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Antiproliferative and antitumour activity of saponins from *Astragalus glycyphyllos* on myeloid Graffi tumour

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ABSTRACT

Ethnopharmacological relevance: Astragalus glycyphyllos L. has been extensively used in Bulgarian folk medicine as an antihypertensive, diuretic, anti-inflammatory, anti-tumour, in cases of cardiac insufficiency, renal inflammation, calculosis, etc.

Aim of the study: To evaluate the possible *in vitro/in vivo* anti-proliferative/anti-tumour activity of a purified saponins' mixture (PSM) obtained from the plant.

Materials and methods: Viability and proliferative activity of the Graffi myeloid tumour cells was assessed by MTT test. The morphological alterations were visualized and analysed by fluorescent microscopy after intravital double staining. An *in vivo* model of Graffi tumour bearing hamsters was used to examine the influence of PSM on transplantability, tumour growth, survival and mortality as well as to observe pathomorphological changes.

Results: Graffi tumour cells were sensitive to purified saponins' mixture after 24 and 48 h treatment. The treatment induced a statistically significant decrease of the viability/proliferation of the Graffi tumour cells. These effects were concentration- and time-dependent. Fluorescent microscopy studies showed that these anti-proliferative effects were connected to the induction of apoptosis. The *in vivo* study showed the presence of a stromal component, single mononuclear cells in the stroma. Multiple incorrect mitotic figures were observed in the tumour tissue from the control group. Well-formed stroma with accumulation of mononuclear cells and mitotic cells were found in the group, treated with PSM. The tumour weight was decreased in the group, treated with PMS.

Conclusion: The results indicate that PSM exhibited *in vitro/in vivo* antiproliferative/anti-tumour effects.

1. Introduction

About 70% of the molecules used worldwide are derived from plants, making herbal drugs an invaluable source of remedies for humanity. Triterpenoid saponins are one of the largest and most varied groups of secondary metabolites, possessing low toxicity. They have been shown to exert numerous activities on malignant cells both *in vitro* and *in vivo*. Many triterpenoid saponins have been isolated from species of genus *Astragalus* L. (Fabaceae) during the last years. There are a lot of methods for quantitative analysis of saponins. Liquid chromatography coupled with high resolution mass spectrometry is proved to be the most suitable and accurate method for determination of saponin content in plant substances, herbal preparations and in biological fluids (Tang et al., 2015).

The information about saponins' antiproliferative, cytotoxic and immune stimulating properties is growing ever since. A lot of research is

Abbreviations: AO, acridine orange; DAPI, 4′,6-diamidino-2-phenylindole; EtBr, ethidium bromide; FITC, fluorescein isothiocyanate; GTBH, Graffi tumour bearing hamsters; HE, haematoxylin/eosin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PI, propidium iodide; PSM, purified saponins' mixture.

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still conducted to elucidate the pharmacological action of *Astragalus*-derived saponins, frequently based on data from the traditional medicine (Ionkova et al., 2014; Krasteva et al., 2016; Pistelli, 2002).

There are 29 *Astragalus* species, distributed in Bulgarian flora (Asyov et al., 2012) from which only *Astragalus glycyphyllos* L. (Liquorice Milk-Vetch) is used in folk medicine of the country. It is an herbaceous perennial plant with crawling rhizomes and roots, spread on rocky sites at forest meadows and open territories in forests and bushes, mainly in mountain regions in Bulgaria, up to 1800 m. a. s. l. (Asyov et al., 2012; Valev, 1976). The species has been extensively used as an antihypertensive, diuretic, anti-inflammatory, anti-tumour, etc. The herb is administered as an infusion in cases of cardiac insufficiency, renal inflammation, calculosis, increased arterial and venous blood pressure, tachycardia, as an adjuvant therapy in cases of cancer (Nikolov, 2006). In France *A. glycyphyllos* is applied as an emollient, diuretic, and refreshing agent. People of the Caucasus use the leaves and seeds of this species in cases of urolithiasis, oliguria, scrofula, dermatitis and as a laxative. Along the Volga River the plant is used to treat nervous diseases. In Belarus a decoction from its aerial parts is applied internally to treat leucorrhoea, uteroptosis, stomach diseases and dysentery; topically in cases of fungal scalp infection. Similar decoction from the herb is used in Ukraine as a laxative, diuretic, expectorant, against sexually transmitted diseases, rheumatism and dermatitis (Lysiuk and Darmohray, 2016). Carpathian people apply this decoction as diuretic, as an expectorant, for rheumatism, arthralgia, diarrhoea, dermatitis, syphilis; in gynaecology to stimulate labour and to accelerate separation of the placenta. In clinical trials a 10% infusion of *A. glycyphyllos* displayed hypotensive, anticoagulant and diuretic activity. Hydroalcoholic extract from the species exerted antibacterial, anti-trichomonas and yeast-static activity. An ether extract of the aerial parts showed *in vitro* anti-tumour effects. *A. glycyphyllos* is regarded to act pharmacologically similar to *A. dasyanthus* by Russian folk medicine (Lysiuk and Darmohray, 2016).

