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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 membranaceous, 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[5] 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.[6-9] 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

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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.[11]

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.[12] 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]$^-$). Compound **4** (m/z 461.1067

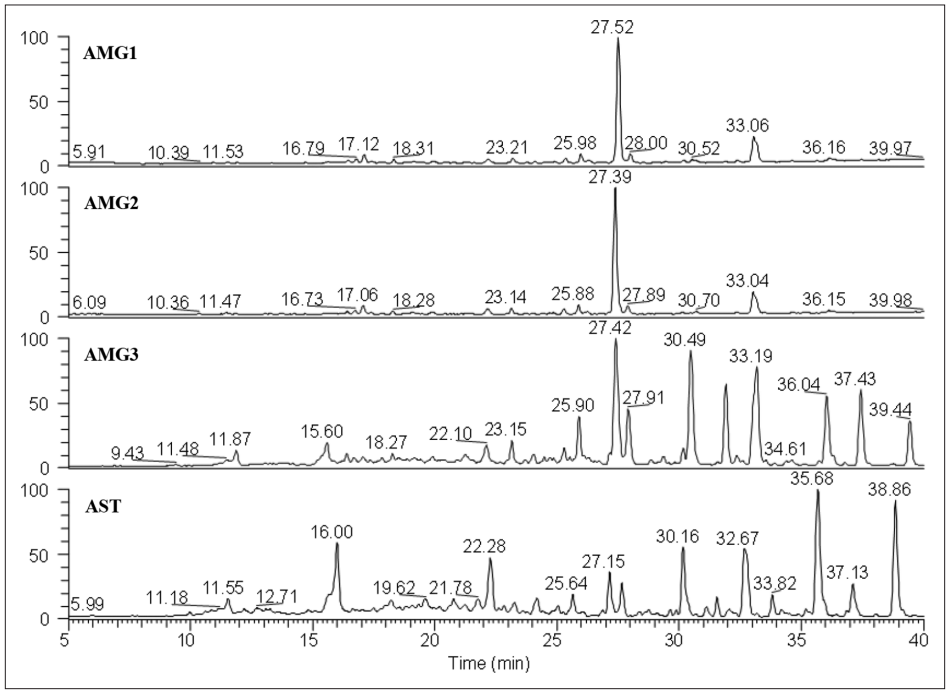

Figure 1. High resolution liquid chromatography-electrospray ionization-mass spectrometry total ion current chromatograms of the AMG1, AMG2, AMG3 , and *Astragalus membranaceus* root extract (AST).

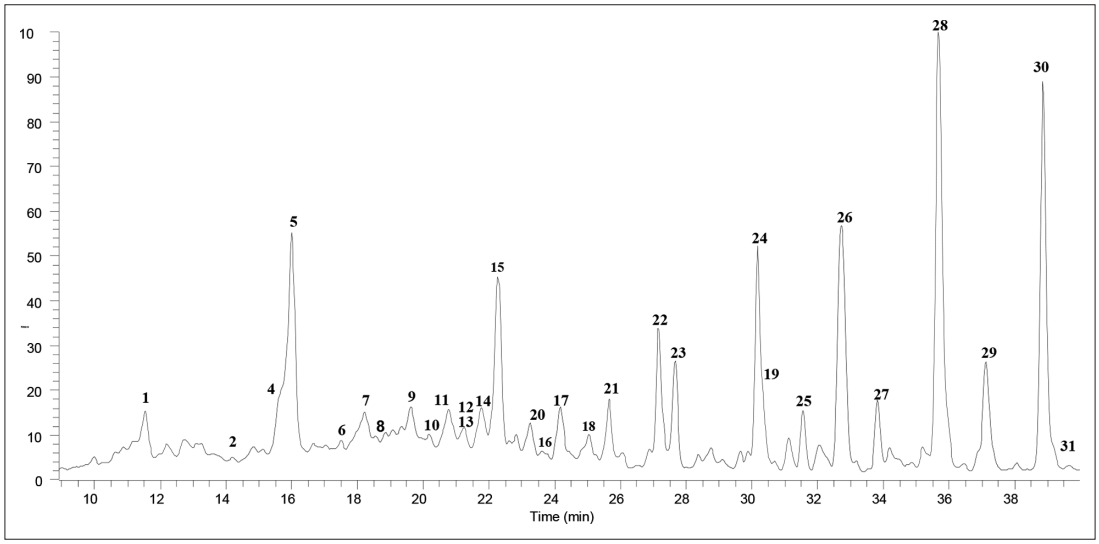

Figure 2. High-performance liquid chromatography-high resolution electrospray ionization-mass spectrometry profile of the polyphenol derivatives and astragalosides in *Astragalus membranaceus* root extract (AST).

