Chemical Profiling of *Astragalus membranaceus* Roots (Fish.) Bunge Herbal Preparation and Evaluation of Its Bioactivity

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Abstract

*Astragalus membranaceus* (Fish.) Bunge is a perennial herb distributed in the northern part of China, and its roots, namely, Hang qi, are included as a natural ingredient in dietary supplement formulations commonly used to treat different disorders such as respiratory infections, diabetes, and heart failure. The availability of a simple method for the determination of the quality of *Astragalus* herbal preparations could be a challenging issue for commercial purposes. In this study, a liquid chromatography–mass spectrometry (LC–MS)/MS based approach was used to characterize specialized metabolite recovery of 3 commercial hydroalcoholic extracts of *A. membranaceus* (AMG1, AMG2, AMG3) in addition to a hydroalcoholic extract of *A. membranaceus* root (AST). The hypoglycemic effect, cholinesterase inhibition, and antioxidant activities were also evaluated. Thirty-one compounds, of which 19 polyphenols and 12 saponins, were identified. The extracts were also quantified by using a sensitive and selective Q-Trap system for their content in flavonoids and astragalosides, selecting astragaloside I and IV as chemical markers. From our results, AMG3 preparation (Axtragyl) was the most abundant in terms of both specialized classes of metabolites, showing a fingerprint similar to that of AST. Interestingly, tested enzyme inhibition ability of flavonoids, daidzein (¹¹) and formononetin (¹⁹), reported a higher α-glucosidase inhibition in comparison with that of acarbose used as positive control. The in silico study clarified the interactions among the molecules and the importance of having a free hydroxy group. Moreover, Axtragyl was able to exert protective effects in Caco-2 cells treated with hydrogen peroxide, confirming its ability as a potential protective agent in intestinal injury.

Keywords

*Astragalus membranaceus*, Fabaceae, flavonoids, saponins, LC-MS, bioactivity

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*Astragalus* L. (Fabaceae family) includes more than 2500 different species and represents one of the most important genus of the flowering plants.¹,² Many *Astragalus* species are largely used as herbal preparations in Asia and provide an important economic source in many regions of this continent. In the traditional Chinese medicine (TCM), the dried roots of several species are considered remedies for the treatment of many diseases such as diabetes mellitus, hypertension, cirrhosis, nephritis, and inflammations.³ Different pharmacological properties are reported for *Astragalus* root extracts, particularly hepatoprotective, immunostimulant, antioxidant, neuroprotective, anti-inflammatory, and antiviral activities.⁴ *Astragalus membranaceus* (Fish.) Bunge is a perennial herb distributed in the northern part of China, and its roots, namely, Hang qi, are a natural dietary supplement ingredient commonly used for immunomodulation and to treat a wide variety of diseases and body disorders such as respiratory infections, diabetes, and heart failure. Recent studies highlighted the potential application for peripheral neuroprotection and pain relief.⁶⁻⁸ The major components of Hang qi extracts are polysaccharides, flavonoids, and saponins, named astragalosides.¹⁰

The availability of a simple method for the determination of the quality of *Astragalus* herbal preparations could be a
challenging issue for commercial purposes. Several methods for the analysis of highly complex compound mixtures of *A. membranaceus* roots are currently used. The aim of this work was to study the phytochemical profile by a liquid chromatography–mass spectrometry (LC–MS) method of three commercial hydroalcoholic root extracts of *A. membranaceus* (AMG1, AMG2, AMG3) in addition to a hydroalcoholic extract of *A. membranaceus* root (AST). In particular, the goal was to analyze the main secondary metabolites, polyphenols and saponins, of the different commercial samples of hydroalcoholic *A. membranaceus* root extracts in order to identify the ones with the most abundant bioactive compounds quantity. Moreover, the antioxidant, anticholinesterase, and inhibition of α-glucosidase and α-amylase activity of these herbal preparations were also assessed using in vitro cell-free assays, together with the in silico interaction of the most active compounds. Finally, the possible protective role played by samples on the injurious effect of reactive oxygen metabolites (ROM) against the intestinal epithelium, using Caco-2 human cell line, was investigated.

**Results and Discussion**

**Qualitative and Quantitative Analyses**

To evaluate the phytochemical profile of the 3 different commercial 50% hydroalcoholic extracts of *A. membranaceus* roots (AMG1, AMG2, AMG3) and *A. membranaceus* roots (AST), high resolution (HR)-LC-electrospray ionization (ESI)-MS analyses were carried out. The HR-LC-ESI-MS total ion chromatogram (TIC) of each extract is reported in Figure 1. Chromatograms showed a similar qualitative fingerprint in all samples but different amounts of compounds. The first step was the qualitative determination of the main classes of specialized metabolites; compound identification was achieved through accurate precursor ions and MS tandem experiments. As reported in the literature, the main compounds found in *A. membranaceus* are flavonoids, isoflavonoids, pterocarpans, and saponins.