Phytochemical investigation of *A. glycyphyllos* led to isolation of six saponins and their structures were partially elucidated (Elenga et al., 1986). After acid hydrolysis of a saponin mixture, obtained from the aerial parts of the plant, soyasapogenol B and 3β,22β,24-trihidroxyolean-12-en-19-one were identified (Elenga et al., 1987). Cycloartane saponins askendoside C and F (Linnek et al., 2008) and an epoxycycloartane saponin lactone (Shkondrov et al., 2020) were later isolated from the species. Several flavonoids (kaempferol, quercetin, isorhamnetin-3-*O*-glucoside, apigenin-7-*O*-arabinosyl-glucoside, kaempferol-3-*O*-xylosyl-glucoside, kaempferol-7-*O*-galactoside and rutin) (Krasteva et al., 2016) and a rare flavonoid – camelliaside A (Shkondrov et al., 2020) were identified in *A. glycyphyllos*. Polysaccharides' mixture, obtained from overground parts of the species was investigated in models of enzyme and non-enzyme-induced lipid peroxidation on isolated rat liver microsomes and showed good antioxidant activity (Kondeva-Burdina et al., 2016). *In vivo* pharmacological investigation reviled that a defatted extract obtained from *A. glycyphyllos* possessed good antioxidant and hepatoprotective effects, similar to those of silymarin (Shkondrov et al., 2015). As an ongoing research on *A. glycyphyllos* a purified saponins' mixture (PSM) was investigated for possible anti-proliferative/anti-tumour activity on Graffi myeloid tumour cells *in vitro* and on tumour formation *in vivo*.

2. Materials and methods

2.1. Plant material and preparation of PSM

The aerial parts of *A. glycyphyllos* were collected in flowering stage from Vitosha Mountain, Bulgaria, in June 2018. The species was identified by Dr. D. Pavlova from the Faculty of Biology, Sofia University, where a voucher specimen was deposited (№ SO-107613). The plant material was dried to 10% moisture, which is acceptable for herbal drugs (Zhang et al., 2019). Then 500 g were extracted *via* percolation with 80% methanol and the extract was evaporated to dryness. The resulting solid extract was suspended in lukewarm water and it was successively and exhaustively extracted with dichloromethane, ethyl acetate and n-butanol. The dried n-butanol extract was separated by column chromatography over Diaion HP-20 eluted with increasing percent methanol. Ten main fractions were collected (I-X) and analysed by HPLC. According to UHPLC-MS fractions VIII and IX, rich in saponins had the same composition. They were combined to gain quantity for the *in vivo* study and named PSM.

2.2. Analysis of PSM

UPLC-HRESI/MS analysis of PSM was carried out using Q Exactive™ Plus Orbitrap mass spectrometer with a HESI ion source (ThermoFisher Scientific, Bremen, Germany) in ultra-high resolution mode (70 000, at *m/z* 200) and UPLC system (Dionex UltiMate 3000 RSLC, ThermoFisher Scientific, Bremen, Germany). The operating conditions of the heated electrospray ionization (HESI) source ionization device operating in the positive ionization mode were: 3.5 kV voltage and 320 °C capillary temperature, 25 units of carrier gas flow and 5 units of dry gas flow. All other detector parameters were set in such a way as to obtain the most intense signal from $[M-H]^-$. Nitrogen was used to atomize the samples. UPLC separations were performed on a Kromasil C18 column (1.9 μm, 2.1 × 50 mm, Akzo Nobel, Sweden) at 30 °C. A mobile phase consisting of 0.1% HCOOH (A) and MeCN + 0.1% HCOOH (B) and a flow rate of 0.3 mL/min was used. Gradient elution (10 min) was performed as follows: 90% A for 0.5 min, then decrease to 5% A for 5 min, isocratic with 5% A for 0.5 min, return to 90% A for 0.1 min and 3.9 min equilibration at 90% A prior to the next injection. The major compound was a triterpene saponin 17(*R*),20(*R*)-3β,6α,16β-trihydroxycycloartanyl-23-carboxylic acid 16-lactone 3-O-β-D-glucopyranoside. It was quantified as 60% (w/w) of the PSM (Supplementary file). The saponin was previously isolated and structurally elucidated by NMR and MS. The HRESIMS spectrum of the major component showed adducts $[M+H]^+$ (at $m/z = 579.3526$, calcd. 579.3533), $[M+Na]^+$ (at $m/z = 601.3341$, calcd. 601.3353) and $[M + HCOO]^-$ (at $m/z = 623.3441$, calcd. 623.3432), consistent with the structure of the epoxycycloartane saponin lactone (Shkondrov et al., 2020).

2.3. In vitro methods

A stock solution of the PSM in ethanol with concentration 10 mg/mL was prepared and stored at 4 °C. Immediately prior the *in vitro* experiments, the stock solution was further diluted with RPMI-1640 containing 10% foetal bovine serum (FBS) to obtain solutions with the desired test concentrations of PSM for cell culture treatment.

The primary culture from Graffi tumour cells was obtained by a standard procedure (Toshkova et al., 2010), from solid myeloid Graffi tumour tissue. Tumour tissue was taken aseptically from a hamster with well-developed subcutaneous tumour. The tissue was cleaned of necrotic masses, disintegrated mechanically and washed with phosphate buffered saline (PBS), pH 7.4 by centrifugation at 1000×*g* for 5 min at 4°C. The cells were cultivated in RPMI-1640 medium complemented with 10% FBS (Gibco, Austria), 100U/mL penicillin and 0.1 mg/mL streptomycin in 25 mL tissue culture flasks (Orange Scientific) at 37 °C, 95% humidity and 5% CO_2. Subcultivation was performed 2–3 times a week at a 1:2 to 1:3 split ratio. Trypsin-EDTA solution was applied for detachment of the adherent cells. Cells in an exponential phase of growth were used for the *in vitro* experiments.

