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## Antiprotease activity of selected Slovak medicinal plants

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Fifty-six methanol extracts obtained from the barks, flowers, leaves and stems of 30 Slovak trees, bushes and herbs used in the traditional medicine of the Small Carpathians, Slovakia, have been screened for antiprotease (trypsin, thrombin and urokinase) activity using chromogenic bioassay. In this study, 14 extracts showed the strong inhibition activity to protease trypsin with IC<sub>50</sub> values below 10 µg/mL. The highest inhibition activities were observed for methanol extracts of *Acer platanoides* IC<sub>50</sub> = 1.8 µg/mL, *Rhus typhina* IC<sub>50</sub> = 1.2 µg/mL and *Tamarix gallica* IC<sub>50</sub> = 1.7 µg/mL. However, the results of extracts tested on thrombin were generally different from those observed for trypsin. The most marked inhibition activity to thrombin were estimated for extracts of *Castanea sativa* IC<sub>50</sub> = 73.2 µg/mL, *Larix decidua* IC<sub>50</sub> = 96.9 µg/mL and *Rhus typhina* IC<sub>50</sub> = 20.5 µg/mL. In addition, *Acer platanoides* and *Rhus typhina* were the only extracts which showed inhibition activity to urokinase with IC<sub>50</sub> = 171.1 µg/mL and IC<sub>50</sub> = 38.3 µg/mL, respectively. In addition, *Rhus typhina* showed the broadest spectrum of inhibition activity to all tested serine proteases and seems to be a prospective new source of natural products as inhibitors of serine proteases.

### 1. Introduction

During the last 30 years, the systematic screening of thousands of plants has led to the isolation of numerous compounds with various biological activities (Jantova et al. 2001). Today, with the specter of antibiotic resistance, emerging infectious diseases, and cancers, medicinal plants and phytochemicals continue to provide new structural leads for the chemotherapeutic industry (Balunas and Kinghorn 2005; Setzer and Setzer 2003). Natural compounds have practical advantages with regard to availability, suitability for oral application, regulatory approval and mechanism of action (Tsuda et al. 2004). About 50% of the drugs introduced to the market during the last 20 years are derived directly or indirectly from small biogenic molecules (Vuorela et al. 2004). On the other hand, despite the promise of these alternative drug discovery methods, there is still a shortage of lead compounds progressing into clinical trials. This is especially the case in therapeutic areas such as oncology where natural products play a central role in lead discovery (Butler 2005). Despite the recent interest by pharmaceutical companies and funding organizations in molecular modelling, combinatorial chemistry techniques, medicinal plants, remain an important source of new drugs, new leads, and new chemical entities (Balunas and Kinghorn 2005). Due to the biodiversity, chemodiversity and still unexplored resources of the plant kingdom, plants and trees growing in Slovakia have been screened in order to find new substances with inhibitory activity towards serine proteases. Serine proteases have been shown to play a multifarious role in health and disease (Walker and Lynas 2001). They are important for normal physiological functions in the body, including digestion, normal blood vessel maintenance, angiogenesis, clot

formation and dissolution, bone remodelling and ovulation (Wang 2001; Debela et al. 2006). However, their breakdown can lead to a variety of pathologies, including cancer, pancreatitis and thrombosis (Jedinak et al. 2006). A very prospective strategy on how to control proteases seems to be the development of selective low-molecular weight inhibitors from natural sources with possible lower toxicity. Thus, inhibition of protease activity by these inhibitors represents a promising strategy in combating these pathologies.

### 2. Investigations, results and discussion

A total of 56 extracts derived from 19 plant species collected from the Small Carpathian of Slovakia were screened for the inhibitory activity against three serine proteases (trypsin, thrombin and urokinase) included in regulation of different physiological and pathological processes in human. The results of the antiprotease activity are given in the Table. For a positive control, extract from *Camellia sinensis* was used, a rich source of polyphenols known for their antiprotease activity (Jedinak et al. 2006; Benelli et al. 2002).

