Antiurease activity of plants growing in the Czech Republic

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The antiurease activity of the aqueous extracts of 42 plants growing in the Czech Republic was investigated. A phenol–hypochlorite reaction was used for the determination of ammonia produced by urease. The inhibitory activity of the extracts at a concentration of 0.2 mg/mL varied from 17.8% to 80.0%. Extracts from six Potentilla species expressed inhibitory activity against jack bean urease. They were further investigated for their phenolic constituents and the major compounds were subjected to molecular docking. The results revealed that both jack bean urease and Helicobacter pylori urease were inhibited by quercetin-3-O-b-D-galactopyranoside-6-O-gallate (1), myricetin-3-O-b-D-glucuronide (2), tiliroside (3) and B-type procyanidin (4). The antiurease activity of the investigated Potentilla species is probably due to the presence of complex phenolic constituents such as flavonoid glycosides and catechin dimers.

Keywords: docking; phenolic constituents; phenol–hypochlorite; Potentilla species; urease

1. Introduction

Urease (urea amidohydrolase; EC 3.5.1.5) catalyses the hydrolysis of urea to produce carbon dioxide and ammonia (Mobley et al. 1995). Ureases occur in plants, bacteria, fungi, algae and invertebrates (Krajewska 2009). The presence of urease is a known virulence factor for a number of bacteria. Helicobacter pylori is a ureolytic bacteria that infects the intestinal tract and causes gastric and duodenal ulcers. The inhibition of urease would prevent these bacteria from alkalisling their environment. The well-known inorganic urease inhibitors, hydroxamic acid and its derivatives, have been shown to be reversible, slow-binding inhibitors of both plant and bacterial ureases (Kobashi 1994; Mobley et al. 1995; Upadhay 2012). Unfortunately, acetyloxhydroxamic acid is associated with severe adverse effects (Thomas & Tolley 2008). A number of plant extracts have been investigated for their antiurease activity (Ghous et al. 2010). The aim of this work was to find new and potentially stronger plant urease inhibitors and identify compounds that are responsible for the inhibitory activity.