The effect of PSM on the viability and proliferative activity on Graffi myeloid tumour cells was assessed by MTT test (Mosmann, 1983). The MTT test is based on the ability of viable cells to metabolize the yellow tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to a purple formazan product. The concentration of the formed formazan is proportional to the mitochondrial enzyme activity and respectively to the cell viability. Graffi tumour cells at a concentration of 1×10^5 cells/mL in RPMI-1640 medium with 10% FBS were

placed in 96-well plates (100 μL/well) and cultivated for 24 h at 37 °C and 5% CO_2 for good adhesion and growth. The cells were then treated with eight different concentrations of PSM (4, 8, 16, 31, 63, 125, 250 and 500 μg/mL) and incubated for 24 and 48 h. Untreated cells were used as a control. At the end of the incubation, the culture medium was removed and 100 μL of MTT (3-(4,5-dimethylthyazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution (0.5 mg/mL) was added to each well. The plates were incubated additionally for 3 h in a CO_2 incubator. The resulting formazan crystals were dissolved by a lysing solution (DMSO: Ethanol, v/v 1:1, 100 μl/well). The optical density (OD) of the dissolved formazan was measured at 570 nm and 620 nm (as a referent wavelength) by ELISA spectrophotometer (TECAN, SunriseTM, Salzburg, Austria). The cell viability was calculated as follows: CV (%) = OD_{540} (experiment)/OD_{540} (control) x 100.

The cytomorphological alterations induced by PSM in the Graffi tumour cells were visualized and analysed by fluorescent microscopy after double staining with acridine orange (AO)/ethidium bromide (EtBr), 4′,6-diamidino-2-phenylindole (DAPI) and Annexin V- fluorescein isothiocyanate (FITC) and propidium iodide (PI). The freshly stained tumour cells were immediately examined under a fluorescent microscope (Leica DM 5000B, Wetzlar, Germany).

Cytomorphological alterations of Graffi tumour cells, treated with PSM were studied after double intravital staining with fluorescent dies AO and EtBr according to a standard procedure (Wahab et al., 2009). Graffi tumour cells at concentration of $1x10^5$ cells/mL in RPMI-1640 medium with 10% FBS, were plated on sterile glass lamellas in 24-well plates and were incubated for 24 h at 37 °C and 5% CO_2 in a CO2 incubator for formation of cellular monolayer. On the next day, the cells were treated with PSM in a concentration equal to the IC_{50} value measured on 24 h by the MTT test (50 μg/mL) and then were incubated for additional 24 h. Untreated tumour cells were used as a control. After incubation, the cells were washed twice with PBS and stained with equal volumes of the fluorescent dyes AO (10 μg/mL PBS) and PI (10 μg/mL PBS).

Fluorescent staining with DAPI was used for examination of the nuclear morphology of treated and untreated (control) Graffi tumour cells (Radhika et al., 2010). Graffi tumour cells ($1x10^5$ cells/mL in RPMI-1640 medium with 10% FBS), were placed on sterile glass lamellas in 24-well plates and cultivated in a CO2 incubator to form a monolayer. After overnight incubation, the cells were treated with 50 μg/mL PSM (concentration equal to IC_{50} value measured on 24 h by MTT test) and cultivated for additional 24 h. Untreated Graffi tumour cells were used as a control. After incubation, the glass lamellas were washed twice with PBS to remove the tumour cells debris. The staining of the cells with DAPI (5 μg/mL in methanol) was performed after fixation with 3% paraformaldehyde. All samples were mounted on microscope slides with Mowiol (Sigma Aldrich, Germany) and kept in the dark until observation.

For assessment of the apoptosis inducing ability of PSM, Annexin V Apoptosis Detection Kit: sc-4252 AK (Santa Cruz Biotechnology, USA) was used. Graffi tumour cells were cultivated on glass lamellas and treated with PSM for 24 h. The culture medium was removed, the cells were washed with PBS, fixed with 3% paraformaldehyde and stained with a solution of Annexin V - fluorescein isothiocyanate (FITC) and propidium iodide (PI), according to the manufacturer's protocol. The samples were incubated for 15 min in the dark at room temperature.

2.4. In vivo methods

Graffi mouse leukaemia virus was adapted and maintained *in vivo* in hamsters as solid tumor by s.c. inoculation of 1–2 x 10^6 viable tumour cells. Transplantation of the tumour in all experimental animals was achieved by single s.c. inoculation of 5 × 10^4 viable Trypan blue excluded tumour cells in the interscapular area. The tumour was 100% transplantable and 100% lethal for hamsters (Toshkova et al., 1997).

Thirtythree two month-old Golden Syrian hamsters of both sexes, weighing 80–100 g were used. They were bred and grown under standard conditions, adopted by the Bulgarian Food Safety Agency. For the examination of the PMS-protective effect, the animals were separated in two experimental groups each of 12. Design of the experiment: Group 1 – injected s. c. with Graffi tumour cells on first day and treated p. o. with PSM (3 x per week, 7 mg/100 g b. w.) for two weeks (Krasteva et al., 2004); Group 2 (control) – hamsters injected s.c. with Graffi tumour cells on first day, untreated.