In the negative ion mode mass spectrum of compounds 1-19 (Figure 2), the observed pattern is in agreement with those of polyphenol derivatives. Compounds 1-2, 4-7, 9, 11-12, 14-15, 17, and 19 belong to isoflavonoid class (Table 1). Compounds 7, 15, and 19 were formononetin derivatives characterized as formononetin 7-O-glucoside (7, \(m/z\) 429.1227 [M-H]-), formononetin O-glucoside-malonate (15, \(m/z\) 515.1195 [M-H]-), and formononetin aglycone (19, \(m/z\) 267.0648 [M - H]-), by the subsequent loss of a hexose (162 Da) and a malonyl group (248 Da), respectively. The same identification criteria were used to identify the other isoflavones 1, 5, 14, as calycosin derivatives with different glycosylation moieties, based on their MS, MS/MS analyses, and comparison with the literature data. Compounds 1 and 5 were characterized as calycosin 7-O-glucoside (\(m/z\) 445.1130 [M - H]-) and calycosin O-glucoside-malonate (\(m/z\) 531.1141 [M - H]-), respectively; furthermore, compound 14 was identified as calycosin aglycone (\(m/z\) 283.0605 [M - H]-).

**Figure 1.** High resolution liquid chromatography-electrospray ionization-mass spectrometry total ion current chromatograms of the AMG1, AMG2, AMG3, and Astragalus membranaceus root extract (AST).
\[ [M - H]^- \] showed a similar fragmentation pattern compared with calycosin 7-O-glycoside and was identified as pratensein 7-O-glucoside, as previously reported in \( A. \) membranaceus roots, while compound 9 \( (m/z 547.1093 \quad [M - H]^-) \) was identified as pratensein O-glucoside-malonate. The peak ions at \( m/z 463.1586 \quad (t_R \quad 14.52 \) and 21.13 minutes) can be attributed to isomucronulat glucosides isomers (compounds 2 and 12), while compounds 6 and 17 were identified as isomucronulat O-glucoside-malonate isomers \( (m/z 549.1600, [M - H]^-) \). Moreover, compound 11 \( (m/z 253.0499 \quad [M - H]) \) was identified as daidzein. Compounds 3, 13, and 18 were identified as flavonoid derivatives. Notably, compound 3 \( (m/z 579.1709 \quad [M - H], \quad t_R \quad 15.44 \) minutes) showed a fragmentation pattern profile in agreement with naringin; compounds 13 \( (t_R \quad 21.63 \) minutes) and 18 \( (t_R \quad 24.91 \) were identified as rhamnocitrin O-glucoside \( (m/z \quad 461.1067 \quad [M - H]) \) and rhamnocitrin O-glucoside-malonate \( (m/z \quad 547.1093 \quad [M - H]) \), respectively. Pterocarpans 8, 10, and 16 were identified as 9,10-dimethoxypterocarpane-glucoside-xiloside \( (m/z \quad 593.1852 \quad [M - H]) \), 9,10-dimethoxypterocarpane-glucoside \( (m/z \quad 461.1365 \quad [M - H]) \), and 9,10-dimethoxypterocarpane-glucoside-malonate \( (m/z \quad 547.1456 \quad [M - H]) \), respectively, from their MS, MS/MS fragmentation, and literature data.

