Studies on the antioxidant properties of extracts from the roots and shoots of two Scutellaria species in human blood plasma

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We determined the in vitro antioxidant activity of methanolic extracts from the shoots and roots of Scutellaria species (S. altissima and S. alpina) against the action of strong oxidants: hydrogen peroxide (H₂O₂) and H₂O₂+Fe²⁺ (donor of hydroxyl radicals) on plasma proteins and lipids. Lipid peroxidation in human plasma was measured by the level of thiobarbituric acid reactive species (TBARS). Protein oxidation was measured by quantitation of thiol group. We observed that the extracts (5–50 µg ml⁻¹) containing phenolic compounds from both Scutellaria species distinctly reduced oxidation of lipids and proteins in human plasma treated with H₂O₂. These results also indicated that the extracts have a protective effect against oxidative damage to the human plasma lipids and proteins by induced hydroxyl radical. The main components of the plant materials analysed were flavonoids, present as aglycones (luteolin) or glycosides (cyanoside, baicalin, wogonoside). In all of the extracts, the phenylethanoid verbascoside was also found. The properties of the tested plant extracts were also compared with the action of a well characterised commercial antioxidant polyphenolic extract from the berries of Aronia melanocarpa (Aronox®). The comparative studies indicated that the analysed plant extracts were comparable to or even more effective in reducing the oxidation processes than the A. melanocarpa extract. The present study suggests that natural extracts from S. altissima and S. alpina have antioxidant activities and, therefore, may be beneficial in the prevention of diseases related to oxidant stress, such as cancer, cardiovascular, and inflammatory diseases.

Key words: antioxidant activity, flavonoid content, oxidative stress, Scutellaria alpina, Scutellaria altissima

Received: 27 November 2014; revised: 29 January, 2015; accepted: 23 March, 2015 available on-line: 26 May, 2015

INTRODUCTION

Numerous studies have shown that reactive oxygen species, including hydroxyl radical (•OH), singlet oxygen (¹O₂), superoxide anion (O₂⁻), peroxyl radical (ROO•), and polycyclic aromatic hydrocarbons (PAH) are highly reactive and toxic molecules that are generated in metabolically active cells. They are very dangerous and can cause oxidative damage to lipids, proteins, including enzymes, and DNA. They have also been linked to the pathogenesis of oxidative diseases, such as atherosclerosis, diabetes, ischemia, Alzheimer’s disease, cancer, and ageing (Halliwell & Gutteridge, 1989).

It is well known that antioxidants can scavenge free radicals. However, some synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), which are the most widely added to food, have recently been restricted because of serious concerns about their carcinogenic potential (Buxiang & Fukuhara, 1997; Sasaki et al., 2002). Therefore, attention has turned to natural products to find natural antioxidants to replace synthetic food additives.

The Scutellaria genus, a member of the mint family, includes about 300 species commonly known as skullcaps, and is widespread in Europe, the United States of America, and East Asia. Many species of this genus and the phenolic compounds isolated from them are widely used in folk and conventional medicine. Studies show that the Scutellaria plants and their active compounds exhibit diverse pharmacological actions, such as antitumor, anti-angiogenesis, hepatoprotective, antioxidant, anticonvulsant, antibacterial, and antiviral activities. They have been used for the treatment of some types of dermatitis, inflammatory, and cardiovascular diseases (Shang et al., 2010).

