Angiotensin-converting enzyme (ACE), neutral endopeptidase (NEP) and aminopeptidase N (APN) are zinc metallopeptidases located on the outer membrane of different cells (entoenzymes). They play an important role in the metabolism of a number of regulatory peptides of human nervous, cardiovascular, inflammatory, and immune systems. ACE (EC 3.4.15.1) is a dipeptidyl carboxypeptidase that converts angiotensin I to angiotensin II (AngII) and degrades kinins. ACE plays a central role in the rennin-angiotensin-aldosterone system (RAAS) (1). Neutral endopeptidase (NEP; EC 3.4.24.11) catalyses the degradation of variety of renal and CNS-active peptides including substance P, bradykinin, enkephalins, atrial natriuretic peptides, endothelin, and AngII. Selective NEP inhibitors further enhance the increase of atrial natriuretic peptide (ANP) concentration in plasma and urinary excretion of ANP, cyclic GMP and sodium (1, 2). Dual ACE/NEP inhibitors were developed and investigated as a new therapeutic approach in the treatment of hypertension, heart failure and other cardiovascular diseases (3). Interestingly, recently performed research revealed that changes of neutral endopeptidase expression are implicated in the progression of prostate cancer (4). In contrast to NEP and ACE, rather specifically acting enzymes, aminopeptidase N (APN; EC 3.4.11.2) is an α-aminoacylpeptide-hydrolase with a low substrate specificity. The change in expression of APN plays an important role in the invasion and metastasis of cancer cells, and in immuno-modulating activities (5, 6).

The aim of this study was to evaluate inhibitory potency of several plant extracts on metallopeptidases. The medicinal plants selected for this trial are used as diuretics in the treatment of urinary tract diseases or in carciiovascular diseases. *Bucco folium*, *Callunae flos*, *Equiseti herba*, *Graminis rhizoma*, *Hernariae herba*, *Juniperi fructus*, *Linariae herba*, *Maydis stigma*, *Onondis radix*, *Orthosphonis folium*, *Ribes nigri folium*, and *Taraxaci herba* were used as diuretics. *Orthosphonis* and *Ribes nigri* leaves, *Bursae pastoris*, and *Leonuri herbes* have also hypotensive activity (8, 9, 10). *Epilobii angustifolii herba* is used in folk medicine for benign prostate hyperplasia (BPH) (8, 9).

To our knowledge, no study concerning inhibition of metallopeptidases activity by those medical plants has been carried out till now. Presented results may in part clarify the pharmacological activity of those extracts, and support their use in phyto-medicine.

**EXPERIMENTAL**

**Material**

L-leucine-p-nitroanilide, Hip-L-His-L-Leu, Suc-L-Ala-L-Ala-Phe-7-amino-4-methylcoumarin (SAAP-AMC), phosphoramidon and aminopeptidase (leucine aminopeptidase, type IV-S from porcine kidney microsomes) were purchased from Sigma-Aldrich. Ophthalaldehyde was obtained from Merck. Lisinopril was a gift from the Drug Institute (Poland). The source of NEP and ACE was a boar sperm preparation.
Plan material


Preparation of extracts

5 g of powdered plant material was extracted with 70% methanol (1:10, w/v) in an ultrasonic water bath for 1 h at 40°C. After methanol evaporation, the water residue was lyophilized.

Before determination of enzymes activity, lyophilized extracts were dissolved in phosphate or HEPES buffer.

Determination of enzymes activity

Determination of the activity of ACE was performed according to Bormann and Melzig (7). *Hip-His-L-Leu* solution (20 µl, 24 mM in water) was added to 30 µl of phosphate-buffer (83 mM K2HPO4 + 326 mM NaCl, pH 8.3), 50 µl of test extracts solution and 200 µl of ACE (1:300). The reaction was incubated for 30 min (37°C) then stopped with 0.4 M NAOH (1000 µl). Methanolic o-phthalaldehyde solution (2%, 100 µl) was added to produce the fluorescence His-Leu-o-phthalaldehyde complex. This mixture was incubated for 10 min in the absence of light, and terminated by addition of 2 M HCl (300 µl). Fluorescence was measured at λ<sub>excit</sub>=365 nm and λ<sub>emiss</sub>=500 nm. The inhibition rate was calculated by comparison with the control without an inhibitor, considering the absorbance of light by test extracts.

A two-step assay, according to Bormann and Melzig (7) was performed for the determination of the activity of NEP. Lisinopril (50 µl, 8 µM), 50 µl *SAAP-AMC* (400 µM) and 350 µl HEPES-buffer (50 mM + 154 mM NaCl, pH 7.4) with and without test extracts were added and mixed. The first enzymatic reaction was started by addition of 50 µl of APN solution (1:5000 in HEPES-buffer) and incubated for 60 min (37°C). Addition of 800 µl of acetone stopped the reaction. The samples were measured spectrophotometrically at 405 nm to determine the p-nitroaniline formed. The inhibition rate was calculated by comparison with the control without an inhibitor, considering the absorbance of light by test extracts.

Statistics

All results are represented as the mean ± standard error of the mean (SEM) of a least three independent experiments (each performed on duplicate samples). Statistical analysis was performed by Student’s t-test, p<0.05. IC<sub>50</sub> values were obtained from dose-effect curves by linear regression.