Compounds 20-31 were identified comparing their high-performance liquid chromatography (HPLC) elution order, HR-MS, and HR-MS/MS data with those previously reported (Figure 2, Table 1). In agreement with previous studies, the chemical composition of the three commercial samples revealed the presence of 10 astragalosides. Compound 20 \( (m/z \quad 945.5022 \quad [M - H]^-, \quad t_R \quad 22.83 \) minutes) showed product ions in MS/MS experiment at \( m/z \quad 813 \quad [M - H - 132]^- \) and 783 \quad [M - H - 162]^- due to the loss of a pentose and a hexose unit, respectively, showing that these two sugar units were both terminal. Other fragments were observed at \( m/z \quad 621 \quad [M - H - 162 - 162]^- \) and 489 \quad [M - H - 132 - 162 - 162]^- identifying 20 as astragaloside VII. Compound 21 \( (m/z \quad 954.5022 \quad [M - H]^-, \quad t_R \quad 25.64 \) minutes) was tentatively attributed to astragaloside VI based on its MS, MS/MS analysis, and comparison with the literature data. Full MS spectra of compound 22 displayed a deprotonated molecule \( [M - H]^- \) at \( m/z \quad 783.4490; \) MS/MS ion spectrum showed ions at \( m/z \quad 651 \quad [M - H - 132], \) 621 \quad [M - H - 162], and 489 \quad [M - H - 162 - 132]^- due to the loss of a glucopyranosyl unit followed by one xylopyranosyl moiety. The identification of 22 as astragaloside IV was confirmed by injection of the standard compound. A very similar fragmentation profile was shown by MS/MS of compound 23 \( (t_R \quad 27.64 \) minutes) obtained at \( m/z \quad 783.4490 \) \quad [M - H]^- \) producing ion at \( m/z \quad 621 \quad [M - H - 162]^- \) and 489 \quad [M - H - 162 - 132]^-, leading to characterize 23 as puerarin III. MS/MS analysis of 3 different ion peaks at \( m/z \quad 825.4610 \quad [M - H]^- \) proved the loss of one acetyl group followed by one hexose; using an injection of a standard compound, the peak at \( t_R \quad 30.16 \) minutes was identified as astragaloside II \( (24), \) while compounds 25 and 27 at \( t_R \quad 31.55 \) and 32.80 minutes, respectively, were characterized as isoastrogladoside II and its isomer. Three different ion peaks at \( m/z \quad 867.4733 \quad [M - H]^- \) were observed; using an injection of standard compounds, the peak at \( t_R \quad 35.68 \) minutes was identified as isoastragaloside I \( (28), \) while compounds 29 and 31 minutes at \( t_R \quad 37.10 \) and 39.16 minutes, respectively, were tentatively identified, based on full mass and retention time, as isoastragaloside I and its isomer differing for the position of an acetyl group. The peak 30 was marked as acetylasortagaloside I with a deprotonated ion at \( m/z \quad 909.4829 \quad [M - H]^- \) and a fragment ion at \( m/z \quad 849 \quad [M - H - 60]^-. \) Compound 26 \( (t_R \quad 32.67 \) minutes) was identified as soyasaponin I based on its deprotonated ion peak at \( m/z \quad 941.5081 \quad [M - H]^-, \) fragment ion at \( m/z \quad 795 \quad [M - H - 146]^-, \) and literature data (Table 1).
| No | t_R (min) | Formula | MW (Da) | MS/MS | AMG 1 | AMG 2 | AMG 3 | AST | Compound Notes |
|----|-----------|---------|---------|-------|-------|-------|-------|-----|----------------|
| 1  | 11.67     | C_{22}H_{22}O_{10} | 445.1130 | 283 [M − H − 162] − | +     | +     | +     | +   | Calycosin-7-O-glucoside |