Flavonoids are the main compounds responsible for the biological activities of the Scutellaria species. The most important compounds isolated from them are baikalin, baicalin, wogonoside, and wogonin. For example, baicalin (Fig. 1a), a flavone glycoside detected in S. altissima L. (Beshko et al., 1975) and S. alpina L. (Kikuchi et al., 1991), exhibits anti-allergic properties and a scavenging effect in protecting erythrocyte membrane from free radical injury (Gao et al., 1999). It protects against aflatoxin-B1-induced liver mutagenesis (de Boer et al., 2005) and H₂O₂-induced liver damage (Zhao et al., 2006). Chang et al. (2002) reported that baicalin inhibits the proliferation of various human hepatoma cell lines. This compound inhibits HIV-1 infection and replication by blocking HIV-1 reverse transcriptase (Kitamura et al., 1998). Wogonoside (Fig. 1b) has a strong activity against lipid peroxidation and an inhibitory effect on histamine and IgE production (Lim, 2003). It inhibits histamine and leukotriene release (Chen et al., 2009), and can also inhibit lipopolysaccharide-induced angiogenesis (Gao, 2009).

Additionally, certain Scutellaria species produce phenylethanoids (verbascoside and martynoside) (Shang et al., 2010). Verbascoside (Fig. 1e) is a very active member of the phenylethanoid group, which exhibits a wide...
spectrum of biological activities, including antibacterial (Shoyama et al., 1986), antiviral (Kernan et al., 1998), anti-leukemic, and cytotoxic activity against a murine cell line (Pettit et al., 1990). The potent anti-inflammatory action of verbascoside is attributed to its ability to inhibit production of pro-inflammatory chemokines at both the transcriptional and translational levels (Georgiev et al., 2012), the activity of cyclooxygenase-2 (COX-2) in a mouse peritoneal macrophage model (Díaz et al., 2004), and of calcineurin, an important regulator of T-cell mediated inflammation (Prescott et al., 2011). Furthermore, an antioxidant activity of verbascoside has previously been described: it has been reported to display radical scavenging activity against diphenylpicrylhydrazyl (DPPH) and hydroxy and superoxide anions (Gao et al., 1999; Kyriakopoulou et al., 2001).

In this paper the antioxidant activity of methanolic extracts from the shoots and roots of S. altissima and S. alpina was determined in vitro. Human plasma contains potential sites for radical formation and destruction. For example, the oxidative stress may alter the hemostatic system, i.e., oxidative damage to plasma proteins involved in blood coagulation may lead to changes in hemostatic process. The ability of extracts from the shoots and roots of S. altissima and S. alpina to protect directly blood plasma components remains poorly recognized. Furthermore, Ultrahigh Performance Liquid Chromatography (UPLC) techniques were employed for quantitative analysis of the main compounds in the extracts from both Scutellaria species: verbascoside, baicalin, wogonoside, luteolin, and cynaroside (luteolin-7-glucoside) (Fig. 1a–c). In these experiments, we also compared the action of the plant extracts tested with the effects of a commercial extract of Aronia melanocarpa (Aronox®), which has various biological activities, including antioxidative properties (Olas et al., 2008; Kędzierska et al., 2009).

MATERIALS AND METHODS

Plant material. The plants used in the studies were grown for two years in field conditions in the Medical Plant Garden of the Department of Pharmacognosy at the Medical University of Łódź. Voucher specimens have been deposited at the Department of Biology and Pharmaceutical Botany, Medical University of Łódź. Seeds of S. altissima were provided by the Garden of Medicinal Plants in Wrocław (Poland) and seeds of S. alpina were provided by the Botanical Garden of the Institute of Ecology and Botany in Vácártót (Hungary). For the experiments, we used the roots and aerial parts of the plants.

Preparation of extracts. The lyophilized plant material (1 g) was pre-extracted with chloroform overnight. After filtration the plant material was extracted three times with 30-ml portions of methanol:water (7:3) for 15 min each in an ultrasonic bath. The extracts were combined and evaporated under reduced pressure.

Phytochemical analysis. The concentration of the main compounds in the extract was determined using an UPLC Agilent Technologies 1290 Infinity UPLC apparatus equipped with a diode array detector (DAD). The mobile phase consisted of 0.1% formic acid in acetonitrile (v/v; solvent A) and 0.1% formic acid in water (v/v; solvent B). A gradient program was applied as follows: 0–15 min from 20 to 50% solvent A, 15.1–17 min 99% solvent A at a flow rate of 0.3 ml min⁻¹. Details of the UPLC procedure were as described previously.

