition. Inhibitory substances in screened extracts may act as specific competitive or non-competitive inhibitors or may inhibit the enzyme by binding to the alosteric active center and changing the secondary and tertiary structure of the enzyme. The latter type of inhibitors is rather unspecific and could also inhibit other enzymes [1, 11].

From the species investigated in our study, only few data exists in the literature concerning their anti HIV-1 RT activity. Inhibitory activity of *Glycyrrhiza glabra* [2] and *Chelidonium majus* [4, 10] was reported. Euphorbiaceae is a family with a relatively high number of HIV-1 RT inhibitory species [2, 3, 10, 13]. Extracts from *Euphorbia amygdaloides* and *Euphorbia cyparissias* exhibited approx. 80% inhibition. *Euphorbia myrsinites* contains two tetracyclic diterpene triesters that are inhibitors of HIV-1 RT [3]. *Calendula officinalis* exhibited inhibitory activity that was previously reported [5] and confirmed in our survey (67.1%).

As methanolic-aqueous extracts without any subsequent purification and enrichment procedures were used in our study, the obtained results are remarkable and may indicate the presence of strong inhibitory compounds to be further analyzed.

4. Experimental

4.1. Materials

Daunomycin, methanol, tannic acid and DMSO were obtained from Merck, polyamide (MN-Polyamid SC 6) was obtained from Macherey Nagel, Reverse Transcriptase Assay, non radioactive was purchased from Roche Diagnostics.

4.2. Preparation of plant extracts

The collected plants or their parts were dried (at room temperature for 48 h and subsequently 2 h at 40 °C) and pulverized. The powder (2 g of each) was extracted with 10 ml of 50% methanol. Procedure was as follows: maceration at room temperature for 1 h, extraction in the ultrasonic bath for 15 min, maceration for 12 h and repetition of the procedure in the ultrasonic bath. Extracts were filtered and solvent was evaporated to dryness under low pressure. Dry extract (20 mg) was dissolved in 1 ml of sterile DMSO (10% v/v aqueous solution) and used immediately.

Extracts were prepared from areal parts (stem, leaves and flowers included) with the following exceptions: *Artemisia absinthium* (leaves), *Sambucus nigra* (leaves), *Sambucus racemosa* (fruits), *Glycyrrhiza glabra* (roots), *Juglans regia* (leaves), *Allium sativum* (bulbs), *Citrus paradisi* (fruits), *Taxus baccata* (leaves), *Asplenium ruta-muraria* (green parts), *Asplenium trichomanes* (green parts), *Polypodium vulgare* (green parts).

4.3. HIV-1 RT assay

The assay was performed according to the instructions given by the manufacturer. HIV-1 RT (2.5 ng) and the sample dissolved in DMSO were added in a microtiter plate well. The reaction was started with addition of the reaction mixture containing RNA template/primer hybrid and DIG-dUTP, biotin-dUTP and dTT nucleotides. The mixture was incubated for 1 h at 37 °C. The microtiter plate was washed several times with washing buffer and anti DIG-POD conjugated antibodies were added. The mixture was incubated again for 1 h at 37 °C.

After the incubation period, the wells were washed with washing buffer and the ABTS substrate was added. Absorbance was measured with a Biolise microtiter plate reader at 405 nm. The inhibition rates were calculated in comparison to the inhibitor-free control and corrected in comparison to the enzyme free control.

4.4. Detection and removal of tannins

Both procedures were performed according to Tan et al. [10] and Cardellina et al. [11]. Qualitative determination of tannins was based on the precipitation with gelatine and NaCl after the addition of extract in comparison to the standard solutions of tannic acid.

Tannins were removed from the samples using a column filled with polyamide. The extract (5 mg) was dissolved in 10 µl of DMSO and applied to a column (1.6 × 6 cm) filled with 200 mg of polyamide powder that was soaked in water overnight. Elution was performed with water (1 ml), 50% methanol (1 ml) and absolute methanol (2 × 2.5 ml). Eluate was collected, combined and evaporated to dryness.

4.5. Statistics

Data are presented as mean ± SEM from one experiment with three parallel samples. The IC_{50} values were estimated after transformations of dose-effect curves for the three selected extracts only.

References

1 De Clercq, E: J. Med. Chem. **38**, 2491 (1995)
2 Vlietinck, A. J.; De Bruyne, T.; Apers, S.; Pieters, L.: Planta Med. **64**, 97 (1998)
3 Ng, T. B.; Huang, B.; Fong, W. P.; Yeung, H. W.: Life Sci. **61**, 933 (1997)
4 Colombo, M. L.; Bossio, E.: Pharmacol. Res. **33**, 127 (1996)
5 Kalvatchev, Z.; Walder, R.; Garzaro, D.: Biomed. Pharmacother. **51**, 176 (1997)
6 Lin, Y. M.; Anderson, H.; Flavin, M. T.; Pai, Y. H.; Mata-Greemwood, E.; Pengsuparp, T.; Pezzuto, J. M.; Schinazi, R. F.; Hughes, S. H.; Chen, F. C.: J. Nat. Prod. **60**, 884 (1996)
7 Yoshida, T.; Ito, H.; Hatomo, T.; Kurata, M.; Nakanishi, T.; Inada, A.; Murata, H.; Inatami, Y.; Matsura, N.; Ono, K.; Nakane, H.; Noda, M.; Lang, F. A.; Murata, J.: Chem. Pharm. Bull. **44,** 1436 (1996)
8 Rimando, A. M.; Pezzuto, J. M.; Farnsworth, N. R.; Santisuk, T.; Reutrakul, V.; Kawanishi, K.: J. Nat. Prod. **57**, 896 (1994)
9 Serkedzhieva, I. et al.: Acta Microbiol. Bulg. **22**, 16 (1988)
10 Tan, G. T.: J. Nat. Prod. **54**, 143 (1991)
11 De Clercq, E.: Clin. Microbiol. Rev. **10**, 674 (1997)
12 De Clercq, E.: Clin. Microbiol. Rev. **8**, 200 (1995)
13 el-Mekkawy, S. et al.: Chem. Pharm. Bull. (Tokyo) **43**, 641 (1995)

Received June 25, 1999
Accepted July 30, 1999

Aleš Mlinarič, M. Pharm.
Department of Pharmaceutical Biology
Faculty of Pharmacy
Aškerčeva 7
1000 Ljubljana
Republic of Slovenia