| 2  | 14.52     | C_{23}H_{28}O_{10} | 463.1586 | 301 [M − H − 162] − | +     | +     | +     | +   | Isomucronulatol-7-O-glucoside |
| 3  | 15.71     | C_{26}H_{32}O_{14} | 579.1709 | 271 [M − H − 162 − 146] − | +     | +     | +     | +   | Naringin |
| 4  | 16.17     | C_{27}H_{32}O_{14} | 549.1600 | 301 [M − H − 248] − | +     | +     | +     | +   | Pratensein-7-O-glucoside |
| 5  | 17.59     | C_{26}H_{30}O_{13} | 429.1227 | 267 [M − H − 162] − | +     | +     | +     | +   | Isomucronulatol-7-O-glucoside-malonate |
| 6  | 18.35     | C_{25}H_{24}O_{13} | 475.1093 | 299 [M − H − 248] − | +     | +     | +     | +   | Formononetin-7-O-glucoside |
| 7  | 18.74     | C_{22}H_{22}O_{11} | 461.1067 | 299 [M − H − 294] − | +     | +     | +     | +   | 9,10-Dimethoxypterocarpane-3-O-xylosylglucoside |
| 8  | 19.71     | C_{22}H_{22}O_{11} | 539.1852 | 299 [M − H − 248] − | +     | +     | +     | +   | Pratensein-7-O-glucoside-malonate |
| 9  | 20.04     | C_{25}H_{24}O_{13} | 429.1227 | 267 [M − H − 162] − | +     | +     | +     | +   | Formononetin-7-O-glucoside-malonate isomer |
| 10 | 20.90     | C_{15}H_{10}O_{4}  | 253.0499 | 224     | +     | +     | +     | +   | Daidzein |
| 11 | 21.13     | C_{22}H_{28}O_{10} | 463.1586 | 301 [M − H − 162] − | +     | +     | +     | +   | Isomucronulatol-7-O-glucoside-malonate |
| 12 | 21.63     | C_{22}H_{22}O_{11} | 461.1067 | 299 [M − H − 248] − | +     | +     | +     | +   | Formononetin-7-O-glucoside-malonate |
| 13 | 21.90     | C_{26}H_{30}O_{13} | 429.1227 | 267 [M − H − 162] − | +     | +     | +     | +   | Calycosin |
| 14 | 22.36     | C_{26}H_{30}O_{13} | 539.1852 | 299 [M − H − 248] − | +     | +     | +     | +   | Calycosin |
| 15 | 22.63     | C_{22}H_{22}O_{11} | 461.1067 | 299 [M − H − 248] − | +     | +     | +     | +   | Isomucronulatol-7-O-glucoside-malonate |
| 16 | 23.26     | C_{27}H_{32}O_{14} | 549.1600 | 301 [M − H − 248] − | +     | +     | +     | +   | Pratensein-7-O-glucoside-malonate |
| 17 | 24.26     | C_{27}H_{32}O_{14} | 593.1852 | 299 [M − H − 294] − | +     | +     | +     | +   | 9,10-Dimethoxypterocarpane-3-O-xylosylglucoside |
| 18 | 24.91     | C_{22}H_{22}O_{11} | 461.1067 | 299 [M − H − 248] − | +     | +     | +     | +   | Calycosin |
| 19 | 30.32     | C_{16}H_{12}O_{5}  | 253.0499 | 224     | +     | +     | +     | +   | Daidzein |
| 20 | 22.83     | C_{16}H_{12}O_{4}  | 253.0499 | 224     | +     | +     | +     | +   | Formononetin |
| 21 | 25.64     | C_{26}H_{30}O_{13} | 429.1227 | 267 [M − H − 162] − | +     | +     | +     | +   | Astragaloside VII |
| 22 | 27.15     | C_{47}H_{78}O_{19} | 945.5022 | 813 [M − H − 132] −, 783 [M − H − 162] −, 765 [M − H − 162] −, 621 [M − H − 162 − 162] −, 489 [M − H − 162 − 132 − 162] −, 383 [M − H − 162 − 132 − 18 − 18] − | +     | +     | +     | +   | Astragaloside VI |
| 23 | 30.16     | C_{43}H_{70}O_{15} | 825.4610 | 783 [M − H − 42] −, 765 [M − H − 60] −, 663 [M − H − 162] −, 603 [M − H − 60 − 162] −, 489 [M − H − 60 − 162 − 132 − 42] − | +     | +     | +     | +   | Astragaloside II |
| 24 | 31.55     | C_{43}H_{70}O_{15} | 825.4610 | 765 [M − H − 60] −, 603 [M − H − 60 − 162] − | +     | +     | +     | +   | Isoastragaloside II |