![Figure 1. Chemical structure of phenolic compounds most abundant in extracts of S. altissima and S. alpina](image)

(Grzegorczyk-Karolak et al., 2013). The detection wavelength was set at 320 nm. Baicalin, wogonoside, luteolin, cynaroside, and verbascoside were identified by comparison of their retention times, UV spectra, and mass spectra with those of standards. The compound contents were expressed as mg g⁻¹ of dry weight (wt).

Chemicals. Baicalin was provided by Sigma-Aldrich, wogonoside by ChemFace, luteolin and cynaroside by Roth, and verbascoside by Phytoplan.

Dimethylsulfoxide (DMSO), 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), thiobarbituric acid (TBA), and H₂O₂ were purchased from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade and were provided by commercial suppliers.

The total concentration of phenolics in the commercial A. melanocarpa extract used in this study was 309.6 mg g⁻¹ of extract, including phenolic acids (isomers of chlorogenic acid; 149.2 mg g⁻¹ of extract), anthocyanins (cyanidin-3-galactoside, cyanidin-3-glucoside, cyanidin-3-arabinoside, cyanidin-3-xylloside; 110.7 mg g⁻¹) and flavonoids (quercetin glycosides; 49.7 mg g⁻¹) (Olas et al., 2008; Kędzierska et al., 2009). The HPLC separation of the phenolic-rich extracts from berries of A. melanocarpa has been described (Olas et al., 2008; Kędzierska et al., 2009).

Human plasma. Fresh human plasma was obtained from medication-free, regular donors at the blood bank (Łodż, Poland). Samples of human plasma were incubated under the following conditions with plant extracts at final concentrations of 0.5–50 mg dry extract per ml of plasma (15 min, at 37°C). Stock solutions of dried S. altissima and S. alpina extracts were made in 50% DMSO. The final concentration of DMSO in samples was less than 0.05% and its effects were determined in all experiments. Stock solution of the A. melanocarpa extract (commercial product: Aronox® by Agropharm Ltd, Poland; batch no. 020/2007k) was made in H₂O at a concentration of 5 mg ml⁻¹.
Table 1. Inhibitory effects of extracts from shoots and roots of *S. altissima* and *S. alpina* and a commercial plant extract of *A. melanocarpa* on plasma lipid peroxidation.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Inhibition of lipid peroxidation induced by H$_2$O$_2$ (%)</th>
<th>Inhibition of lipid peroxidation induced by H$_2$O$_2$+Fe$^{2+}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. altissima</em> shoots</td>
<td>25.3 ± 3.7*</td>
<td>46.6 ± 2.5*</td>
</tr>
<tr>
<td><em>S. altissima</em> roots</td>
<td>30.2 ± 6.3*</td>
<td>44.9 ± 4.4*</td>
</tr>
<tr>
<td><em>S. alpina</em> roots</td>
<td>32.6 ± 3.0*</td>
<td>58.7 ± 4.1*</td>
</tr>
<tr>
<td><em>A. melanocarpa</em> fruits</td>
<td>14.5 ± 5.3*</td>
<td>46.5 ± 5.9*</td>
</tr>
</tbody>
</table>

Samples of plasma preincubated with the plant extracts (50 µg ml$^{-1}$, 15 min, at 37°C) were treated with H$_2$O$_2$ or H$_2$O$_2$+Fe$^{2+}$. The results are mean values ± S.E. of 7–9 independent experiments.