Fourteen extracts showed strong inhibition activity to protease trypsin, which is involved in pathogenesis of pancreatitis (Liddle and Nathan 2004) with IC<sub>50</sub> values below 10 µg/ml. The highest inhibition activities were observed for methanol extracts of *Acer campestre* IC<sub>50</sub> = 5.8 µg/ml/leaves, *Acer platanoides* IC<sub>50</sub> = 1.8 µg/ml/bark, *Aesculus hippocastanum* IC<sub>50</sub> = 5.1 µg/ml/bark, *Betula pendula* IC<sub>50</sub> = 4.1 µg/ml/bark, *Rhus typhina* IC<sub>50</sub> = 1.2 µg/ml/leaves and *Tamarix gallica* IC<sub>50</sub> = 1.7 µg/ml/bark. All these extracts showed much higher

**Table: Inhibitory effects of Small Carpathian plant extracts on serine proteases**

Family	Plant	Extract	IC <sub>50</sub> µg/ml of trypsin	IC <sub>50</sub> µg/ml of thrombin	IC <sub>50</sub> µg/ml of urokinase
Aceraceae	<i>Acer campestre</i> L.	L	5.8 ± 0.1	-	-
		B	14.8 ± 0.2	-	-
	<i>Acer platanoides</i> L.	L	25.2 ± 0.2	-	-
		B	1.8 ± 0.1	-	171.1 ± 0.2
Anacardiaceae	<i>Rhus typhina</i> L.	B	5.8 ± 0.1	66.3 ± 0.2	38.3 ± 0.2
		L	1.2 ± 0.2	20.5 ± 0.2	43.8 ± 0.3
		F	6.3 ± 0.1	50.3 ± 0.3	233.0 ± 0.3
Araliaceae	<i>Hedera helix</i> L.	S	-	-	-
		L	-	-	-
Asteraceae	<i>Tanacetum vulgare</i> L.	L	23.4 ± 0.3	-	-
		F	31.8 ± 0.2	-	-
	<i>Taraxacum officinale</i> Web.	L	-	-	-
Betulaceae	<i>Alnus glutinosa</i> L.	L	38.1 ± 0.3	-	-
		B	879.1 ± 0.2	-	-
	<i>Betula pendula</i> Roth.	B	4.1 ± 0.1	-	-
		L	13.4 ± 0.2	-	-
Caprifoliaceae	<i>Sambucus ebulus</i> L.	L	80.3 ± 0.2	365.0	-
	<i>Sambucus nigra</i> L.	L	96.5 ± 0.2	-	-
		B	33.7 ± 0.2	-	-
Equisetaceae	<i>Equisetum arvense</i> L.	S	-	-	-
Fagaceae	<i>Castanea sativa</i> Mill.	L	24.4 ± 0.2	216.8 ± 0.2	-
		B	8.0 ± 0.3	73.2 ± 0.3	-
	<i>Quercus petraea</i> L.	B	12.6 ± 0.2	-	-
		L	9.8 ± 0.1	-	-
Hippocastanaceae	<i>Aesculus hippocastanum</i> L.	L	889.8 ± 0.4	-	-
		B	5.1 ± 0.2	-	-
Juglandaceae	<i>Juglans regia</i> L.	L	42.6 ± 0.2	-	-
		B	11.3 ± 0.3	-	-
Magnoliaceae	<i>Magnolia x soulangiana</i> Soul.-Bod.	L	-	-	-
Oleaceae	<i>Fraxinus excelsior</i> L.	B	-	-	-
		B	318,8 ± 0.1	545.3 ± 0.3	-
		L	84.5 ± 0.2	170.1 ± 0.3	-
	<i>Syringa vulgaris</i> L.	L	-	-	-
		B	-	-	-
Pinaceae	<i>Larix decidua</i> Mill.	L	133.7 ± 0.2	349.0 ± 0.3	-
		B	6.2 ± 0.3	96.9 ± 0.2	-
	<i>Pinus sylvestris</i> L.	L	10.9 ± 0.1	-	-
		B	15.2 ± 0.2	-	-
Plantaginaceae	<i>Plantago lanceolata</i> L.	L	199.4 ± 0.3	-	-
Rosaceae	<i>Crataegus oxyacantha</i> L.	L	25.1 ± 0.1	-	-
		B	17.5 ± 0.2	-	-
	<i>Fragaria vesca</i> L.	L	123.0 ± 0.4	-	-
		B	9.4 ± 0.1	-	-
	<i>Prunus spinosa</i> L.	L	970.2 ± 0.2	882.5 ± 0.2	-
		L	17.0 ± 0.3	414.2 ± 0.3	-
		S	8.7 ± 0.2	-	-
Salicaceae	<i>Populus nigra</i> L.	B	35.8 ± 0.1	-	-
		L	46.5 ± 0.3	-	-
Salicaceae	<i>Salix alba</i> L.	L	43.9 ± 0.1	-	-
		B	219.7 ± 0.3	-	-
Scrophulariaceae	<i>Linaria vulgaris</i> Mill.	L	-	-	-
		S	-	-	-
Tamaricaceae	<i>Tamarix gallica</i> L.	L	5.3 ± 0.1	-	-
		B	1.7 ± 0.1	-	-
Theaceae	<i>Camellia sinensis</i> L.	L	9.6 ± 0.2	136.9 ± 0.3	276.6 ± 0.2
Tiliaceae	<i>Tilia platyphyllo</i> Scop.	B	17.7 ± 0.2	-	-
		L	-	-	-
	<i>Quercetin (standard)</i>		1.8 ± 0.1	10.8 ± 0.1	24.1 ± 0.1
	<i>Nafamostat mesylate</i>		0.0005 ± 0.00001	0.0005 ± 0.00001	0.0265 ± 0.001
	<i>(control)</i>				