[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,[12] while compound **9** (m/z 547.1093 [M − H]$^-$) was identified as pratensein *O*-glucoside-malonate. The peak ions at m/z 463.1586 (t_R 14.52 and 21.13 minutes) can be attributed to isomucronulatol glucosides isomers (compounds **2** and **12**), while compounds **6** and **17** were identified as isomucronulatol *O*-glucoside-malonate isomers (m/z 549.1600, [M − H]$^-$). Moreover, compound **11** (m/z 253.0499 [M − H]$^-$) was identified as daidzein. Compounds **3**, **13**, and **18** were identified as flavonoid derivatives. Notably, compound **3** (m/z 579.1709 [M − H]$^-$, t_R 15.44 minutes) showed a fragmentation pattern profile in agreement with naringin[13]; compounds **13** (t_R 21.63 minutes) and **18** (t_R 24.91) were identified as rhamnocitrin *O*-glucoside (m/z 461.1067 [M − H]$^-$) and rhamnocitrin *O*-glucoside-malonate (m/z 547.1093 [M − H]$^-$), respectively. Pterocarpans **8**, **10**, and **16** were identified as 9,10-dimethoxypterocarpane-glucoside-xiloside (m/z 593.1852 [M − H]$^-$), 9,10-dimethoxypterocarpane-glucoside (m/z 461.1365 [M − H]$^-$), and 9,10-dimethoxypterocarpane-glucoside-malonate (m/z 547.1456) [M − H]$^-$), respectively, from their MS, MS/MS fragmentation, and literature data.[12]

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,[14,15] the chemical composition of the three commercial samples revealed the presence of 10 astragalosides. Compound **20** (m/z 945.5022 [M − H]$^-$, t_R 22.83 minutes) showed product ions in MS/MS experiment at m/z 813 [M − H − 132]$^-$ and 783 [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 621 [M − H − 162 − 162]$^-$ and 489 [M − H − 132 − 162 − 162] identifying **20** as astragaloside VII. Compound **21** (m/z 945.5022 [M − H]$^-$, t_R 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 783.4490; MS/MS ion spectrum showed ions at m/z 651 [M − H − 132]$^-$, 621 [M − H − 162]$^-$, and 489 [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 27.64 minutes) obtained at m/z 783.4490 [M − H]$^-$ producing ion at m/z 621 [M − H − 162]$^-$ and 489 [M − H − 162 − 132]$^-$, leading to characterize **23** as astragaloside III. MS/MS analysis of 3 different ion peaks at m/z 825.4610 [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 30.16 minutes was identified as astragaloside II (**24**), while compounds **25** and **27** at t_R 31.55 and 32.80 minutes, respectively, were characterized as isoastragaloside II and its isomer. Three different ion peaks at m/z 867.4733 [M − H]$^-$ were observed; using an injection of standard compounds, the peak at t_R 35.68 minutes was identified as astragaloside I (**28**), while compounds **29** and **31** minutes at t_R 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 acetylastragaloside I with a deprotonated ion at m/z 909.4829 [M − H]$^-$ and a fragment ion at m/z 849 [M − H − 60]$^-$. Compound **26** (t_R 32.67 minutes) was identified as soyasaponin I based on its deprotonated ion peak at m/z 941.5081 [M − H]$^-$, fragment ion at m/z 795 [M − H − 146]$^-$, and literature data (Table 1).[16]

Table 1. Chromatographic and MS Data of Compounds **1**-**31** Detected in AMG1, AMG2, AMG3, and *Astragalus membranaceus* Root Extract (AST).