The level of oxidative stress biomarkers (TBARS and thiol groups) in untreated human plasma was low: 0.74 ± 0.11 mM TBARS and 0.363 ± 0.011 mM thiol groups. The addition of H$_2$O$_2$, or H$_2$O$_2$+Fe$^{2+}$ to plasma induced oxidative alterations in lipids and proteins (Figs. 2 and 3). The lower concentrations (0.5 and 5 µg ml$^{-1}$) of the extracts from *S. altissima* shoots and roots, and from *S. alpina* shoots did not substantially inhibit the lipid peroxidation induced by H$_2$O$_2$ (Fig. 2A). However, the highest concentration of the extracts (50 µg ml$^{-1}$) significantly reduced the plasma lipid peroxidation induced by H$_2$O$_2$ and especially by H$_2$O$_2$+Fe$^{2+}$ (Figs. 2A and B). The decrease in lipid peroxidation was quantified by measuring the concentration of malondialdehyde (MDA) and thiobarbituric acid-reactive substances (TBARS) using the molar extinction coefficients for MDA: e=156 000 M$^{-1}$ cm$^{-1}$ and for TBARS: e=13 600 M$^{-1}$ cm$^{-1}$ (Ando & Steiner, 1973a; Ando & Steiner, 1973b; Rice-Evans et al., 1991).
The level of thiol groups in plasma proteins increased in plasma treated with the highest concentration (50 µg ml⁻¹) of extracts from *S. altissima* shoots and roots in the presence of *H₂O₂* reached about 60% (Fig. 2B, Table 1). Furthermore, all plant extracts tested effectively diminished oxidation of thiol groups in plasma proteins, with a statistically significant effect for all the concentrations used versus control II (plasma with oxidation inducer and without extract) (Figs. 3A and B).

The results obtained in our studies were compared to the effect of a commercial plant extract from *A. melanocarpa* (50 µg ml⁻¹). This comparison revealed that the inhibition of oxidation (lipid peroxidation induced by *H₂O₂* + Fe²⁺) caused by the extracts from both *S. altissima* and *S. alpina* is similar to that of the *A. melanocarpa* extract (Table 1), and in the case of *H₂O₂* as oxidizing agent even higher. Table 2 shows that the level of thiol groups in plasma proteins protected by the highest concentration (50 µg ml⁻¹) of *S. altissima* (shoots and roots) and of *S. alpina* (shoots and roots) extracts in the presence of *H₂O₂* + Fe²⁺ was higher than for the *A. melanocarpa* extract (50 µg ml⁻¹). The most effective extract in this study was that from the roots of *S. alpina*. The phytochemical analysis of the extract showed a high level of flavonoids typical for the *Scutellaria* genus baicalin and wogonoside, 24.01 and 11.27 mg g⁻¹ dry wt, respectively (Table 3). The baicalin content was similar in the roots of *S. altissima*, while the levels of wogonoside and the other compounds of interest (verbascoside and luteolin) were 2–4 times lower than in *S. alpina* (Table 3). This could be the cause of the slightly higher antioxidant activity that was found for *S. alpina* roots, although it was not always statistically significant (Tables 1 and 2). The inhibition of the lipid oxidation induced by *H₂O₂* + Fe²⁺ was 43.7% for *S. altissima* root extract, and 58.7% for the *S. alpina* one (Table 1). In the case of the level of thiol groups in plasma proteins in the presence of *H₂O₂* + Fe²⁺, it was

Table 2. Comparative effects of extracts from shoots and roots of *S. altissima* and *S. alpina* and a commercial plant extract of *A. melanocarpa* on the level of thiol groups in plasma proteins.

