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Antiproliferative effects of a flavonoid and saponins from *Astragalus hamosus* against human tumor cell lines

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ABSTRACT

The antiproliferative effects of a flavonol glycoside rhamnocitrin 4'- β -D-galactopyranoside and mixture of two saponins, obtained from *Astragalus hamosus* L. (Fabaceae), were tested in a panel of human tumor cell lines, using the MTT-dye reduction assay. The saponin mixture caused concentration-dependent inhibition of malignant cell proliferation, while the flavonoid exerted only marginal effects. These data were corroborated by an ELISA test to assess the characteristic for apoptosis DNA-fragmentation after treatment with the tested compounds. At equipotent concentrations the saponin mixture was found to be superior inducer of apoptosis as compared to the flavonoid.

KEY WORDS: *Astragalus hamosus*, Fabaceae, flavonoids, saponins, MTT-assay, apoptosis.

INTRODUCTION

Astragalus hamosus L. (Fabaceae) (European milk-vetch) is an annual herb widely distributed in Europe (East, Southeastern and Southwestern Europe), Africa and Asia (Arabian Peninsula, Western Asia, Caucasus, Middle Asia, Indian subcontinent). The plant has a well documented use in folk medicine as a carminative, astringent, emetic, diuretic, emollient, demulcent, aphrodisiac and laxative. *A. hamosus* has also been described useful in treating inflammatory states and malignant tumors (1). Our previous phytochemical investigation on this species resulted in the isolation of a new flavonol glycoside rhamnocitrin 4'- β -D-galactopyranoside (2). Other chemical studies of the plant have indicated the presence of peregrinose and azukisaponin V, which were found not cytotoxic against a variety of human cancer cells. However, dose-related modulation of lymphocyte proliferation was observed (3).

The present paper reports investigations on the cytotoxic activity of newly isolated rhamnocitrin 4'- β -D-galactopyranoside and mixture of two triterpene saponins from *A. hamosus* in a panel of human tumor cell lines. Additionally their ability to induce apoptotic cell death in the T-cell leukemia-derived SKW-3 cell line was determined as well.

MATERIALS AND METHODS

Plant material, extraction and isolation

The plant material was collected in June in North-Eastern parts of Bulgaria and identified by Dr D.

Pavlova (Department of Botany, Faculty of Biology, Sofia University).

Air-dried aerial parts of the plant (1 kg) were defatted with n-hexane and extracted with MeOH/H₂O (9:1) and (1:1). The extracts were filtrated, concentrated and successively partitioned with CHCl₃, EtOAc and n-BuOH. A flavonol glycoside (Fl₁) was isolated by Sephadex LH-20 column chromatography and crystallization with MeOH from the ethyl acetate. The structure of the compound was elucidated as 7-O-methyl-kaempferol 4'- β -D-galactopyranoside (rhamnocitrin 4'- β -D-galactopyranoside) (Fig. 1) by chemical and spectral methods. Details of isolation and identification of the compound have been published previously (2).

The n-BuOH extract was subjected to on Sephadex LH-20 column chromatography eluting with MeOH to give six main saponin fractions (C₁-C₆). Fractions C₅ were flash chromatographed on a silica gel with CHCl₃/MeOH/H₂O (18:11:2) followed by column chromatography on a RP-18 silica gel (solvent H₂O/MeOH (1.5:3.5)) to afford a mixture of saponins 1 and 2 (52 mg).

Cell lines and culture conditions

The panel of human tumor cell lines used in this study included the acute myeloid leukemia HL-60, its multidrug-resistant sub-line HL-60/Dox, the T-cell leukemia SKW-3, and the multiple myeloma derived cell lines RPMI-8226, U-266 and OPM-2. They were

supplied from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The cells were maintained in a controlled environment (RPMI-1640 medium, supplemented with 10 % heat-inactivated fetal calf serum and 2mM L-glutamine, at 37°C in a 'Heraeus' incubator with 5% CO₂ humidified atmosphere). In order to keep the cell cultures in log phase cellular suspension aliquots were supplemented with fresh RPMI-1640 medium two or three times per week. HL-60/Dox was maintained in medium containing 0.2 µM doxorubicin in order to sustain their MDR phenotype. 1 week prior to the cytotoxicity determination however they were kept in a drug-free medium in order to avoid synergistic interactions between the anthracyclines and the tested compounds. The stock solution of the tested compounds was prepared in ethanol and consequently diluted in RPMI-1640. At the final dilutions obtained the concentration of the solvent never exceeded 0.5 %.