Data are the mean ± SD of triplicate determinations  
 - not detected (activity over 1000 µg/ml)  
 L – leaves, F – flower, B – bark, S – stems

inhibition activity than the positive control, an extract from a green tea *C. sinensis*  $IC_{50} = 9.6 \mu\text{g/ml/leaves}$ . Moreover, *A. platanoides*, *R. typhina* and *T. gallica* showed stronger or comparable inhibition activity to trypsin than standard flavonoid quercetin  $IC_{50} = 1.8 \mu\text{g/ml}$ , which is a potent trypsin inhibitor (Jedinak et al. 2006). In addition, the inhibitory activities of *A. hippocastanum* and *B. pendula* correspond with the use of these plants in Slovak traditional medicine for diseases of the gastrointestinal tract (Kresanek and Krejca 1988). Strong trypsin inhibitory activity of extracts from *A. campestre*, *A. platanoides* and *R. typhina* are reported for the first time. Moreover, we also studied the antiprotease activity of extracts considering the selective effect.

From the results, there is evidence of considerable protease inhibition activity in selected extracts. The extracts of *A. hippocastanum*, *B. pendula*, *Q. petraea*, and *T. gallica* showed strong selective inhibition activity to trypsin. No significant inhibition activities of these extracts were observed to thrombin and urokinase. Therefore, these extracts may be potential sources of trypsin inhibitors.

On the other hand, the results of extracts tested on thrombin as one of the possible drug targets in treatment of thromboembolic disorders (Katira et al. 2005) are generally different from those observed for trypsin. The most marked inhibition activity to thrombin were estimated for extracts of *Castanea sativa*  $IC_{50} = 73.2 \mu\text{g/ml/bark}$ , *Larix decidua*  $IC_{50} = 96.9 \mu\text{g/ml/bark}$  and *Rhus typhina*  $IC_{50} = 20.5 \mu\text{g/ml/leaves}$ . However, only moderate inhibition activity compared to trypsin was observed for these extracts. The highest inhibition activity was observed for *R. typhina* which showed six times higher inhibition activity than the positive control, *C. sinensis*. On the other hand, *R. typhina* showed weaker inhibition activity to thrombin than the standard quercetin. Moreover, *C. sativa* and *L. decidua* are used in Slovak traditional medicine for treatment of respiratory diseases (Kresanek and Krejca 1988) and marked inhibition activity to thrombin of *C. sativa*, *L. decidua* and *R. typhina* are reported for the first time.

In addition, *Acer platanoides* and *Rhus typhina* were the only extracts which showed inhibition activity to urokinase as a rational drug target for the treatment of cancer and metastasis (Katz et al. 2000). *R. typhina* showed the best inhibition activity to urokinase  $IC_{50} = 38.3 \mu\text{g/ml/bark}$ , which was seven times higher than that of the positive control, however weaker than standard quercetin. Furthermore, *R. typhina* showed the broadest spectrum of inhibition activity to all tested serine proteases. Marked inhibition activity of *A. platanoides* and *R. typhina* are reported for the first time. *R. typhina* belongs to a genus (*Rhus*) that contains over 250 individual species of flowering plants in the family Anacardiaceae (Rayne and Mazza 2007). The research of sumac to date indicates a promising potential for this plant family. It has been reported that *R. typhina* contains mainly tannins (Niemetz and Gross 2005) and the flavonoids fustin, quercetin, sulfuretin, fisetin, which could be responsible for the antioxidant and antitumorogenic activity of Staghorn sumac (Bate-Smith 1962; Plouvier 1970; Young 1976). Thus, the chemical constituents found in previous studies may also be responsible for the observed antiprotease activity of *R. typhina*. In addition, molecular docking studies demonstrated that polyphenols can be accommodated in specific binding sites found on serine proteases in the proximity of the active site, whereas larger polyphenols were reported to non-specifically hinder the catalytic pocket of serine proteases (Cucciolini et al. 2009; Maliar et al. 2004). Therefore, derivation of such a polyphenol-based molecule capable of modulating numerous serine proteases involved in complex cascade mechanisms, such as carcinogenesis, blood coagulation cascade or digestive processes could lead to development of multitarget inhibitor of