2. Results and discussion

A total of 42 aqueous plant extracts were assayed for their antiurease activity using the phenol–hypochlorite method. It was found (Table S1) that the extracts at a concentration 0.2 mg/mL...
possessed at least some antiurease activity (17.8–80.0% inhibition). Water extracts were prepared in order to obtain water-soluble compounds, as urease works only in aqueous media. Water extract from *Chrysanthemum balsamita* expressed significant antiurease activity (80.0%). Several extracts indicated inhibitory activity higher than the standard of acetohydroxamic acid (72.6% inhibition): *Allium ursinum* (79.2%), *Hyssopus officinalis* (74.3%), *Meum athamanticum* (74.0%), *Potentilla argentea* (76.0%), *Salvia sclarea* (78.0%), *Solidago virgaurea* (75.6%) and *Yucca filamentosa* (79.0%). Seven plant extracts from the family Lamiaceae showed an activity that varied from 61.1% to 74.3%. Four species from *Potentilla* genus (*Potentilla anserina*, *Potentilla erecta*, *Potentilla fruticosa* and *Potentilla argentea*) revealed inhibitory activity similar to the standard (72.7–73.7%), while two other species (*Potentilla arenaria* and *Potentilla aurea*) expressed lower activity (44.4% and 56.9%, respectively) (Table S1). The genus *Potentilla* (Rosaceae) comprises more than 300 species and is widely distributed in Northern temperate regions (Delgado et al. 2000). Plants from the genus *Potentilla* possess many biological activities (Tomczyk & Latte 2009). Herbs from *Potentilla* genus are commonly used in the treatment of gastric ulcers. In most cases (70%), gastric ulcers are caused by *H. pylori* infection (Sung et al. 1995). Urease is one of its virulence factors and allows the bacteria to survive under strong acidic conditions of the stomach. It is possible that *Potentilla* extracts can inhibit *H. pylori* through the inhibition of urease. This fact would explain the use of *Potentilla* spp. in treating gastric ulcers. Phytochemical analysis of water extracts of *Potentilla* species revealed the presence of complex phenolic compounds such as tannins and flavonoids (Table S2). The compounds were separated on Ascentis Express RP-Amide column and characterised based on UV–Vis, mass spectra, MS fragmentation behaviour and comparison with the literature (Poon 1998; Tomczyk & Latte 2009; Kajdzanoska et al. 2010; Moilanen et al. 2013). The major peak (Figure S2) in four *Potentilla* species (*P. anserina*, *P. argentea*, *P. erecta* and *P. fruticosa*) was identified as quercetin-3-O-β-D-galactopyranoside-6′-gallate (1) which has been previously isolated from *P. fruticosa* (Ganenko et al. 1991). UV spectrum with absorption maxima 254 and 368 nm, molecular ion in the negative mode at *m/z* 615 with two fragments at *m/z* 463 to M-galloyl residue and *m/z* 301 (M-galloyl, M-hexose) confirmed its presence. Major peaks in *P. arenaria* were assigned to isorhamnetin-diglucuronide with [M−H]− *m/z* 667 and luteolin-diglucuronide with [M−H]− *m/z* 637. Quercetin-3-O-β-D-galactopyranoside-6′-gallate (1) was also found in a significant amount. The UV spectra were typical for this type of compounds. Major peak in *P. aurea* extract was assigned to myricetin-3-O-β-D-glucuronide (2). Absorption maxima were 256 nm for band B and 354 nm for band A, [M−H]− *m/z* 493 with 317 fragment that corresponds with the molecular weight of the myricetin aglycone. This compound has been previously isolated from *P. anserina* (Tomczyk & Latte 2009). It was also found as a minor constituent in *P. anserina* and *P. arenaria*. Other dominant peaks were attributed to flavonoid glycosides. Quercetin, kaempferol, luteolin and isorhamnetin were identified as aglycones with various sugar units (hexose, pentose or glucuronic acid). Tiliroside (3) that has been previously identified in *Potentilla* species (Xue et al. 2005; Tomczyk & Latte 2009) was found in *P. anserina*, *P. argentea* and *P. fruticosa*. A number of flavonoid glycosides were found in all the extracts. The highest number was found in *P. fruticosa* extract. A number of HHDP-glucose (hexahydroxydiphenyl-glucose), bis-HHDP-glucose and HHDP-galloyl-glucose molecules were found in all the extracts. HHDP- and galloyl glucoses are the biosynthetic precursors for ellagitannins; therefore, they are present in the tannin-containing extracts (Moilanen et al. 2013). Catechin dimers (B-type procyanidins (4)) were also found in small amounts in the aerial part of all *Potentilla* species. Major compounds 1–4 (Figure S1) were subjected to molecular docking. They exhibited perfect fitting inside the binding pocket of jack bean urease (Figure 1) with good docking scores (−5.9 kcal/mol for 1, −3.6 kcal/mol for 2, −5.6 kcal/mol for 3 and −5.7 kcal/mol for 4). The protein–ligand interaction profile revealed the importance of the hydrogen bonding between the phenolic hydroxyl groups and the amino acid residues of the
active site. This binding demonstrates the ability of these key structures to interact with the enzyme, which supports the experimental findings. Because of the known relationship between the plant and *H. pylori* ureases, we docked our key molecules into the predicted binding pocket of the bacterial enzyme. The molecules exhibited better fitting (Figure S3) and docking scores (−6.9 kcal/mol for 1, −7.2 kcal/mol for 2, −8.9 kcal/mol for 3 and −6.9 kcal/mol for 4). We compared the 2D interaction fingerprints (Figure S4) of compound 1 binding modes in both the enzymes. The galloyl moiety goes deep into the small pocket of the jack bean urease and the flavonoid moiety is facing an electrostatic rich pocket. While in case of *H. pylori* urease, the galloyl moiety does not fit into the deeper pocket and it forms a strong hydrogen bonding ALA 120. The flavonoid moiety fits well in a hydrophobic cleft. In general, we noticed higher binding affinity of these compounds towards *H. pylori* urease.

3. Experimental

3.1. General

HPLC separation was performed on Ascentis Express RP-Amide column (column length 100 mm × 2.1 mm i.d., particle size 2.7 μm) at a temperature of 40°C using an Agilent 1100 instrument equipped with Diode Array Detector (DAD) (Agilent Technologies, Böblingen, Germany). The injection volume was 5 μL and the UV detector was set to 254, 280 and 350 nm. The mobile phase for the gradient elution was a mixture of acetonitrile and 0.2% formic acid,
and the flow rate was 0.3 mL/min. For LC–MS detection, a Hewlett Packard Agilent 1100 Series LC/MS (Agilent Technologies) equipped with DAD and Mass Spectroscopy Detector trap with an electrospray ion source was used. Chromatographic separation was performed on the same column as mentioned earlier. The nebulising and drying gas was high-purity nitrogen ($t = 350^\circ$C) flowing at a rate of 10 L/min, the nebuliser pressure was 40 psi and the needle voltage was 3.5 kV. The full mass scan covered the range from $m/z$ 50 to 1000. Full scan mass spectra and MS/MS of the selected pseudomolecular ion were collected in the negative mode.

3.2. Plant materials

Plant materials were collected in the flowering period from May to August 2012 in the Medicinal Herbs Centre of Masaryk University and in the arboretum of Mendel University of Agriculture and Forestry in Brno, Czech Republic. The materials were identified by Dr Milan Žemlička from the Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic. A voucher specimen (PAC-07–PAC-54) has been deposited at the herbarium of the Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic. The plant material was air-dried. One gram of each part of the plant material was well crushed, mixed with 20 mL of water and put in a microwave extractor (Milestone, Shelton, CT, USA) for 30 min at 120°C. The liquid was then filtered using a cotton filter and the residual water was removed using a freeze-dryer (CHRIST, Osterode, Germany). The lyophilised samples were dissolved just before testing in distilled water to a concentration of 1 mg/mL (stock solution).