MTT dye reduction assay

The antiproliferative/cytotoxic activity of the compounds was assessed using the standard MTT-dye reduction assay, as described by Mossman (4) with minor modifications (5). The results were expressed as survival fraction (% of untreated control).

Apoptosis assay

The DNA fragmentation as a quantitative merit of the ability of tested compounds to induce apoptosis was detected using a commercially available "Cell Death Detection" ELISA kit (Roche Applied Science), according to the manufacturers instructions. Each test was run in triplicate.

Statistics

The data processing included the Student's t-test with $p \leq 0.05$ taken as significance level, using Origin Plot and GraphPad Prism software for PC. The experimental data were fitted to sigmoidal dose-response curves and the corresponding IC₅₀ values were calculated using non-linear regression analysis (GraphPad Prism) as endpoints to assess the relative cytotoxic/antiproliferative potency of the tested compounds.

RESULTS

The antiproliferative effects of rhamnocitrin 4'-β-D-galactopyranoside (FI₁) and mixture of saponins 1 and 2 were evaluated in a panel of human tumor cell lines, using the standard MTT-dye reduction assay, after 72 h continuous exposure of the cells. Throughout the investigations the clinically applied natural product derivative etoposide was used as a positive cytotoxic control.

The tested compounds inhibited the proliferation of

the malignant cells in a concentration-dependent manner, which allowed the construction of dose-response curves (not shown) and the calculation of the corresponding IC₅₀ values (concentrations causing 50% decrease of cell viability), summarized in Table 1.

The saponin mixture generally proved to exert superior antiproliferative activity, although its relative potency was lower than that of the referent anticancer drug. Among the panel of cell lines tested the myeloid cells HL-60 demonstrated higher sensitivity relative to the lymphoid cell line SKW-3, while the multiple myeloma-derived cell lines were far less responsive. Interestingly the multi-drug resistant HL-60/Dox was significantly more susceptible to the effect of the saponin mixture as compared to the sensitive parent line.

The flavonoid compound demonstrated only marginal inhibitory effects against most of the cell lines and actually IC₅₀ value was determined only against the T-cell leukemia SKW-3.

Figure 2 presents the level of DNA-fragmentation in SKW-3 cells after 12 or 24 h treatment with equi-effective concentrations (half-IC₅₀) of the novel compounds or etoposide. Evident from the results the saponin mixture caused significant time-dependent increase in the oligonucleosomal content of SKW-3 cells. The flavonoid was less potent in this respect causing significant DNA-fragmentation only after the longer incubation period.

DISCUSSION

This study describes the evaluation of the antiproliferative potential of a saponins mixture and of a new flavonoid, isolated from *Astragalus hamosus*, against a panel of human tumor cell lines, representative for some common neoplastic diseases. The results clearly demonstrate that the saponin mixture and to a lesser extend the flavonoid exert inhibitory effects against malignant cells. These results are in unison with the growing number of articles demonstrating the antiproliferative and cytotoxic potential of *Astragalus* extracts and pure compounds (6-8).

Apart from the exploration of the anticancer potential of the tested compounds another major issue which we addressed was to test their ability to retain activity in resistant cell lines. HL-60/Dox cells have been established via continuous selection in doxorubicin-containing medium and are characterized by strong expression of MRP-1 a member of the ATP-binding cassette family of membrane transporter. MRP-1 mediates an ATP-dependent efflux of chemically