No	t_R (min)	Formula	$[M-H]^-$	MS/MS	AMG 1	AMG 2	AMG 3	AST	Compound
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	15.44	$C_{27}H_{32}O_{14}$	579.1709	271 $[M-H-162-146]^-$	+	+	+		Naringin
4	15.71	$C_{22}H_{22}O_{11}$	461.1067	299 $[M-H-162]^-$				+	Pratensein 7-*O*-glucoside
5	16.17	$C_{25}H_{24}O_{13}$	531.1141	283 $[M-H-248]^-$				+	Calycosin 7-*O*-glucoside-malonate
6	17.59	$C_{26}H_{30}O_{13}$	549.1600	301 $[M-H-248]^-$				+	Isomucronulatol *O*-glucoside-malonate isomer
7	18.35	$C_{22}H_{22}O_9$	429.1227	267 $[M-H-162]^-$			+	+	Formononetin 7-*O*-glucoside
8	18.74	$C_{28}H_{34}O_{14}$	593.1852	299 $[M-H-294]^-$	+	+	+	+	9,10-Dimethoxypterocarpane 3-*O*-xylosylglucoside
9	19.71	$C_{25}H_{24}O_{14}$	547.1093	299 $[M-H-248]^-$				+	Pratensein 7-*O*-glucoside-malonate
10	20.04	$C_{23}H_{26}O_{10}$	461.1365	299 $[M-H-162]^-$	+		+	+	9,10-Dimethoxypterocarpane 3-*O*-glucoside
11	20.90	$C_{15}H_{10}O_4$	253.0499	224	+	+	+	+	Daidzein
12	21.13	$C_{23}H_{28}O_{10}$	463.1586	301 $[M-H-162]^-$	+	+	+	+	Isomucronulatol *O*-glucoside isomer
13	21.63	$C_{22}H_{22}O_{11}$	461.1067	299 $[M-H-162]^-$			+	+	Rhamnocitrin *O*-glucoside
14	21.90	$C_{16}H_{12}O_5$	283.0605	268 $[M-H-15]^-$	+	+	+	+	Calycosin
15	22.36	$C_{25}H_{24}O_{12}$	515.1195	267 $[M-H-248]^-$				+	Formononetin 7-*O*-glucoside-malonate
16	23.32	$C_{26}H_{28}O_{13}$	547.1456	299 $[M-H-248]^-$				+	9,10-Dimethoxypterocarpane 3-*O*-glucoside-malonate
17	24.26	$C_{26}H_{30}O_{13}$	549.1600	301 $[M-H-248]^-$				+	Isomucronulatol *O*-glucoside-malonate isomer
18	24.91	$C_{25}H_{24}O_{14}$	547.1093	299 $[M-H-248]^-$				+	Rhamnocitrin *O*-glucoside-malonate
19	30.32	$C_{16}H_{12}O_4$	267.0648	252$[M-H-15]^-$	+	+	+	+	Formononetin
20	22.83	$C_{47}H_{78}O_{19}$	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 VII
21	25.64	$C_{47}H_{78}O_{19}$	945.5022	783 $[M-H-162]^-$	+	+	+	+	Astragaloside VI
22	27.15	$C_{41}H_{68}O_{14}$	783.4490	651 $[M-H-132]^-$, 621 $[M-H-162]^-$, 489 $[M-H-162-132]^-$ 471 $[M-H-162-132-18]^-$	+	+	+	+	Astragaloside IV
23	27.64	$C_{41}H_{68}O_{14}$	783.4490	621 $[M-H-162]^-$, 489 $[M-H-162-132]^-$ 383 $[M-H-162-132-18-18]^-$	+	+	+	+	Astragaloside III
24	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
25	31.55	$C_{43}H_{70}O_{15}$	825.4610	765 $[M-H-60]^-$, 603 $[M-H-60-162]^-$	+	+	+	+	Isoastragaloside II

(Continued)