(Continued)
Successively, quantitative analysis of the flavonoids and astragalosides fraction was performed in all samples. The flavonoids were quantified as formononetin, whereas astragaloside IV was used to express the saponins amount. In Table 2, the composition of total flavonoids and astragalosides as a percentage of 100 g of extracts is reported. Results of the quantitative analysis indicated that in the AMG3 (Axtragyl), saponins are more abundant compared with the other samples, whereas the flavonoids are the most represented in AST sample compared with the other three, although the flavonoids content in AMG3 was higher than in AMG1 and AMG2. Astragaloside I and astragaloside IV, being the most representative among the saponins, were selected and quantified in all samples as a marker of quality of the commercial batches. In order to obtain accurate data regarding their amounts, a selective and sensitive ultra-performance liquid chromatography-ESI-QTrap-MS/MS method was developed after the direct introduction of the standard to optimize the signal of the analyte. Data were acquired in multiple-reaction monitoring (MRM) mode in triplicate, and the results of quantitative analysis of astragaloside I and astragaloside IV in all the samples are the following: 0.0004 and 0.04 mg/g for AMG1, 0.0003 and 0.011 mg/g for AMG2, 0.24 and 0.84 mg/g for AMG3, and 0.16 and 0.13 mg/g for AST.

### α-Glucosidase and α-Amylase Inhibition Activity

Since *A. membranaceus* root extracts are used in the TCM for the treatment of diabetes mellitus, the hypoglycemic activity of all extracts in comparison to some selected pure compounds was investigated. α-Glucosidase and α-amylase are key enzymes of dietary carbohydrate digestion in humans. Inhibitors of these enzymes may be effective in retarding carbohydrate digestion and glucose absorption to suppress postprandial hyperglycemia. Several molecules are currently used in the clinical practice as antidiabetic drugs including acarbose. These drugs work well by slowing the action of certain enzymes that break down starches and carbohydrates into sugars, but they have known side effects. Several natural compounds have been already demonstrated to possess a similar or even higher ability to inhibit these enzymes than acarbose, that, in this study, has been used as positive control.18

### Table 1. Continued

| No. | AMG 1 | AMG 2 | AMG 3 | AST |
|-----|-------|-------|-------|-----|
| 26  | 32.67 | 32.80 | 35.68 | 37.10 |
| 27  | 32.67 | 32.80 | 35.68 | 37.10 |
| 28  | 32.67 | 32.80 | 35.68 | 37.10 |
| 29  | 32.67 | 32.80 | 35.68 | 37.10 |
| 30  | 32.67 | 32.80 | 35.68 | 37.10 |
| 31  | 32.67 | 32.80 | 35.68 | 37.10 |

| Compound | Formula | [M − H]− | MS/MS AMG 1 | AMG 2 | AMG 3 | AST |
|----------|---------|----------|-------------|-------|-------|-----|
| Soyasaponin I | C48H78O18 | 941.5081 | 795 [M − H − 146]−, 653 [M − H − 146 − 162]−, 491 [M − H − 146 − 162 − 194]− | + | + | + |
| Isoastragaloside II isomer | C45H72O16 | 867.4733 | 707 [M − H − 146]−, 655 [M − H − 146 − 162]−, 503 [M − H − 146 − 162 − 194]− | + | + | + |
| Astragaloside I | C45H72O16 | 867.4733 | 707 [M − H − 146]−, 655 [M − H − 146 − 162]−, 503 [M − H − 146 − 162 − 194]− | + | + | + |
| Isoastragaloside I isomer | C47H74O17 | 909.4829 | 749 [M − H − 146]−, 655 [M − H − 146 − 162]−, 491 [M − H − 146 − 162 − 194]− | + | + | + |
| Acetylastragaloside I | C45H72O16 | 867.4733 | 707 [M − H − 146]−, 655 [M − H − 146 − 162]−, 503 [M − H − 146 − 162 − 194]− | + | + | + |

MS, mass spectrometry.

### Table 2. Content in Percentage (%) of Flavonoids and Astragalosides in 100 g of AMG1, AMG2, AMG3, and *Astragalus membranaceus* Root Extract (AST).

| Sample | Flavonoids | Astragalosides |
|--------|------------|----------------|
| AMG1   | 0.014%     | 0.027%         |
| AMG2   | 0.011%     | 0.056%         |
| AMG3   | 0.12%      | 0.44%          |
| AST    | 0.26%      | 0.36%          |
AMG3 and AMG1 demonstrated the highest inhibitory activity on α-amylase among tested extracts (half-maximal inhibitory concentration [IC₅₀] 495.6 ± 23.8 µg/mL and 679.3 ± 22.1 µg/mL, respectively) but, as expected, lower than acarbose (IC₅₀ 3.5 ± 0.2 µM) (Table 3). No activity was observed in the α-glucosidase assay for all the extracts. Among the pure compounds, the astragalosides were inactive (data not shown), while the isoflavone aglycones showed only a mild activity against α-amylase enzyme at test concentrations. According to previous studies, 19-21 daidzein (11) and its methylated analog formononetin (19) reported higher α-glucosidase inhibition (IC₅₀ 12.9 ± 0.1 µM and 201.7 ± 63.4 µg/mL, respectively) compared with acarbose used as standard (IC₅₀ 126.2 ± 8.1 µM). The chemical structure affects the activity of the compounds and the affinity for the enzyme. 22 The methyl substitution of 4′-OH in 19 seems to negatively influence the activity compared with 11. Moreover, other polyphenols showed negligible activity (data not shown).