Tumour transplantability (TT%) was determined for each experimental group on days 14 and 18 as a ratio between the number of tumour bearing hamsters (TBH) and the number of all hamsters in each group. Inhibition of tumour growth (ITG) – the tumour size in mm (the mean arithmetical value of two perpendicular tumour diameters) was determined by a caliper for each animal on days 10, 12, 16, 20 and 22 after tumour transplantation. The ITG was calculated according to the formula: ITG = $[(A\text{-}B)/A]$x100, where A is the mean arithmetical value of tumour diameters (mm) in TBH without PSM treatment and B is the mean arithmetical value of tumour diameters (mm) in PSM-treated TBH. The protective effect was examined by the mean survival time (MST) and mortality percentage (M) as well. Mortality (%) was followed on days 20, 24, 30, 34, 38 and 40 after tumour transplantation.

Experiments with animals cannot be replaced by experiments with tissue culture lines. The number of experimental animals was reduced as much as possible, depending on statistical significance. All the experimental procedures were conducted in accordance with the NIH guidelines of the care and use of laboratory animals.

Tumours were removed from animals after death and fixed in 10% neutral buffered formalin. For the preparation of histopathological slides tissues with thickness of about 1 cm were washed under running tap water for 24 h, dehydrated continually with increasing concentrations of alcohol from 50% to 100%. The samples were then diaphonized in xylene and embedded in paraffin, melted in a thermostat at a temperature of 60 °C. Tissues were cut to 5 μm sections, deparaffinized and hydrated using xylene and graded alcohol concentration (to 50%), and stained with haematoxylin and eosin. Histopathological changes were studied using light microscope Euromex BioBlue.

2.5. Statistics

The *in vitro* results were expressed as mean value ± SD (standard deviation). The statistical analysis of the results was performed by one-way analysis of variance (ANOVA), followed by *post hoc* test (Bonferroni) with GraphPad PRISM, version 5 (GraphPad Software Inc., San Diego, USA). The values of * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ were accepted statistically significant. I*n vivo* study results were compared with the control group and analysed by the Student's *t*-test. Data were presented as the mean ± SD and $p < 0.05$ was accepted as statistically significant.

3. Results

3.1. Effect of PSM on graffi tumour cells in vitro

The aim of this study was to assess in dynamics the cytotoxic effect of purified saponins' mixture on the 24th and 48th hour. The results showed statistically significant antiproliferative and cytotoxic effects of the tested PMS. The cell viability values measured at both time intervals showed similar concentration-dependency and no signs for reversibility of the cytotoxic action was found. PSM induced statistically significant decrease of cell viability ($p < 0.01$), compared to the control (non-treated Graffi tumour cells), on the 24 h at concentrations higher than 31 μg/mL. Cell viability values (expressed as % of the control) were: 32.97 ± 3.86% (for PSM concentration 63 μg/mL); 0.56 ± 0.169% (125 μg/mL); 1.015 ± 0.03% (250 μg/mL) and 1.598 ± 0.36% (500 μg/mL). On the 48 h, statistically significant decrease of cell viability was established at seven of the eight concentrations tested. The lower PSM

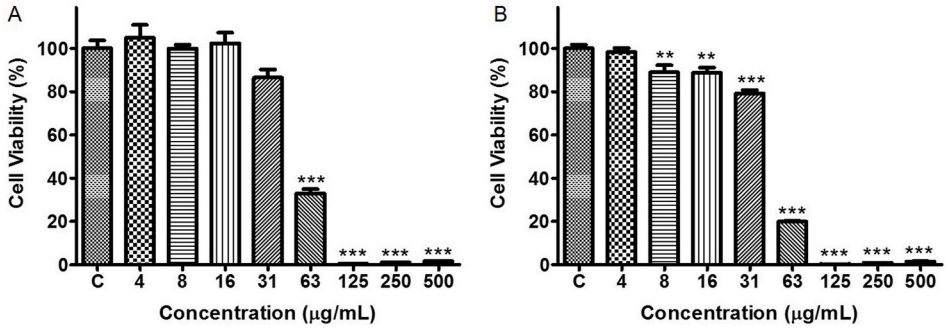

Fig. 1. Effect of different PSM concentrations on the cell viability of Graffi tumour cells, after 24 h (A) and 48 h (B) exposure evaluated by MTT test; ** ($p < 0.01$); *** ($p < 0.001$).

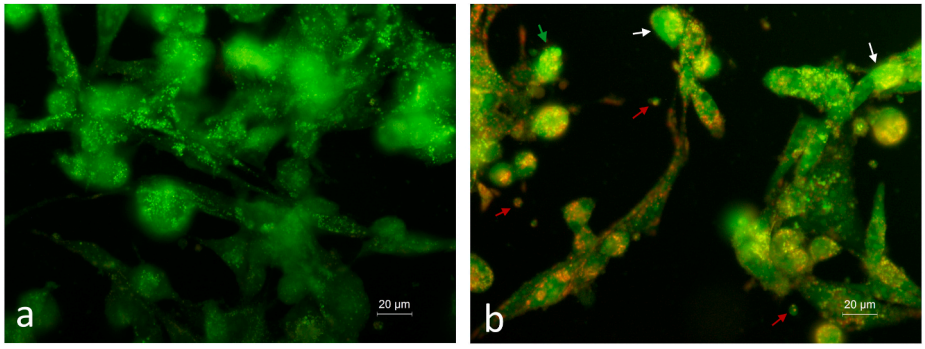

Fig. 2. Morphological changes in Graffi tumour cells cultured in the presence of PSM (50 μg/mL) for 24 h, followed by AO and EtBr double staining. Fluorescence micrographs of: (a) untreated Graffi cells, and (b) cells treated with 50 μg/mL PSM; scale bar = 20 μm; white arrow – chromatin condensation; red arrow – apoptotic bodies; green arrow – fragmented nucleus. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

concentrations (4, 8, 16 and 31 μg/mL) decreased the viability of Graffi tumour cells to values ranging from 88.94 ± 6.58% to 79.16 ± 3.32%, while the higher concentrations strongly decreased the viability: 19.92 ± 1.01% (for PSM concentration 63 μg/mL); 0.315 ± 0.07% (125 μg/mL); 0.89 ± 0.1% (250 μg/mL) and 1.5 ± 0.36% (500 μg/mL). The IC_{50} values were: 51.67 μg/mL and 43.38 μg/mL for the 24 h and 48 h, respectively (Fig. 1).