Table 1. Continued

No	t_R (min)	Formula	$[M-H]^-$	MS/MS	AMG 1	AMG 2	AMG 3	AST	Compound
26	32.67	$C_{48}H_{78}O_{18}$	941.5081	795 $[M-H-146]^-$, 633 $[M-H-146-162]^-$, 439 $[M-H-146-162-194]^-$, 421 $[M-H-146-162-194-18]^-$	+	+	+	+	Soyasaponin I
27	32.80	$C_{43}H_{70}O_{15}$	825.4610	765 $[M-H-60]^-$, 603 $[M-H-60-162]^-$	+	+	+	+	Isoastragaloside II isomer
28	35.68	$C_{45}H_{72}O_{16}$	867.4733	807 $[M-H-60]^-$, 747 $[M-H-60-60]^-$, 645 $[M-H-60-162]^-$	+	+	+	+	Astragaloside I
29	37.10	$C_{45}H_{72}O_{16}$	867.4733	807 $[M-H-60]^-$, 645 $[M-H-60-162]^-$			+	+	Isoastragaloside I
30	38.86	$C_{47}H_{74}O_{17}$	909.4829	849 $[M-H-60]^-$			+	+	Acetylastragaloside I
31	39.16	$C_{45}H_{72}O_{16}$	867.4733	807 $[M-H-60]^-$, 645 $[M-H-60-162]^-$		+	+	+	Isoastragaloside I isomer

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).

	Content (%)	
Sample	Flavonoids	Astragalosides
AMG1	0.014%	0.027%
AMG2	0.011%	0.056%
AMG3	0.12%	0.44%
AST	0.26%	0.36%

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.

a-Glucosidase and a-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.[17] 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 3. α-Amylase and α-Glucosidase Inhibition of AMG1, AMG2, AMG3, *Astragalus membranaceus* Root Extract (AST), and Pure Compounds.

	α-Amylase inhibition (IC_{50} μg/mL)	α-Glucosidase inhibition (IC_{50} μg/mL)
AMG1	679.3 ± 22.1[a]	-
AMG2	4302.5 ± 77.4[b]	-
AMG3	195.6 ± 23.8[c]	-
AST	10.1 ± 0.9[*]	-
11	-	12.9 ± 0.1[a]
19	-	201.7 ± 63.4[b]

IC_{50}, half-maximal inhibitory concentration.
*In this case, it was not possible to reach the IC_{50} value; the results were expressed as % α-amylase inhibition obtained testing extract at 50 μg/mL.
Acarbose IC_{50} 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.

AMG3 and AMG1 demonstrated the highest inhibitory activity on α-amylase among tested extracts (half-maximal inhibitory concentration [IC_{50}] 495.6 ± 23.8 μg/mL and 679.3 ± 22.1 μg/mL, respectively) but, as expected, lower than acarbose (IC_{50} 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 tested concentrations. According to previous studies,[19-21] daidzein (**11**) and its methylated analog formononetin (**19**) reported higher α-glucosidase inhibition (IC_{50} 12.9 ± 0.1 μM and 201.7 ± 13.4 μM, respectively) compared with acarbose used as standard (IC_{50} 226.2 ± 8.1 μM) (Table 3). 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 a-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-rated 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-rated solution has the "double ring" (7-hydroxychromen-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 IC_{50} 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 opportunity 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 (IC_{50} 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).