**Molecular Docking of Daidzein and Formonoetin Into Human Small Intestine α-Glucosidase**

To get further insight into the inhibitory effects of compounds 11 and 19 on α-glucosidase activity, the structure of the C-terminal domain of human small intestine α-glucosidase was used in docking studies. The docking procedure provides the treatment of conformationally flexible ligand within the active site of the protein with flexible side chains. The results showed that both compounds have the ability to bind to the active sites of α-glucosidase with notable estimated binding energies of −8.9 and −8.3 kcal/mol, respectively. In the top-ranked binding pose solution of the docking with 11, the phenol group enters deep down in the tightest part (a cleft) of the substrate binding pocket in the vicinity to the active site residues and the phenol hydroxyl group makes a hydrogen bond with D1279 (Figure 3a), whereas the second-ranked solution has the “double ring” (7-hydroxycromen-4-one) moiety penetrates into this cleft. In sharp contrast, 19 in the highest-ranking docking solution has the “double ring” moiety inserted in this position of the cleft (Figure 3b) and no solution has the methylated phenol group in the cleft. The best docking solutions of 11 and 19 can be comparable to the position of acarbose found in the crystal structure of human α-glucosidase used in docking; this molecule has the valienamine group in the cleft of the binding pocket (Figure 3a-b). In conclusion, these results demonstrate that both compounds are highly likely to interact in analogy to acarbose with the active site of α-glucosidase. In particular, the phenol group of 11 preferably binds the active site but also the “double ring” has this ability, whereas the additional methylation of the phenol group of 19 prevents its binding to the cleft and therefore only its “double ring” can bind. These differences in binding might explain why 11 has an IC₅₀ value around 16 times lower than that of 19 (Table 3).

**Cholinesterase Inhibition Activity**

According to the published studies on the protective role of *A. membranaceus* in nervous cell models, the modulation of cholinesterase enzyme activity was also investigated. 23 Cholinesterase enzymes, acetylcholinesterase (AChE) in particular, are an important target for the treatment of several neurodegenerative disorders including Alzheimer’s disease (AD). Nowadays, prevention of acetylcholine degradation in synapses is one of the most accepted palliative therapy opportunities for neuroprotection. 24 Since the introduction of the first cholinesterase inhibitor in 1997, most clinicians consider cholinergic drugs such as galantamine used in this study as the reference drug, as first-line pharmacotherapy for mild and moderate AD. 25

In the present study, the inhibition of AChE and butyrylcholinesterase (BChE) enzymes was evaluated for all extracts and pure compounds (Table 4). AMG3 reported the highest AChE inhibition (IC₅₀ 27.9 ± 5.1 µg/mL) at tested concentrations. All extracts showed a similar value of inhibition vs BChE (Table 4). On the contrary, pure compounds showed a low value of inhibition of AchE activity and no activity in the BChE inhibition assay (data not shown).

| Compounds | α-Amylase inhibition (IC₅₀ µg/mL) | α-Glucosidase inhibition (IC₅₀ µg/mL) |
|-----------|----------------------------------|-------------------------------------|
| AMG1      | 679.3 ± 22.1a                    | -                                   |
| AMG2      | 4302.5 ± 77.4b                   | -                                   |
| AMG3      | 195.6 ± 23.8c                    | -                                   |
| AST       | 10.1 ± 0.9f                      | -                                   |
| 11        |                                  | 12.9 ± 0.1a                         |
| 19        |                                  | 201.7 ± 63.4b                       |

IC₅₀, half-maximal inhibitory concentration.

*In this case, it was not possible to reach the IC₅₀ value; the results were expressed as % α-amylase inhibition obtained testing extract at 50 µg/mL.

Acarbose IC₅₀ 9.59 ± 0.90 and 350.3 ± 12.6 µg/mL vs α-amylase and α-glucosidase respectively. Significant differences (*P<0.05*) are represented with different letters.
Figure 3. Docking of daidzein (11) and formononetin (19) in the C-terminal domain of human small intestinal α-glucosidase. Daidzein (a) (in sticks with carbons in cyan) and formononetin (b) (in sticks with carbons in orange) docked into the binding pocket of α-glucosidase (gray spheres with interacting residues in sticks with carbons in green) close to the active site residues (D1420 and D1526) and where acarbose (displayed as lines with carbons in yellow) binds.
Antioxidant Activity

Several studies reported a protective effect of *A. membranaceus* preparations in intestinal injury models. In our study, the antioxidant activity of the four samples was evaluated in differentiated Caco-2 cells. However, preliminary, the cytotoxic potential of the extracts was evaluated in human peripheral blood mononuclear cells (PBMC) from healthy donors. All extracts did not cause any toxic activity at 150 µg/mL. Then, to investigate ROS-induced cytotoxic effects in Caco-2 cells, increasing amounts of H$_2$O$_2$ were added to the medium, bathing the apical side of the cells, and after incubation, cellular alterations were evaluated. Incubation of cells in the presence of a molar concentration of H$_2$O$_2$ resulted in a decrease in Caco-2 viability; after 20 hours of treatment with 10 mmol/L H$_2$O$_2$, about 25% loss of cell viability was observed. Then, the protective effect of the extracts against H$_2$O$_2$-induced injury to the intestinal Caco-2 cells was investigated. When cells were pretreated with each sample before being challenged with 10 mmol/L H$_2$O$_2$, a moderate decrease in cell viability was observed, indicating that the extracts at a dose of 150 µg/L are able to reduce the H$_2$O$_2$-induced toxicity. Among all tested extracts, AMG3 (Axtragyl) was able to improve cell viability even at the lowest tested concentrations (Table 5). Nevertheless, further experiments on different cell model assays will be performed to confirm obtained results, together with in vivo studies directed to the assessment of other biological properties related to *A. membranaceus* root extract.

