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Phytochemical analysis of ethyl acetate extract from *Astragalus corniculatus* Bieb. and brain antihypoxic activity

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Dry ethyl acetate extract containing flavonoids was obtained from above-ground parts of *Astragalus corniculatus* Bieb. Seven flavonoids were isolated and identified as rutin, hyperoside, isoquercitrin, narcissin, quercetin, kaempferol and isorhamnetin for the first time. The extract was found to be practically non-toxic (acute oral toxicity > 5 g kg^{-1} in mice). The extract was investigated for antihypoxic activity in two models of experimental hypoxia – haemic and circulatory. Antihypoxic activity was especially pronounced in the model of circulatory hypoxia. This effect may be attributed, at least in part, to the presence of flavonoids in the extract.

Keywords: Astragalus corniculatus Bieb. (*Fabaceae*), flavonoids, antihypoxic activity

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Mostly flavone and flavonol glycosides and their aglycones displaying different biological activities were isolated from *Astragalus* spp. The most interesting properties of flavonoids are their antioxidative, vasodilatory and antimicrobial traits (1–3).

Astragalus corniculatus Bieb. (*Fabaceae*) is found in North Bulgaria and distributed in Southeastern Romania, South Ukraine, Moldova, *etc.* Protective effect of *Astragalus corniculatus* saponins against myeloid Graffi tumor in hamsters was demonstrated in an *in vivo* model (4).

There have been no reports on the chemical constituents of this plant to date. The aim of the present study was to obtain a dry ethyl acetate extract from above ground parts of *Astragalus corniculatus* containing flavonoids and to test it for acute oral toxicity and brain antihypoxic activity.

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EXPERIMENTAL

Plant material

Astragalus corniculatus herbs were collected in July 1999 near the town of Svishtov, Bulgaria, and identified by Dr. D. Pavlova. A voucher specimen was deposited in the Herbarium of the Faculty of Biology, Sofia University, Bulgaria (SO95265).

Chemicals and apparatus

The UV-spectra were recorded on a SPECORD UV-VIS spectrometer with diagnostic shift reagents (5).

Analytical TLC was carried out on silica gel plates (Kieselgel G, F_{254}, type 60, Merck, Germany), eluted with ethyl acetate/acetic acid/water (100:10:40) (A) for glycosides, chloroform/methanol (9:1) (B) for aglycones and acetone/chloroform/methanol/water (75:10:10:5) (C) for sugars. Visualization of flavonoids was done by spraying with 1% methanolic solution of diphenylboric acid aminoethyl ester (NST) and of sugars by aniline/hydrogen phthalate reagent followed by heating at 110 °C for 3–5 min. TLC comparison was done with standard samples (Merck) before and after acid hydrolisis.

Column chromatography was performed on cellulose (Whatman, Germany), Polyamid S (Riedel-de Haën, Germany) and Shephadex LH-20 (Pharmacia, Sweden).

Preparative PC was conducted on Filtrac No 7 paper using *n*-butanol/acetic acid/water (40:10:22) as the mobile phase.

Preparative TLC on silica gel plates (Kieselgel 60, 0.5 mm thick), eluted with ethyl acetate/formic acid/water (100:10:40), detection at 365 nm (or after spraying with NST).

Acid hydrolysis was carried with 2 mol L^{-1} hydrochloric acid (100 °C, 2 h) and yielded the expected sugars and aglycones.

Extract preparation

Air-dried plant material (800 g) was powdered and extracted with 50% ethanol under reflux. After the removal of ethanol *in vacuo,* the aqueous residue was consecutively treated with chloroform and ethyl acetate. Ethyl acetate extract was evaporated to dryness to give a solid residue (14 g), which was suspended in 5% DMSO (dimethyl sulfoxide) in water for pharmacological experiments.