RESULTS AND DISCUSSION

*Bucco folium, Callunae flos, Epilobi angustifolii herba, Orthosiphonis folium, and Ribes nigri folium* extracts showed more than 50% inhibition of the tested peptidases activity at 100 µg/ml. Other extracts were much less active (Table 1). Between...
tested enzymes, the neutral endopeptidase was inhibited in the higher extend and in a dose depend manner (Figure 1). Other than Orthosiphonis folium, all active extracts are rich in tannins. Bucco folium, Callunae floss, and Ribes nigri folium contain mainly proanthocyanidins while Epilobii angustifolii herba is rich in ellagitannins (8). The inhibition of ACE by tannins, especially by oligomeric procyanidins is well established (11, 12). Orthosiphonis folium exhibited no activity against ACE but only showed NEP and APN inhibition. Blackcurrant leaves are rich in prodelphinidin dimmer and trimer, which probably demonstrated a higher affinity to neutral endopeptidase and aminopeptidase N than to the angiotensin-converting enzyme (13). Our previous study revealed a high inhibition of NEP activity by oenothein B (IC₅₀=20 µM), a macrocyclic ellagitannin isolated from Epilobii herba. On the other hand the ellagitannin had low activity against ACE (IC₅₀=250 µM) and APN (IC₅₀=165 µM) (14). Ueno et al. have also isolated an ellagitannin: geraniin from Phyllanthus niruri L. as a weak ACE inhibitor (15). Although tannins are known as protein-binding agents, Zhu et al. explained the fact than tannins may bind to protein ligands in a specific manner (16). They tested 20 phenolic compounds representative of proantocyanidins and gallic acid/hexahydroxydiphenic acid derivatives for their ability to inhibit binding the of specific radioactive-labelled ligands to 16 receptors. The most strongly inhibited receptors were β-adrenergic, 5TH₁, and opiate receptors. Some of the compounds showed selectivity for a single or coupled receptors indicating that the activity cannot be explained only by phenolic-protein binding (16). Tannins seem to be responsible for the activity of tested extracts. However the synergistic activity of other compounds cannot be excluded. Polyphenols such as flavonoids are known to inhibit metallopeptidases (7). Significantly, both ellagitannins and procyanidins are bioavailable and may contribute to the activity of herbal remedies in vivo (17, 18). Orthosiphonis stamineus contains highly methoxyolated flavonoids (sinensetin), diterpene esters, and caffeic acid derivatives (rosmarinic acid) (8). In the Bormann and Melzig study on the inhibition of metallopeptidases by flavonoids and related compounds, sinensetin showed no activity, whereas rosmarinic acid was able to inhibit both NEP and ACE (7). Methylripariochromene A, a benzochromene isolated from the leaves has been shown to decrease systolic blood pressure and to increase urinary volume and excretion of Na⁺, K⁺, and Cl⁻ in hypertensive rats after oral administration (10). Orthosiphonis extract activity against ACE and NEP may be due to the presence of a benzochromene derivative or the synergetic activity of several compounds.

**CONCLUSIONS**

Our preliminary investigation demonstrates that pharmacological effects of water-alcoholic extracts of herbal remedies used as diuretics (Ortho-

Table 1. Inhibition of metallopeptidases

<table>
<thead>
<tr>
<th></th>
<th>Inhibition [%] ± SD at 100 µg/ml (IC₅₀ µg/ml)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>ACE</td>
</tr>
<tr>
<td><strong>Bucco fol.</strong></td>
<td>58±4 (78)</td>
</tr>
<tr>
<td><strong>Bursae pastoris hba</strong></td>
<td>13±3</td>
</tr>
<tr>
<td><strong>Callunae fl.</strong></td>
<td>66±8 (69)</td>
</tr>
<tr>
<td><strong>Epilobii ang. hba</strong></td>
<td>16±4</td>
</tr>
<tr>
<td><strong>Equiseti hba</strong></td>
<td>–</td>
</tr>
<tr>
<td><strong>Graminis rh</strong></td>
<td>26±2</td>
</tr>
<tr>
<td><strong>Hernariae hba</strong></td>
<td>–</td>
</tr>
<tr>
<td><strong>Juniperi fr.</strong></td>
<td>15±2</td>
</tr>
<tr>
<td><strong>Lunuri hba</strong></td>
<td>18±8</td>
</tr>
<tr>
<td><strong>Linariae hba</strong></td>
<td>10±4</td>
</tr>
<tr>
<td><strong>Maydis st.</strong></td>
<td>–</td>
</tr>
<tr>
<td><strong>Itononidis rx</strong></td>
<td>22±4</td>
</tr>
<tr>
<td><strong>Orthosiphonis fol.</strong></td>
<td>40±5 (&gt;200)</td>
</tr>
<tr>
<td><strong>Ribes nigri fol.</strong></td>
<td>11±6</td>
</tr>
<tr>
<td><strong>Taraxaci hba</strong></td>
<td>–</td>
</tr>
</tbody>
</table>

– no inhibition
siphonis folium, Bucco Folium, Callunae flos and Ribes nigri folium) and hypotensives (Orthosiphonis folium and Ribes nigri folium) might be related to the inhibition of metallopeptidases. Further studies are needed to determine wich compounds (flavonoids, phenolic acids, tannins or others) may contribute to the pharmacological activity.

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