Conclusions

Herbal extracts should be standardized to ensure safety, quality, and efficacy. In our study, LC–MS/MS-based approach was used to characterize specialized metabolite recovery in 3 different commercial samples of *A. membranaceus* (hydroalcoholic roots extracts) AMG1, AMG2, and AMG3 and compared with the freshly produced hydroalcoholic extract of *A. membranaceus* roots (AST). Thirty-one compounds, of which 19 polyphenols and 12 saponins were identified showing that all extracts possess the typical polyphenols and saponins pattern of *A. membranaceus* roots. Among the extracts, AMG3 commercial sample (Axtragyl) resulted in a higher content of bioactive compounds proven to confer protective benefits. AMG3 showed a fingerprint comparable to that of AST confirming the high degree of reproducibility of the manufacturing process. Regarding the biological assays, the most interesting results come from flavonoids, particularly in the α-glucosidase inhibition activity test, where 11 was found to be up to 20-fold more potent than acarbose. The in silico study elucidated the interaction between 11 and the importance of free hydroxy group: our results showed that the phenol group of 11 is able to preferably bind the active site even though there is a possibility of interaction that concerns the “double ring”.

Experimental

Reagents

Solvents for extraction were purchased from Sigma Chemicals Company (Milan, Italy). LC–MS grade solvents were purchased by Romil Ltd Pure Chemistry (Cambridge, GB). For the quantitative HPLC analysis, the following standards were used: astragaloside IV, European Pharmacopeia (EP) reference standard (97.8%), astragaloside I, Phyproof reference substance ≥98.0% (HPLC), and formononetin, analytical standard.
the injection volume 10.0 µL.

Plant Material

The roots of *A. membranaceus* (Fish.) Bunge were collected from plants cultivated in Sichuan province and Gansu province (China). Axtragyl (Giellepi Health Science, Lissone, Italy) is an *A. membranaceus* root extract manufactured using a selective extraction process that ensures a broad spectrum of natural bioactive compounds. Commercial samples of *A. membranaceus* dried roots 50% hydroalcoholic extract, respectively, renamed AMG1, AMG2, and AMG3 (Axtragyl) were provided by Giellepi.

Extraction and Sample Preparation

The dried roots of *A. membranaceus* (500 g) were powdered and extracted with 50% hydroalcoholic mixture by exhaustive maceration for 48 hours (3 × 2.5 L). The extraction solvent was eliminated under vacuum obtaining almost 68 g of dried hydroalcoholic extract (AST). For qualitative and quantitative analyses, 10 mg of AST, AMG1, AMG2, and AMG3 were dissolved in 1 mL of ultrapure methanol, obtaining a concentration of 10 mg/mL, and, after centrifugation for 5 minutes at 13,000 rpm, the supernatant was subjected to LC-ESI-MS analysis.

LC–MS Qualitative Analysis

The separation system adopted was an Accela (Thermo Fisher Scientific, Milan, Italy) HPLC interfaced through an ESI source to a high-resolution mass analyzer (LTQ-Orbitrap XL, Thermo Fisher Scientific, Milan, Italy). The MS data were acquired, in negative ion mode, at first in full-mass and data dependent-scan mode, then, tandem MS experiments were done in order to identify the specialized metabolites. Capillary temperature was set at 350°C, flow rate of sheath gas and auxiliary gas were set at 30.0 and 10 arbitrary units, capillary voltage was −48.0 V. A C18 column (Luna C18 Phenomenex, 100 × 2.0 mm, 2.5 µm) and a binary mobile phase composed of eluent A (ultrapure water–formic acid 0.1% v/v) and eluent B (ultrapure acetonitrile–formic acid 0.1% v/v) were used. The separation conditions are from 10% to 95% of B in 60 minutes. Flow rate was 0.0200 mL/min and the injection volume 10.0 µL.