Phytochemical study

The ethyl acetate extract was chromatographed on a cellulose column, using a 0–95% ethanol linear gradient. Seventy fractions, 60 mL each, were collected and analyzed by TLC on silica gel. Identical fractions were put together: 1–20, 21–40, 41–52, 53–70. Fractions 1–20 were rechromatographed on a Sephadex LH-20 using methanol as eluent. Further purification was achieved by column chromatography on a polyamide, eluting with 0–50% ethanol gradient, followed by preparative PC or TLC. Final purification was carried out on a Shephadex LH-20 with methanol as eluent. Five flavonol

glycosides and three flavonol aglycones were isolated and identified by chromatographic, chemical and spectral methods.

Quantitative determination of flavonoids

The content of flavonoids in ethyl acetate extract, calculated as hyperoside, was determined by spectrophotometry at 320 nm (6). A Hewlett Packard 8452A array diode spectrometer was used for the measurements.

Animals

Animals used in the experiments were male mice, strain H, mass 22–25 g, kept under standard conditions in animal house (water and food *ad libitum*, 12 h dark and light cycle). The following groups of animals were used in the study: control groups and 3 experimental groups for each model, haemic and circulatory hypoxia. Each group comprised 6 animals. Controls were treated with vehicle (5% DMSO in water) in the same volume as the treated animals (0.1 mL per 10 g). No effects of the vehicle were observed.

All the experimental procedures were conducted in accordance with the NIH guidelines of the Care and Use of Laboratory animals.

Acute toxicity

Acute oral toxicity (LD_{50}) was estimated by the up-and-down procedure according to the OECD Test Guideline 425. No behavioral changes or mortality were observed during the period of 14 days.

Haemic hypoxia

Thirty minutes after oral administration of ethyl acetate extract 250, 125 and 62.5 mg kg^{-1}, namely (1/20, 1/40 and 1/80 of LD_{50}), $NaNO_2$ (360 mg kg^{-1}) was applied *i.p.* to each mouse and antihypoxic activity was estimated as the latent time of evidence of hypoxia in minutes according to the method of Roshtina and Ostrovskaya (7).

Circulatory hypoxia

Thirty minutes after oral administration of ethyl acetate extract 125, 62.5 and 31.3 mg kg^{-1}, namely (1/40, 1/80 and 1/160 of LD_{50}), NaF (150 mg kg^{-1}) was applied *i.p.* to each mouse and the antihypoxic activity was estimated in minutes as the latent time of evidence of hypoxia.

Statistical analysis

The LD_{50} data were assessed by the AOT 425 statistical program (Version: 1.0). Antihypoxic activity was expressed relative to the control and was compared by Student's paired *t*-test.

RESULTS AND DISCUSSION

Seven known flavonoids were isolated for the first time from the above-ground parts of *A. corniculatus* Bieb. The compounds were identified as rutin (1), hyperoside (2), isoquercitrin (3), narcissin (4), quercetin (5), kaempferol (6) and isorhamnetin (7) by chromatographic, chemical and spectral methods.

The Rf-values of glycosides **1**–**4** (solvent system A) and aglycones **5**–**7** (solvent system B) are presented in Table I.

UV spectra of isolated flavonoids revealed different profiles, recorded alone or after the addition of some diagnostic ahift reagents (Table I). The spectral data showed flavonol structure with an *o*-dihydroxy group on B-ring for compounds **1**, **2**, **3**, **5**, free hydroxy groups on C-5 and C-7 for all compounds and a substituted hydroxy group on C-3' on B-ring for **4** and **7**.

Total concentration of flavonoids in the extract, calculated as hyperosid, was 3.8%.

The extract is practically non toxic (LD_{50} > 5 g kg^{-1}) in mice. In contrast, the literature data about LD_{50} of pure quercetin (97–98%) show relatively high toxicity (*p.o.* LD_{50} 160 mg kg^{-1} in mice) (8). As mentioned above the total flavonoid content in our extract is only 3.8%, which can explain the difference in LD_{50} values. A statistically significant antihypoxic activity of the extract was established in the experimental model of haemic and circulatory hypoxia in mice. The effect was found to be dose-dependent (Table II) in a range of 250–62.5 mg kg^{-1} (1/20–1/80 parts of LD_{50}) for haemic and 125–31.75 mg kg^{-1} (1/40–1/160 parts of LD_{50}) for circulatory hypoxia. There are literature data that administration of sodium fluoride (substance that induces circulatory hypoxia) increases the blood histamine content and decreases the oxygen carrying capacity (9). The study of Karcher *et al*. (10) shows that a preliminary acute treatment of mice with *Ginkgo biloba* extract, which also contains quercetin, kaempferol and other flavonoids, has a significant protective effect on other forms of hypoxia such as hypobaric hypoxia.