The values measured on the 48th hour were slightly lower than those measured on the 24th hour and the IC_{50} values was comparable. The results indicate that PSM exerted significant concentration and time-dependent antiproliferative and cytotoxic effect on the Graffi tumour cells.

The morphological alterations induced by 50 μg/mL PSM in Graffi tumour cells after 24 h treatment were analysed by three different fluorescent staining methods. Acridine orange (AO) stains both live and dead cells, emitting strong green fluorescence, as a result of the intercalation in the double-strand DNA. As opposed to AO, EtBr can only enter dead and late apoptotic cells with poor membrane integrity, so it stains all dead cells to generate red fluorescence. The early apoptotic cells were characterized by bright green nuclear staining and chromatin condensation in a form of dense green regions and the late apoptotic cells are with orange nucleus with chromatin condensation. The controls (non-treated Graffi tumour cells) showed normal monolayer growth. The cells with polygonal and spindle shape predominated and single cells with oval shape were observed. The cells were uniformly green stained with one or several bright green nucleoli and green coloured granules in the cytoplasm (Fig. 2a). The incubation with PSM lead to changes in both the growth and morphology of the Graffi cells. The cellular monolayer was damaged, cells with oval shape, with bright yellow-green nucleus with chromatin condensation, visible as dense green regions (early apoptotic cells) predominated, and cells with condensation and margination of the chromatin or fragmented nucleus, forming apoptotic bodies (late apoptotic cells) were also found (Fig. 2b).

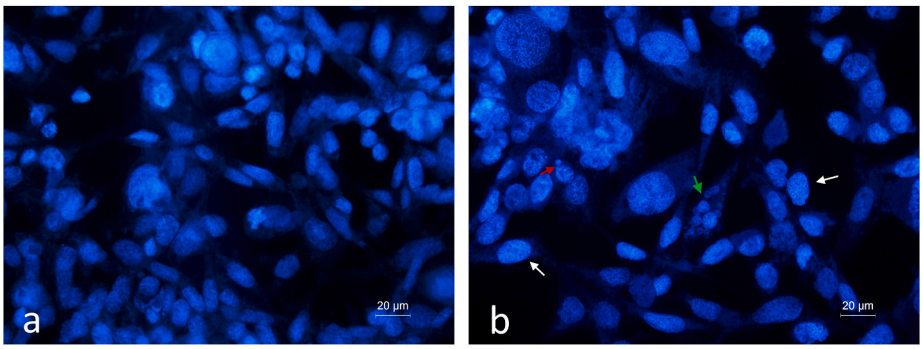

Fig. 3. Morphological changes in the nucleus of Graffi tumour cells cultured in the presence of PSM (50 μg/mL) for 24 h, followed by DAPI staining. Fluorescence micrographs of: (a) untreated Graffi cells, and (b) cells treated with 50 μg/mL of PSM; scale bar = 20 μm; white arrow – chromatin condensation; red arrow – apoptotic bodies; green arrow – fragmented nucleus. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

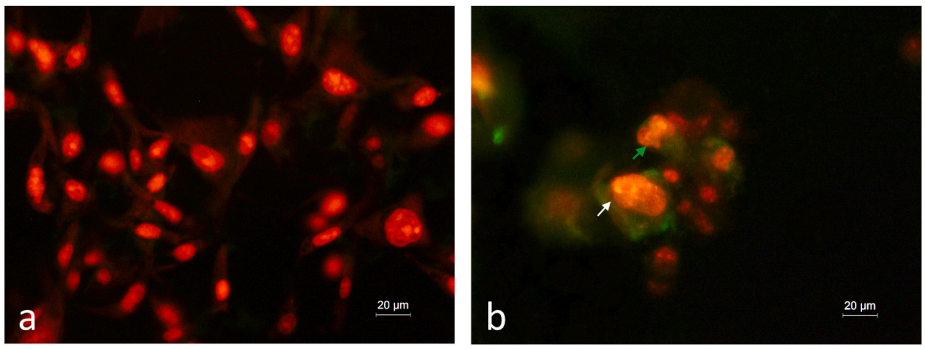

Fig. 4. Morphological changes in the Graffi tumour cells cultured in the presence of PSM (50 μg/mL) for 24 h, followed by AnnexinV-FITC/PI staining. Fluorescence micrographs of: (a) untreated Graffi cells, and (b) cells treated with 50 μg/mL of PSM; scale bar = 20 μm; white arrow – chromatin condensation; green arrow – fragmented nucleus. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