LC–MS Quantitative Analysis

Quantification of astragalosides I and IV was carried out using an API6500 Q-Trap (ABSciex Foster City, CA, USA) coupled with an A NexeraX2 UHPLC apparatus (Shimadzu, USA), working in negative MRM mode. All the instrumental parameters were optimized directly injecting solutions containing pure compounds. Samples were loaded on a Kinetex column (Phenomenex) (C18 100 A, 50 mm × 2.6 mm × 2.1 mm), and compounds were separated using a linear gradient from 30% to 55% of acetonitrile (eluent B) and water containing 0.1% formic acid (eluent A) over 10 minutes. The flow rate was 0.35 mL/minute, and the injection volume was 3 µL for standards and samples. To perform accurate quantitative analyses, 9 points (in the range 0.10-2 µg/mL) calibration curves were built for the two astragalosides. The mean values ± standard deviation from at least three experiments showing similar results were reported.

α-Amylase Inhibition Assay

The α-amylase inhibition assay was performed using the iodine/potassium iodide method. Each sample (25 μL) was mixed with the α-amylase solution (50 μL, 5 U/mL) in phosphate buffer (pH 6.9 with 6 mM sodium chloride) in a 96-well microplate and incubated for 10 minutes at 37°C. Then, the reaction was initiated with the addition of the starch solution (100 μL, 0.1%). Similarly, a blank was prepared by adding a sample solution to all reaction reagents without enzyme solution. The reaction mixture was incubated for 10 minutes at 37°C. The reaction was then stopped with the addition of hydrochloric acid (HCl, 25 μL, 0.1 M). This was followed by the addition of a potassium iodide (KI /0.5 mM) solution (100 μL), 0.1%). The absorbance of the sample and blank was read at 630 nm for 10 minutes. Results are expressed as IC_{50} values (µg/mL for extracts and pure compounds) determined by GraphPad Prism 5 Software (San Diego, CA, USA). When it was not possible to reach the IC_{50} % of enzyme inhibition measured at certain concentrations is reported.

α-Glucosidase Inhibition Assay

Different concentration of each sample was incubated with the α-glucosidase solution (40 µL, 0.1 U/mL) in phosphate buffer (50 µL, 0.1 M, pH 7) for 10 minutes. Then, 40 µL of 0.5 mM 4-nitrophenyl α-D-glucopyranoside were added and incubated for 15 minutes. The reaction was stopped by adding 100 µL of 0.2 M sodium carbonate solution. The enzymatic hydrolysis of the substrate was monitored by the amount of p-nitrophenol released in the reaction mixture at 405 nm. Results are expressed as IC_{50} values (µg/mL for extracts and pure compounds) determined by GraphPad Prism 5 Software (San Diego, CA, USA).

In Silico Molecular Docking

Molecular docking of conformationally flexible 11 and 19 into the structure of the C-terminal domain of human α-glucosidase of the small intestine (PDB ID: 3TOP) was performed with AutoDock Vina. Residues of α-glucosidase with flexible side chain conformation were chosen in its substrate-binding
Cell Cultures

PBMC cells were isolated from buffy coats of healthy donors and cell viability evaluated as reported before.32 Caco-2 cells were maintained in DMEM, containing 200 mL/L FCS, 10 mL/L of 100× nonessential amino acids, 2 mmol/L L-glutamine, 5 × 10⁻⁵ IU/L penicillin, 50 mg/L streptomycin at 37°C in a 5% carbon dioxide atmosphere at 90-100% relative humidity. Cells were grown in 10 cm Petri dishes. For experiments, cells were seeded at a density of 90,000 cells/cm² in the apical compartment of the transwell insert by the addition of the oxidative stress-inducing agents. The cytotoxicity of ROM on Caco-2 was assessed by the viability test of neutral red uptake, performed according to the procedure of Fautz et al.33 After oxidative stress induction, the medium in the insert was removed and replaced with 0.1 mL of fresh medium containing 1.14 mmol/L neutral red. At the end of 3 hours of incubation, the medium was removed and cells were washed twice with PBS; finally, the incorporated neutral red was released from cells by incubation for 15 minutes at room temperature in the presence of 1 mL of cell lysis buffer containing acetic acid (1%, v/v) and ethanol (50% v/v). To measure the dye taken up, the cell lysis products were centrifuged and supernatants spectrophotometrically measured at 540 nm.

Statistical Analysis

Data were expressed as mean ± standard deviation. Statistical analysis was performed by analysis of variance followed by Tukey’s test using GraphPad Prism 5 Software, Inc. (San Diego, CA, USA) and a P-value of 0.05 or less was considered as statistically significant. All measurements were performed by using SPECTROstarNano (BMG Labtech, Ortenberg, Germany).

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