Table I. Chromatographic and UV spectral data of identified compounds

Compd.	R_f-value	UV (λ_{max}, nm)
Rutin (**1**)	0.15	(MeOH) 257, 355; (+NaOMe) 272, 405; (+$AlCl_3$) 275, 422; (+$AlCl_3$/HCl) 265, 396; (+NaOAc) 269, 393; (+NaOAc/H_3BO_3) 260, 372
Hyperoside (**2**)	0.43	(MeOH) 253, 355; (+NaOMe) 323, 405; (+$AlCl_3$) 272, 420; (+$AlCl_3$/HCl) 272, 393; (+NaOAc) 265, 387
Isoquercitrin (**3**)	0.50	(MeOH) 255, 357; (+NaOMe) 278, 407; (+$AlCl_3$) 278, 432; (+$AlCl_3$/HCl) 275, 405; (+NaOAc) 278, 398; (+NaOAc/H_3BO_3) 265, 385
Narcissin (**4**)	0.23	(MeOH) 252, 347; (+NaOMe) 277, 405; (+$AlCl_3$) 279, 355, 407; (+$AlCl_3$/HCl) 279, 345, 400; (+NaOAc) 278, 325, 382; (+NaOAc/H_3BO_3) 253, 347
Quercetin (**5**)	0.27	(MeOH) 254, 367; (+$AlCl_3$) 268, 437; (+$AlCl_3$/HCl) 263
Kaempferol (**6**)	0.52	(MeOH) 267, 365; (+$AlCl_3$) 268, 350, 420; (+$AlCl_3$/HCl) 267, 348, 418
Isorhamnetin (**7**)	0.59	(MeOH) 253, 368; (+$AlCl_3$) 267, 425; (+$AlCl_3$/HCl) 260, 418

Table II. Antihypoxic activity of ethyl acetate extract of Astragalus corniculatus *Bieb. on two models of brain hypoxia*

Haemic hypoxia		Circulatory hypoxia	
Dose (mg kg^{-1}, part of LD_{50})	Activity (%)[a]	Dose (mg kg^{-1}, part of LD_{50})	Activity (%)[a]
Control	100 ± 6	Control	100 ± 5
Group 1 (62.5 mg kg^{-1}, 1/80)	101 ± 7[b]	Group 1 (31.75 mg kg^{-1}, 1/160)	140 ± 9[b]
Group 2 (125 mg kg^{-1}, 1/40)	127.8 ± 13[b,c]	Group 2 (62.5 mg kg^{-1}, 1/80)	195 ± 11[b,c]
Group 3 (250 mg kg^{-1}, 1/20)	208 ± 19[b,c,d]	Group 3 (125 mg kg^{-1}, 1/40)	230 ± 18 [b,c,d]

[a] Data are expressed as mean ± *SD* (n = 6).
Statistically significant difference: [b] $p < 0.05$ compared to control); [c] $p < 0.05$ (compared to group 1); [d] $p < 0.05$ (compared to group 2).

Our results may be supported by the literature data that flavonoids (including quercetin) increase cerebral blood flow and possess antihypoxic activity. The mechanism of this protective action may be due in part to the antioxidant activity as well as to the Ca^{2+} channel blocking effect of quercetin (11–14).

CONCLUSIONS

Our studies indicate that the ethyl acetate extract from *Astragalus corniculatus* Bieb. has a low acute oral toxicity and a remarkable antihypoxic effect, especially in a model of circulatory hypoxia. It is therefore very promissing for further pharmacological and biochemical experiments, which will be focused on evaluating the mechanism of antihypoxic activity.