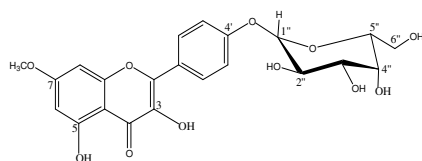


Figure 1. Chemical structure of FI₁

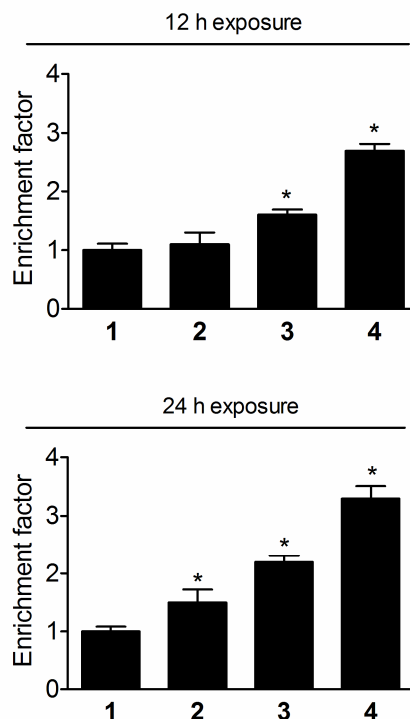


Figure 2. Proapoptotic effects of FI-1, saponin mixture and etoposide in the human T-cell leukemia SKW-3. The enrichment of SKW-3 cytosole with mono- and oligo-nucleosomal DNA fragments was monitored using a 'Cell death detection' ELISA kit (Roche Applied Science) after 12 or 24 h exposure to FI₁ (2), saponins mixture (3) or etoposide (4), vs. the untreated control (1).

* $p < 0.05$ vs. the untreated control (Student's t-test).

diverse agents out of tumor cells and confers them resistant to anthracyclines, Vinca alkaloids, epipodophyllotoxins and other structurally unrelated classes of anticancer drugs (9, 10). In our hands HL-60/Dox cells were found to exert significant collateral sensitivity, i.e. their IC₅₀ values in this cell line were several fold lower relative to those in HL-60. In a dissimilar fashion the semi-synthetic lignan etoposide was less active in HL-60/Dox with a resistance index of ca. 5.

Such collateral sensitivity in HL-60/Dox is characteristic for some plant-derived multi-drug resistance modulators e.g. the alkaloid thaliblastine (11). On this ground our data suggest for further

detailed evaluation of the saponins mixture in chemotherapy-non responsive tumor models.

The flavonoid FI₁ was generally less active than the saponins (with the only exception of SKW-3 cells). Despite this low cytotoxicity however the relative contribution of such compounds to the effects of *Astragalus* in vivo could not be ruled out. It is well appreciated that flavonoids exert immunomodulatory, antiangiogenic effects and furthermore could favorably modify the toxicological and safety profile of anticancer drugs (7).

The DNA fragmentation analysis unambiguously indicate that the induction of programmed cell death (apoptosis) at least partly mediates the established

Table 1. Antiproliferative activity of the newly isolated compounds and etoposide after 72 h continuous exposure (MTT-dye reduction assay)

Cell lines	Origin	IC ₅₀ (µg/ml)		
		Saponin mixture	FI ₁	Etoposide
HL-60	Acute myeloid leukemia	63.4 ± 4.1	> 200.0	4.7 ± 0.9
HL-60/Dox	Acute myeloid leukemia ^a	25.3 ± 4.1	> 200.0	20.8 ± 3.2
SKW-3	T-cell leukemia	84.2 ± 3.9	146.2 ± 7.4	7.5 ± 1.4
RPMI-8226	Multiple myeloma	143.5 ± 9.8	> 200.0	6.8 ± 2.6
OPM-2	Multiple myeloma	126.5 ± 7.6	> 200.0	9.6 ± 3.4
U-266	Multiple myeloma	119.8 ± 7.7	> 200.0	7.2 ± 1.6

^aSelected in doxorubicin-containing medium and characterized by pleiotropic drug resistance, due to the over-expression of MRP-1 efflux pump.

cytotoxicity in SKW-3 cells, whereby this proapoptotic effect is quite more pronounced after treatment with the saponins. The induction of apoptosis is a common mechanism by which antineoplastic drugs kill cancer cells and our data demonstrate the generality of this phenomenon.

CONCLUSION

The anticancer activity potential of diverse *Astragalus* species incl. *A. hamosus* is well established and found to have multimodal mechanisms including immune response modulation, inhibition of angiogenesis and cytotoxicity. Our data further justifies and elucidates the base for the ethnopharmacological use of *Astragalus hamosus* in antineoplastic diseases. The encountered antiproliferative potential of the saponin mixture especially in the multidrug resistant tumor model justifies the further thorough investigation of its antineoplastic potential.

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