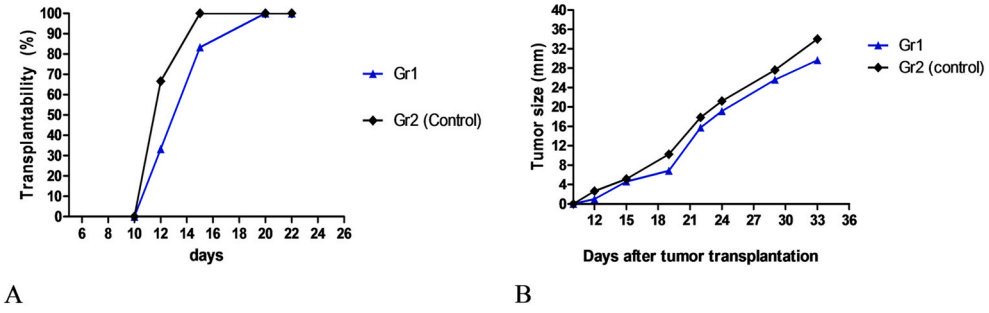

Fig. 5. Tumour transplatability (%): **A** and tumour size in GTBH: **B**.

DAPI staining was used for investigation of the apoptotic changes in the nucleus of the Graffi tumour cells treated with PSM. The typical apoptotic features were condensation, margination of the chromatin and formation of the apoptotic bodies. The control (non-treated Graffi tumour cells) was with intact nuclei, oval in shape and varying in size, with smooth edge and homogenously distributed chromatin (Fig. 3a). Cells at different phases of mitosis were found (Fig. 3a). Changes in the nuclear morphology of the Graffi tumour cells, induced by the PSM treatment, included chromatin condensation (heterogeneously distributed chromatin with granular structure), fragmentation of the nucleus and formation of the apoptotic bodies (Fig. 3b).

At the early stages of apoptosis, changes in the lipid composition of the cell membrane occur. One of these changes is the translocation of phosphatidylserine (PS) from the inner side of cytoplasmic membrane to the surface. The location of PS on the cell membrane was detected by Annexin V-FITC staining, which in combination with PI stain, could differentiate apoptotic from necrotic cells (Zhang et al., 1997). The effect of PSM treatment of Graffi tumour cells and the induction of apoptosis were examined by Annexin V Apoptosis Detection Kit. Control cell showed homogenous red-orange fluorescence, as a result of PI staining, because both control and treated cells were fixed with 3% paraformaldehyde. Green fluorescence was not found (Fig. 4a). In the cell cultures treated with PSM, early apoptotic cells, emitting green fluorescence, as a result of PS binding to Annexin V – FITC and late apoptotic cells with green fluorescent staining of the membranes and orange-red fluorescence of the nucleus with chromatin condensation and fragmented nuclei were observed (Fig. 4b).

3.2. *In vivo effects of PSM*

On the 12th and 22nd days of the onset, appearance of tumours were delayed (tumour transplantability, TT%) and tumour size was smaller in hamsters with PSM therapy. In the control group (untreated animals), tumours appeared 10–14 days after s. c. transplantation of the tumour cells, whereas in PSM-treated hamsters – from 10 and prolonged to 20 days (Fig. 5A). The tumour size was assessed to the 33rd day of the

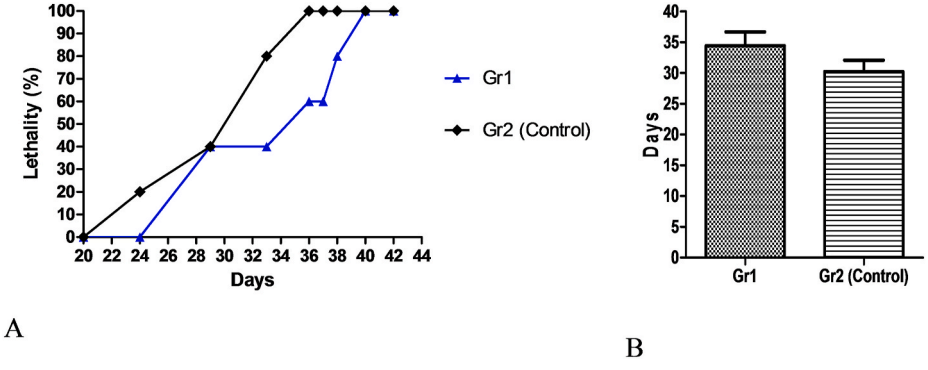

Fig. 6. Lethality (%): **A** and prolonged mean survival time (days): **B** in GTBH.