REFERENCES

1. N. Cook and S. Samman, Flavonoid chemistry, metabolism, cardioprotective effects, and dietary sources, *J. Nutr. Biochem.* **7 (**1996**)** 66–76.
2. T. J. De Paskual and W. Craig, Phytochemicals: guardians of our health, *Am. Diet. Assoc.* **97** (1997) 197–204.
3. I. Krasteva, N. Benbassat and S. Nikolov, Flavonoids from genus *Astragalus* L., *Farmatsiya* (Sofia) **50** (2000) 20–25.
4. I. Krasteva, R. Toshkova and S. Nikolov, Protective effect of *Astragalus corniculatus* saponins against myeloid Graffi tumor in hamsters, *Phytother. Res.* **18** (2004) 255–257.
5. T. J. Mabry, K. R. Markham and M. B. Thomas, *Systematic Identification of Flavonoids*, Springer-Verlag, Berlin-Heidelberg 1970.

6. *European Pharmacopoeia*, 4th ed., Council of Europe, Strasbourg 2002, pp. 742–743.
7. L. Roshtina and R. Ostrovskaya, Effect of piracetam on the body resistance to hypoxia, *Farmacol. Toksikol.* **44** (1981) 210–213.
8. *The Merck Index*, 10th ed., Merck, Rahway 1983, pp. 1160.
9. E. Sumina, V. Shugaev and V. Shugaev, The mechanism of circulatory hypoxia in acute poisoning with sodium fluoride poisoning, *Farmakol. Toksikol.* **41** (1978) 480–482.
10. L. Karcher, P. Zagermann and J. Krieglstein, Effect of an extract of *Ginkgo biloba* on rat brain energy metabolism in hypoxia, *Naynyn–Schmiedebergs Arch. Pharmacol.* **327** (1984) 31–35.
11. T. Beck, M. Abdel-Rahman, G. Bielenberg, H. Oberpichler and J. Krieglstein, *Comparative Study on the Effects of Two Extract Fractions of Ginkgo biloba on Local Cerebral Blood Flow and on Brain Energy Metabolism in the Rat Under Hypoxia, in Pharmacological Study of Cerebral Ischemia* (Ed. J. Krieglstein), Elsevier, Amsterdam 1986, pp. 345–350.
12. R. Meli, G. Authore, G. Di Carlo and F. Capasso, Inhibitory action of quercetin on intestinal transit in mice, *Phytother. Res.* **5** (1990) 201–203.
13. A. Mora, M. Paya, J. Rios and M. Alcaraz, Structure activity relationships of polymetoxyflavones and other flavonoids as inhibitors of non-enzymic lipid peroxidation, *Biochem. Pharmacol.* **40** (1990) 793–797.
14. D. Q. Wang, W. M. Shen, Y. P. Tian, Z. Y. Sun, S. M. Yuan and C. G. Jiang, The effects of the three components isolated from *Astragalus mongolicus* Bunge on scavenging free radicals, *Chin. Pharmacol. Bull.* **10** (1994) 129–132.

SAŽETAK

Fitokemijska analiza etil-acetatnog ekstrakta iz biljke *Astragalus corniculatus* Bieb. i antihipoksični učinak u mozgu

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Iz nadzemnih dijelova biljke *Astragalus corniculatus* Bieb. priređen je suhi etil-acetatni ekstrakt u koje su identificirani sljedeći flavonoidi: rutin, hiperozid, izokvercitrin, narcisin, kvercetin, kempferol i izorhamnetin. Ekstrakt je praktički netoksičan (akutna toksičnsot nakon peroralne primjene > 5 g kg^{-1} kod miševa). Ispitivan je antihipoksični učinak ekstrakta na dva modela eksperimentalne hipoksije – krvne i circulacijske. Antihipoksično djelovanje je jače izraženo kod cirkulacijske hipoksije. Taj učinak se barem djelomično može pripisati flavonoidima.

Ključne riječi: Astragalus corniculatus Bieb. (*Fabaceae*), flavonoidi, antihipoksično djelovanje

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