<table>
<thead>
<tr>
<th>Extract</th>
<th>The level of thiol groups in plasma proteins treated with <em>H₂O₂</em> [mM]</th>
<th>The level of thiol groups in plasma proteins treated with <em>H₂O₂</em> + Fe²⁺ [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control I</td>
<td>0.292 ± 0.006³</td>
<td>0.306 ± 0.002⁴</td>
</tr>
<tr>
<td>Control II</td>
<td>0.043 ± 0.003³</td>
<td>0.031 ± 0.003⁴</td>
</tr>
<tr>
<td><em>S. altissima</em> shoots</td>
<td>0.074 ± 0.007⁵</td>
<td>0.104 ± 0.008⁸</td>
</tr>
<tr>
<td><em>S. altissima</em> roots</td>
<td>0.091 ± 0.007⁵</td>
<td>0.118 ± 0.005⁶</td>
</tr>
<tr>
<td><em>S. alpina</em> shoots</td>
<td>0.089 ± 0.004⁴</td>
<td>0.119 ± 0.003⁴</td>
</tr>
<tr>
<td><em>S. alpina</em> roots</td>
<td>0.119 ± 0.004⁴</td>
<td>0.127 ± 0.005⁵</td>
</tr>
<tr>
<td><em>A. melanocarpa</em> fruits</td>
<td>0.086 ± 0.005⁴</td>
<td>0.055 ± 0.004⁴</td>
</tr>
</tbody>
</table>

Samples of plasma preincubated with the plant extracts (50 mg ml⁻¹; 15 min, at 37°C) were treated with *H₂O₂* or *H₂O₂* + Fe²⁺. Controls I and II have the same meaning as in Fig. 3. The results are mean values ± S.E. of 7–9 independent experiments.
The content of baicalin and wogonoside was much lower in the aerial parts of the plants. In the shoots of S. alpina, we found about 4 mg g⁻¹ dry wt of each compound, but only 0.3 mg g⁻¹ dry wt in S. altissima. The verbascoside content in the shoot extracts of both species were also twice lower than in the roots (Table 3). In contrast, luteolin and its 7-glucoside (cynaroside) levels were higher in shoots than in the roots of both Scutellaria species (Table 3). In the roots of S. alpina, we detected 2.6 mg g⁻¹ dry wt luteolin and its glucoside combined, while in the shoots 4.2 mg g⁻¹ dry wt. The corresponding values for the S. altissima organs were 1.4 and 4.4 mg g⁻¹ dry wt.

DISCUSSION

Recently, much attention has been focused on preventive strategies for oxidative stress and related diseases. Many natural compounds present in the human diet can lower the risk of developing diseases such as cancer, and cardiovascular and neurodegenerative disorders.

Our experiments showed that the polyphenol compounds present in the analysed Scutellaria extracts could protect the body against noxious effects of free radicals due to their ability to chelate and oxidize Fe²⁺ ions and/or to directly scavenge the forming free radicals. In this study, the most effective extract was that from the roots of S. alpina. It contained the highest levels of verbascoside and flavonoids typical for plants of the Scutellaria genus: baicalin and wogonoside. Interestingly, in some assays, the shoot extract of this species had a comparable antioxidant activity, although the content of baicalin and wogonoside were six- and three-fold lower in the shoots (Table 3). A similar relationship has been reported previously for other Scutellaria species, such as S. baicalensis, S. tomentosa, and S. wrightii, for which the levels of the compounds were higher in the roots than in the stems and leaves (Islam et al., 2011). Also verbascoside, which can function as an antioxidant, was present at a two-fold higher level in the roots than in the shoots of S. alpina. Only luteolin and its glucoside (cynaroside) were more abundant in S. alpina shoots than in the roots. This raises the question of how the components present in the analysed extracts are related to their antioxidant properties.

Numerous studies evaluating the strong antioxidant capacity of luteolin has been carried out. Luteolin has the ability to both directly reduce free radicals through the donation of electrons and hydrogen atoms, as well as to chelate transition metals, thereby inhibiting their participation in the Fenton reaction and other transition metal-induced oxidative processes. In addition to its ability to directly quench free radicals and chelate metal ions, luteolin has also been found to enhance the activity of antioxidant enzyme systems, such as glutathione reductase, as well as to inhibit pro-oxidant enzymes, such as cyclooxygenase. This compound and its derivatives have been shown to inhibit in vitro lipid peroxidation (Lee et al., 2002).