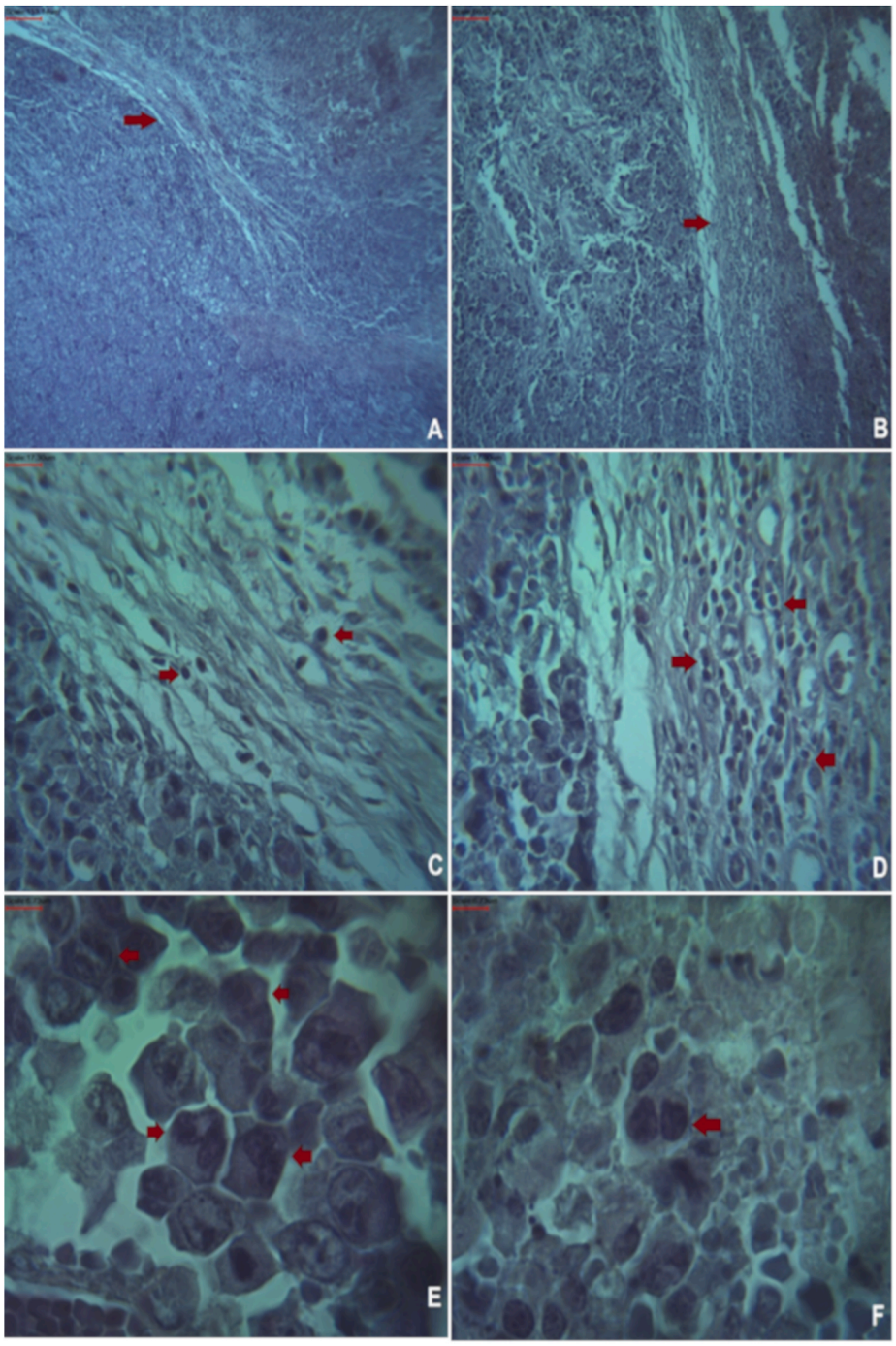

Fig. 7. Histopathology of tumour from a hamster: **A** Control. Presence of a stromal component; **B** Treated with PSM. Well-formed stroma with accumulation of mononuclear cells; **C** Control. Single mononuclear cells into the stroma; **D** Treated with PSM. Numerous mononuclear cells into the stroma; **E** Control. Presence of multiple incorrect mitotic figures; **F** Treated with PSM. Mitotic cells.

transplantation of tumour cells at certain time intervals. Tumours smaller in size were observed in hamsters treated with PSM, compared to control animals (Fig. 5B). The highest lethality rate was observed in the control group (Graffi tumour bearing hamsters, GTBH). The same was significantly lower in PSM-treated animals, both in the period 28–38 days (Fig. 6A). The mean survival time (MST) was 30.2 ± 4.2 days for the GTBH control and 34.4 ± 5.1 days for the animals treated with PSM (Fig. 6B).

The pathological study showed the presence of a stromal component (Fig. 7**A),** single mononuclear cells into the stroma (Fig. 7**C)** and multiple incorrect mitotic figures (Fig. 7**E)** were observed in the tumour tissue from the control group. Well-formed stroma with accumulation of mononuclear cells (Fig. 7**B, D)** and mitotic cells (Fig. 7**F)** were found in the group, treated with PSM.

4. Discussion

The PSM was obtained in relatively large quantity through a classical approach to allow the *in vivo* study. There are many ways to increase quantity in technological means as pre-soaking of the plant material (Zhao et al., 2020). As for saponins such research is still an object of investigation.

The results obtained from the pharmacological study complement other researchers' as well as our previous findings. A purified extract from *Astragalus gombo*, rich in saponins, displayed *in vitro* antiproliferative activity against a panel of human solid tumour cells – HBL-100 and T-47D (breast) and WiDr (colon) (Teyeb et al., 2017). An oleanane type saponin, 3-*O*-[α-L-rhamnopyranosyl-(1 → 2)-β-D-xylopyranosyl-(1 → 2)-β-D-glucuronopyranosyl]-3β,22β,24-trihydroxyolean-12-en-29-oic acid, isolated from *A. angustifolius* was reported to possess minor cytotoxic effect against cervical (HeLa) and human colon (HT-29) cancer cell lines (IC_{50} of 36 and 50 μM) (Gülcemal et al., 2012). A purified saponin mixture from *A. corniculatus* displayed a protective effect against experimental Graffi myeloid tumour in hamsters (Krasteva et al., 2004). In addition on both healthy and Graffi-tumor bearing hamsters treated with the same saponins, the number, migration and phagocytic indexes of peritoneal macrophages (pMøs) and of blood polymorphonuclear leukocytes (PMNs) were increased significantly (Toshkova et al., 2007). The immunostimulating and immunorestoring activity on the T- and B-spleen cells in healthy and Graffi tumour bearing hamsters was also proved (Toshkova et al., 2008). The antiproliferative effect of a mixture of two saponins, obtained from *A. hamosus* against SKW-3 cells was confirmed (Krasteva et al., 2008). The results of the current study support also the literature data about the *in vitro* cytotoxic and anti-tumour activity of the saponins, isolated from genus *Astragalus*, as well as immunomodulating activity of *A. kahiricus* saponins (Ionkova et al., 2014).