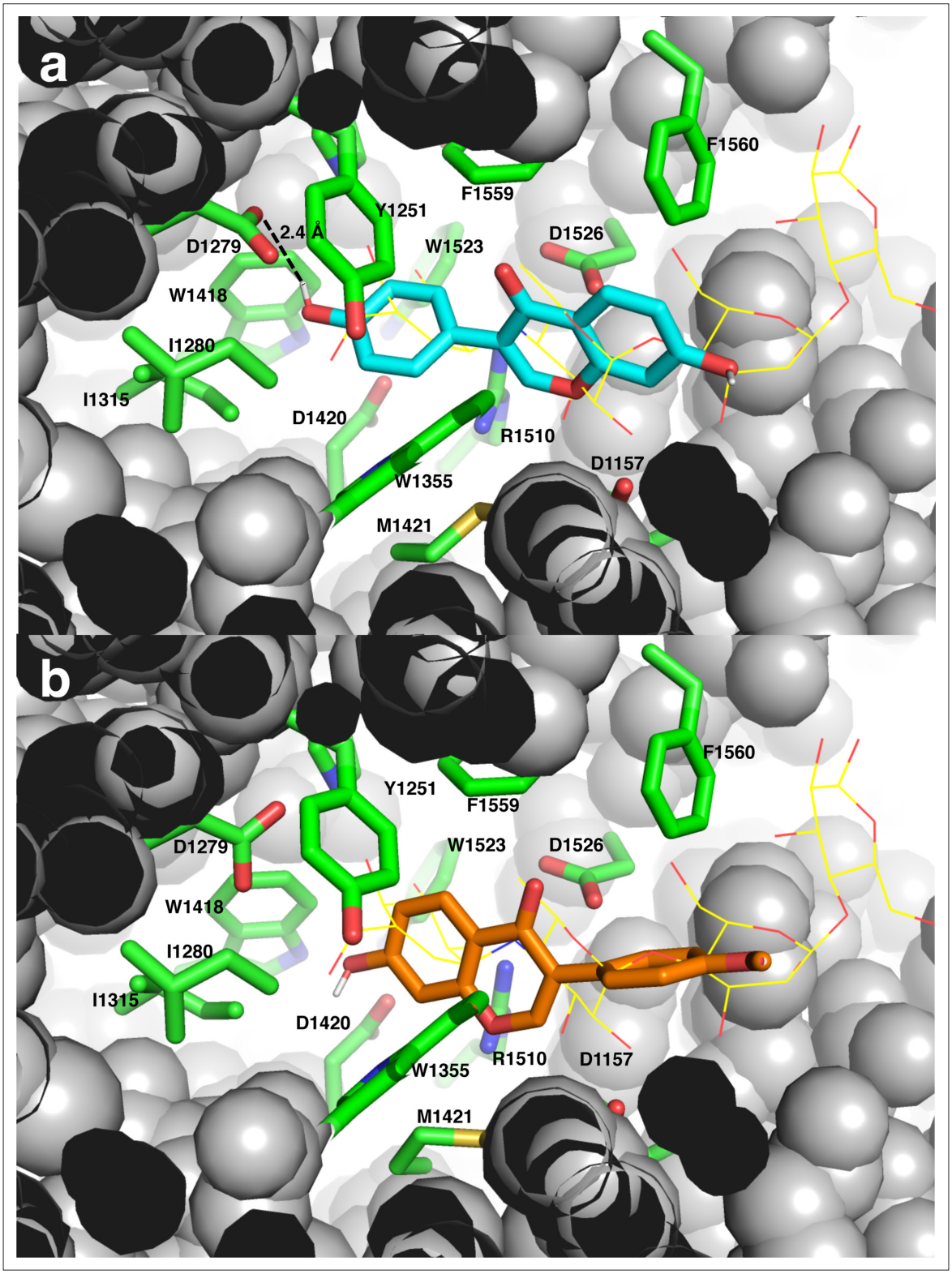

Figure 3. Docking of daidzein (**11**) and formononetin (**19**) in the *C*-terminal domain of human small intestinal α-glucosidase. Daidzein (a) (in sticks with carbons in cyan) and formononetin (b) (in sticks with carbons in orange) docked into the binding pocket of α-glucosidase (gray spheres with 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.

Table 4. AChE and BChE Inhibition of AMG1, AMG2, AMG3, and *Astragalus membranaceus* Root Extract (AST).

	% AChE inhibition (50 μg/mL)	% BChE inhibition (50 μg/mL)
AMG1	16.2 ± 4.3[a]	25.6 ± 0.1[a]
AMG2	-	12.4 ± 7.1[b]
AMG3	27.9 ± 5.1[b]	23.8 ± 0.1[a]
AST	11.7 ± 5.0[a]	27.3 ± 4.0[a]

AChE, acetylcholinesterase; BChE, butyrylcholinesterase; IC_{50}, half-maximal inhibitory concentration.
Percentage of AChE and BChE inhibition obtained testing extracts at 50 μg/mL and of pure compounds at 5 μM; galantamine IC_{50} = 0.2 ± 0.0 and 6.9 ± 0.4 μg/mL vs AChE and BChE, respectively. Significant differences ($P < 0.05$) are represented with different letters.