3.3. Urease inhibition assay – phenol–hypochlorite method

This spectrophotometric assay is based on the detection of ammonia released during urea hydrolysis. Ammonia reacts with phenol–hypochlorite at high pH forming blue indophenol (Weatherburn 1967). A reaction mixture comprising 60 $\mu$L of enzyme solution (jack bean urease type IX from *Canavalia ensiformis*, 15 $\mu$g/mL, purchased from Sigma, St. Louis, MO, USA) and 60 $\mu$L of an inhibitor solution (plant extract at a concentration 1 mg/mL, the concentration in each well was 0.2 mg/mL) was incubated for 3 min at 27°C in 96-well plates. Sixty microlitres of urea water solution (100 $\mu$M) was added to the mixture and incubated for 7 min at 27°C. Urease activity was determined by measuring ammonia production using the phenol–hypochlorite method as described by Weatherburn (1967). Briefly, 60 $\mu$L of phenol reagent (1% (w/v) phenol and 0.005% (w/v) sodium nitroprusside) and 60 $\mu$L of alkali reagent (0.5% (w/v) sodium hydroxide and sodium hypochlorite containing 0.1% active chloride) were added to each well. The increasing absorbance at 630 nm was measured after 30 min, using a Microplate reader (Synergy HT BIOTEK, Higland Park, IL, USA). All reactions were performed in triplicate to a final volume of 300 $\mu$L. Excel 2007 was used for statistical analysis. The standard deviation of each triplicate was <10%.

Percentage inhibitions were calculated using the following formula:

$$UI (\%) = 100 - \frac{A_{\text{testwell}}}{A_{\text{control}}} \times 100.$$

Acetohydroxamic acid (1 mM water solution) was used as a standard urease inhibitor.

3.4. Computational methods

3.4.1. Ligand preparation

We used the Structure PrOtonation and REcognition System (SPORES) (ten Brink & Exner 2009, 2010) for automatic ligand preparation. The atom types, bond orders, connectivity and hybridisation states were assigned from the atomic coordinates. Missing hydrogen atoms were
added. The protonation states were generated, calculated and adjusted according to the calculated pK<sub>a</sub> values. All acceptable tautomers were calculated and held. Stereoisomers were defined.

3.4.2. **Protein preparation**

We used UCSF Chimera (Pettersen et al. 2004) for protein preparation. The crystal structures of the fluoride-inhibited jack bean urease (PDB ID: 4GOA) and of *H. pylori* urease (PDB ID: 1E9Z) were downloaded from the RCSB Protein Data Bank (http://www.rcsb.org/pdb/) (Bernstein et al. 1977). Extraneous atoms, alternate amino acid residue conformations, ligands, ions and solvent molecules were removed. Missing hydrogen atoms and incomplete side chains were added. Atom types were assigned according to Sybyl atom-type labels, and partial charges were adjusted using Amber force field parameters.

3.4.3. **Binding pocket detection**

We used Voronoi tessellation-based open source pocket detection (fpocket) (Le Guilloux et al. 2009) to predict possible ligand-binding pockets (cavities). Based on the calculated pocket score, drugability, solvent accessible surface area (total, polar and apolar), volume and number of alpha spheres, we selected the pocket that exhibited the highest score.

3.4.4. **Molecular docking**

We used the interactive generic evolutionary method for molecular docking tool (Hsu et al. 2011). The binding site was defined by the amino acid residues that were identified by fpocket. The extracted pocket was used for the docking step. The molecular databases were prepared by SPORES and saved in mol2 format. Ligand conformations and orientations relative to the binding pocket were computed using the genetic algorithm in the standard docking method with the following parameters: population size = 200, generations = 80 and number of docking solutions = 3. We generated protein–ligand interaction tables in the form of protein–ligand interaction profiles of electrostatic (E), hydrogen-bonding (H) and van der Waals (V) interactions. The post-docking analysis was performed by k-means and hierarchical clustering methods to get the most useful pharmacological interactions and amino acid residues contributing in these interactions.

4. **Conclusion**

In total, 42 different plant extracts were prepared and tested for their inhibitory activity against jack bean urease *in vitro*. All the extracts expressed some antiurease activity probably due to the presence of phenolics such as flavonoid glycosides and condensed tannins. Extracts from *Potentilla* species that expressed a significant antiurease activity were further investigated for their phenolic constituents. Major compounds were subjected to molecular docking. They fitted in the active site of the enzyme and exhibited an interaction between the active site of jack bean urease and *H. pylori* urease and the compounds, although better docking scores were obtained for *H. pylori* urease. This would explain the inhibitory activity of the extracts. Based on these facts, phenolics such as flavonoid glycosides and catechin dimers are responsible for the antiurease activity observed.

**Supplementary material**

Supplementary material relating to this article is available online.
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