We found that Graffi tumour cells were sensitive to PSM from *A. glycyphyllos*, after 24 and 48 h treatment. PSM induced a statistically significant decrease of the viability/proliferation of the Graffi tumour cells *in vitro*. These effects were concentration- and time-dependent. IC_{50} values for 24 and 48 h were calculated.

Fluorescent microscopy studies indicated that these antiproliferative/anti-tumour effects were connected to the induction of apoptosis in the Graffi tumour cells. After intravital double staining with AO/EtBr of the Graffi cells, treated with PSM, early- and late-apoptotic cells were observed. The majority of cells were late apoptotic with membrane blebbing, the chromatin was condensed and marginated, the nuclei were fragmented and apoptotic bodies were visible.

DAPI has high affinity for binding to adenine-thymine rich clusters of DNA. The binding appear at the small groove of the DNA molecule. It is known that DAPI could also bind to adenine-uracil rich clusters of RNA, but to a lesser extent. The molecule of DAPI can pass through the intact cytoplasmic membrane, which gives the opportunity to investigate the nuclear morphology of both live and fixed cells (Tanious et al., 1992). After DAPI staining of the Graffi cells, treated with PSM, the morphological changes of the nucleus were typical for the apoptosis (non-homogenously distributed chromatin, condensation of the chromatin, nucleus fragmentation and formation of the apoptotic bodies).

Annexin V binds the negatively charged superficial phospholipids, by Ca^{2+}-dependent mechanism, and shows high affinity to the phosphatidylserine. The cells, which show membrane changes (translocation of phosphatidylserine from the internal to the external side of the cell membrane) are coloured in green, while the cells with changed membrane permeability (late apoptotic and necrotic cell as well as fixed cells) show bright red-orange fluoresce, because of PI binding with DNA (Rieger et al., 2011). Cytochemical investigations of Graffi cells, after staining with Annexin V-FITC, showed changes in the cell membrane, which were typical for the apoptosis. Some of the cells were stained in green that is a sign for translocation of phosphatidylserine from the inner to the outer membrane surface, while other cells were with changed membrane permeability (late apoptotic and necrotic) and showed bright red-orange fluorescence, because of PI binding to DNA. The underlying mechanism of numerous solid cancers is the activation of the PI3K/Akt/mTOR signal pathway. The regulation of the growth, survival, motility, as well as drug-resistance is also indicated to be connected with the PI3K/Akt/mTOR cascade. Thus this pathway is considered to be of importance as an attractive target for new anticancer agents (Zhou et al., 2018).

It was established that *A. membranaceus* purified extract inhibited the PI3K/Akt/mTOR signal pathway *in vitro*, resulting in inhibited cell proliferation in MCF-7 (ER+, HER2−), SK-BR-3 (ER−, PR−, HER2+) and MDA-MB-231 (a triple-negative breast cancer (TNBC, ER−, PR−, and HER2−) cancer lines (Zhou et al., 2018). It could be assumed, that the observed anti-tumour effects of the purified saponins' mixture of *A. glycyphyllos* could also involve this signal pathway.

The *in vitro* anti-neoplastic effects of PMS were supported by the *in vivo* results on the tumour formation. The tumour size was decreased in the group, treated with PMS, as well as well-formed stroma with accumulation of mononuclear cells and mitotic cells was observed.

There is little information of anti-tumour effects *in vivo*, connected with saponins isolated from some *Astragalus* species (Ionkova et al., 2014). A saponin fraction from the root of *A. membranaceus*, restored the depressed mitogenic response in tumour-bearing mice *in vivo* (Cho and Leung, 2007). This fraction significantly increased the influx of macrophages into the peritoneal cavity of mice and also slightly prolonged the life-span of tumour-bearing mice. In the same study, the influx of macrophages into the peritoneal cavity and the effector functions of macrophages were found to be enhanced. The administration of saponins *in vivo* could partially restore the lymphocyte blastogenic response in tumour-bearing mice. Injection of mice could markedly enhance the *in vivo* tumoricidal activity of the peritoneal macrophages. Saponins could act as a priming agent for the TNF (tumour-necrotic factor) production in tumour bearing mice *in vivo*.

According our *in vivo* data and the literature data, we can suggest that *in vivo* anti-tumour effects of PMS, might be due to possible immune-modulating and anti-inflammatory activity.

5. Conclusion

The PSM induced dose- and time dependent *in vitro* antiproliferative and cytotoxic effects on the Graffi myeloid tumour cells. The fluorescent microscopy studies revealed marked alterations in the cellular and nuclear morphology of PSM treated Graffi cells. The *in vivo* study confirmed these results. PSM reduced tumour growth, transplantability and mortality rates, improved the histological profile.

Declaration of competing interest

The authors declare no competing interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2020.113519.

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