serine proteases in co-treatment of pathologies without significant toxic effects (Cucciolini et al. 2009). The significance of protease inhibitors from herbal drugs is that they might offer an alternative approach to synthetic inhibitors, which very often have side effects and poor bioavailability. Polyphenols, particularly tannins, regulate digestive enzymes in the gastrointestinal tract by formation of tannin-enzyme complexes (Longstaff and McNab 1991). However, polyphenols are mainly excreted by stool, urine or are absorbed in the small intestine, followed by distribution to the plasma and tissues, where proteases might be regulated by these compounds (Erlund et al. 2000; El Mohsen et al. 2004). Therefore, herbs and medicinal plants containing bioactive molecules can modulate coagulation, fibrinolytic, complement, and digestive processes via protease inhibition, thus preventing disease progression.

In conclusion, present results demonstrate that *R. typhina* may be potential source for developing serine protease inhibitors. Nevertheless, further investigations and identification of the antiprotease active components are needed.

### 3. Experimental

#### 3.1. Chemicals

Quercetin, Tris-HCl, and dimethylsulfoxide (DMSO) were purchased from Fluka, Switzerland;  $N\alpha$ -benzoyl-D,L-arginine-paranitroanilide hydrochloride {BAPNA}, trypsin from porcine pancreas (2000BAEE units/mg), glycine-arginine parnitroanilide dihydrochloride (GAPNA.2HCl), urokinase from human kidney cells (10 000 Plough units/mg),  $N\alpha$ -benzoyl-phenylalanyl-valyl-arginine-paranitroanilide (BPVA-pNA), thrombin (51 NIH units/mg) and Nafamostat mesylate were purchased from Sigma, USA. A photometric microplate reader MRX (Dyner, USA) was used for the *in vitro* enzyme assay and related equipment (Revelation 2.01 software, USA).

#### 3.2. Collection of material from Slovak trees, bushes and herbs

The material from trees, bushes and herbs (fresh drug) was collected (the list is given in the Table) in the period of October/November of 2004 around the town Modra in the Small Carpathian Mountains of Slovakia. The samples were identified according to the Atlas of Medicinal Herbs and Forest Fruits (Kresanek and Krejca 1988) and herbarium voucher specimens were deposited in the Biology Department of the VULM a.s. The green tea (Lipton) was purchased from a local market in Pezinok, Slovakia.

#### 3.3. Extract preparation

The fresh drug (10 g) was extracted in 100 ml of 80% methanol for 24 h at room temperature in the dark. Macerate was filtered, completely evaporated and dissolved in 0.8 ml of methanol and 3.2 ml distilled water, then extracted through Sep-Pak C<sub>18</sub> column (Waters, USA). Active chlorophyll was separated by washing Sep-Pak C<sub>18</sub> column with distilled water (5 ml). Extract was then eluted by 75% methanol, completely evaporated, weighted, dissolved in DMSO (Fluka, Switzerland) and stored at +4 °C.

#### 3.4. Antiprotease bioassay

For determination of trypsin/thrombin/urokinase inhibition activity by herb extracts a photometry method was used, using chromogenic substrates BAPNA for trypsin, GAPNA.2HCl for urokinase, and BPVA-pNA for thrombin. The substrates are cleaved by trypsin, thrombin, urokinase and at the end of the incubation period, the absorption was determined with a plate reader at 410 nm as previously described (Erlanger et al. 1961; Nieuwenhuizen et al. 1977; Jedinak et al. 2006). Each extract was tested at least five times and the  $IC_{50}$  value represents an average of three independent experiments. The value of  $IC_{50}$  was calculated according the equation:

$$\Delta OD = (OD \text{ at } 410 \text{ nm in } 61\text{st minute}) - (OD \text{ in the } 1\text{st minute})$$

OD – optical density

$$\% \text{ of inhibition activity} = ((1 - \Delta OD \text{ sample} / \Delta OD \text{ control}) * 100)$$

$$\log IC_{50} = ((50 - a) / b) \text{ from equation:}$$

$$\% \text{ of inhibition activity} = a + b * \log IC_{50} (\text{sample})$$

a – intercept point of the regression

b – slope of the regression

$$IC_{50} = 10^{\exp(\log IC_{50})}$$

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