Antioxidant Activity

Several studies reported a protective effect of *A. membranaceus* preparations in intestinal injury models.[26] 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_2O_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_2O_2 resulted in a decrease in Caco-2 viability; after 20 hours of treatment with 10 mmol/L H_2O_2, about 25% loss of cell viability was observed. Then, the protective effect of the extracts against H_2O_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_2O_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_2O_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. membranaceous* 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

Table 5. Effect of Cytotoxicity in Caco-2 Cells of AMG1, AMG2, AMG3, and *Astragalus membranaceus* Root Extract (AST).

Compounds	Concentration	Cell viability
Control	-	100%
Hydrogen peroxide	+10 mM/L	73 ± 2.5%[a]
AMG1	+150 μg/mL	83 ± 2.8%[b]
	+100 μg/mL	79 ± 3.2%[b]
	+50 μg/mL	75 ± 3.4%[ab]
AMG2	+150 μg/mL	84 ± 2.8%[b]
	+100 μg/mL	81 ± 2.5%[b]
	+50 μg/mL	77±3.4%[ab]
AMG3	+150 μg/mL	89 ± 2.9%[b]
	+100 μg/mL	85 ± 2.3%[b]
	+50 μg/mL	80 ± 3.4%[b]
AST	+150 μg/mL	90 ± 3.3%[b]
	+100 μg/mL	86 ± 4.3%[b]
	+50 μg/mL	80 ± 2.0%[b]

Values are means ± standard deviation; $N = 3$.
Significant differences ($P < 0.05$) are represented with different letters.

(≥98% by HPLC) from (Sigma-Aldrich, Milano, Italy). Dulbecco's modified Eagle's medium (DMEM), Eagle's minimum essential medium (EMEM), and fetal calf serum (FCS), penicillin–streptomycin, from porcine pancreas and phosphate-buffered saline (PBS) tablets were purchased from Euroclone.

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 linear ion trap coupled 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 C_{18} column (Luna C_{18}, 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) (C_{18} 100 A, 50 mm × 2.6 μm × 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.

a-Amylase Inhibition Assay

The α-amylase inhibition assay was performed using the iodine/potassium iodide method.[27] 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). 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.

a-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.[28] 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)[29] was performed with AutoDock Vina.[30] Residues of α-glucosidase with flexible side chain conformation were chosen in its substrate-binding

site: K1156, D1157, Q1158, P1159, Y1167, Y1251, D1279, I1280, Q1286, I1315, D1317, W1355, W1369, Q1372, K1377, W1418, D1420, M1421, E1423, S1425, F1427, K1460, R1510, W1523, D1526, T1528, D1555, F1559, F1560, Q1561, R1582, H1584, T1586, and I1587.

AChE and BChE Inhibition Assay

The inhibition of AChE was determined based on Ellman's method, as reported by Faraone et al.[31] In this assay, 25 µL of 0.05 U/mL AChE, 125 µL of DTNB (3 mM), 25 µL of buffer B (50 mM Tris–HCl, pH 8 containing 0.1% BSA), and 50 µL of the sample at different concentrations were incubated for 10 minutes. Then, 25 µL of acetylthiocholine iodide (5 mM) was added, and the absorbance was measured at 405 nm after 10 minutes. The BChE inhibition assay was performed in a similar way using 25 µL of butyrylthiocholine chloride (5 mM) as substrate and 0.05 U/mL of BChE as enzyme. Three independent assays were performed in triplicate at different concentrations. Results are expressed as % of enzyme inhibition measured at certain concentrations.

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, 5×10^4 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/cm^2 in a Transwell insert, and the medium (0.1 mL in the insert and 0.8 mL in the well) was changed every 48 hours. Fourteen to 16 days after confluence, the integrity of the monolayer of differentiated cells was monitored according to the method of Hildago et al.[33]

Induction of Oxidative Stress and Neutral Red Assay

An iron-free medium (EMEM) was used for the oxidative stress induction experiments. The oxidative stress was induced in the apical compartment of the transwell insert by the addition of H_2O_2. To assay the capacity of AMG1, AMG2, AMG3, and AST to protect Caco-2 cells from ROM-mediated oxidative injury, cells were preincubated for 4 hours with extracts that were added to the apical side of monolayer. After the end of the preincubation time, the medium was changed before 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.[34] 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 SPECTROstar^{Nano} (BMG Labtech, Ortenberg, Germany).

Declaration of Conflicting Interests

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