It is known that the antioxidant activity of flavonoids is strongly related to their structure. Previous reports indicate that the activity of flavonoids depends on the following conditions: (1) the presence of a 3′,4′-dihydroxystructure in the B ring; (2) the presence of a 2,3-double bond in conjunction with the 4-oxo group in the heterocycle, thus allowing for conjugation between the A and B rings; and (3) the presence of 3- and 5-hydroxyl groups in ring A together with a 4-oxo function in rings A and C (Pulido et al., 2000; Rice-Evans et al., 1996). The greater the number of hydroxyl groups in the rings, especially in ring B, the greater the radical scavenging potency of flavonoids. Luteolin fulfills all of the conditions mentioned above. However, baicalin does not meet the first one and, therefore, it is less effective than luteolin and its derivatives in reacting with the most reactive oxygen species (Gao et al., 1999). The presence of significant amounts of the latter compounds in the S. alpina shoots could explain their strong antioxidant activity despite the low content of other flavonoids (baicalin and wogonoside) in comparison to the root extracts.

Although luteolin derivatives display a significantly greater antioxidant activity than other compounds (baicalin, wogonoside, and verbascoside), this did not explain the potent activity of the S. altissima shoot extract, in which very low levels of baicalin and wogonoside, and only moderately high content of luteolin, cynaroside and verbascoside were found (Table 3). Therefore, in further studies it would be worthwhile to carry out a more detailed analysis concerning other compounds present in the extract that could be associated with its activity.

CONCLUSION

Skullcap extracts added at 50 μg ml⁻¹ to blood significantly reduce oxidative stress. Scutellaria extracts containing compounds such as baicalin, wogonoside, luteolin, cynaroside and verbascoside may have some promising effects in vivo since they are good antioxidants in in vitro models, and their presence in the human diet can have protective effects against lipid and protein peroxidation. They can also be useful as protecting factors against diseases associated with oxidative stress. It is important to underline that in our studies the skullcap extracts were found to be a more effective antioxidant than a commercial extract of aronia known for its antioxidant properties.

Acknowledgments

This work was supported by grant 506/1136 from the University of Lodz. Special thanks to Ms. Monika

Table 3. Content of secondary metabolites (flavonoids and verbascoside) in shoots and roots of S. altissima and S. alpina

<table>
<thead>
<tr>
<th>Extract</th>
<th>verbascoside</th>
<th>baicalin</th>
<th>wogonoside</th>
<th>cynaroside</th>
<th>luteolin</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. altissima shoots</td>
<td>1.38 ± 0.09_a</td>
<td>0.27 ± 0.05_a</td>
<td>0.31 ± 0.04_a</td>
<td>2.33 ± 0.41_a</td>
<td>2.05 ± 0.02_a</td>
</tr>
<tr>
<td>S. altissima roots</td>
<td>2.03 ± 0.21_a</td>
<td>22.57 ± 1.41_b</td>
<td>6.21 ± 0.56_b</td>
<td>0.87 ± 0.08_b</td>
<td>0.57 ± 0.05_b</td>
</tr>
<tr>
<td>S. alpina shoots</td>
<td>3.25 ± 0.08_b</td>
<td>4.18 ± 0.08_b</td>
<td>4.11 ± 0.28_b</td>
<td>1.03 ± 0.24_b</td>
<td>3.21 ± 0.10_b</td>
</tr>
<tr>
<td>S. alpina roots</td>
<td>7.63 ± 0.09_a</td>
<td>24.01 ± 0.35_a</td>
<td>11.27 ± 0.46_a</td>
<td>0.68 ± 0.05_a</td>
<td>1.95 ± 0.07_a</td>
</tr>
</tbody>
</table>

The compound contents are expressed as mg g⁻¹ dry weight of plant material. The results are mean values ± S.E. of 8–9 independent experiments.
Olczyk (student of the Faculty of Biology and Environmental Protection, University of Lodz) for skillful technical assistance.

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