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Steroids from *Helleborus caucasicus* reduce cancer cell viability inducing apoptosis and GRP78 down-regulation.

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Abstract

Helleborus caucasicus (Ranunculaceae) is an endemic plant of the Caucasian flora, widely distributed in West Georgia. Biological activities for the extracts of some *Helleborus* species including *H. caucasicus* have been reported. In this work we found that butanolic extract of the underground parts of *H. caucasicus* and isolated compounds decreased cell viability *in vitro* on cancer cell line of lung origin (Calu-1) in a concentration-dependent manner, compared to the normal cell line. In particular, we identified that furostanol derivative (25S)-22α,25-epoxyfurost-5-ene-3β,11β,26-triol 26-O-β-D-glucopyranoside (**5**), 20-hydroxyecdysone (**6**), and 3β,5β,14β-trihydroxy-19-oxo-bufa-20,22-dienolide 3-O-α-L-rhamnopyranoside, known as deglucohellebrin (**7**) exerted a strong cytotoxic effect on the same cells and on other cancer cell lines (HepG2 and Caco-2) reducing the S-phase entry (compound **6**) and inducing cell apoptosis associated with activation of caspase-3 (compound **7)**. Moreover we demonstrated that **6** and **7** significantly decreased protein expression of GRP78, a general ER-stress marker, suggesting pro-apoptotic functions. These findings indicated that selected compounds from *H. caucasicus* are potential interesting agents in anti-cancer therapy.

Abbreviations

(DMSO)dimethylsulfoxide, (MTT)3-(4,5 dimethylthiazol-2-yl)-2,5diphenyltetrazolium,(BrdU)bromodeoxyuridine, (DEVD)Asp-Glu-Val-Asp, (pNA) p-nitroanilide, (VP16) Etoposide, (THP) thapsigargin.

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1. Introduction

Helleborus species are evergreen, rhizomatous plants belonging to the family Ranunculaceae. For some species, biological activities are reported. A remedy with antalgic and antirheumatic properties based on *Helleborus* extracts has been registered with the name Boicil (US patent) [1]. *H. niger*, the wellknown ornamental plant known as Christmas Rose, is used in homeopathy to treat mental conditions that are marked by lethargy and confusion. Recently, the cytotoxic activity of extracts from *H. niger* has been reported [2].

H. caucasicus is an endemic plant of the Caucasian flora, widely distributed in West Georgia.

Previous studies demonstrated that the polar extract of the roots and rhizomes of this plant showed high cytotoxic activity against human lung cancer (A-549) and human colorectal cancer (DLD-1) cell lines [3]. The butanolic extract of the underground parts of this plant resulted to be rich in steroidal derivatives belonging to different classes, namely furostanol glycosides, spirostanol glycosides, bufadienolides and ecdysteroids [4].

With this study we aimed to evaluate the cytotoxic activities of the butanolic extract and of selected purified compounds from *H. caucasicus* against specific human cancer cell lines, to understand which chemical compounds were potentially bioactive. Moreover, in order to identify new agents in anti-cancer therapy, we investigated the molecular mechanism of action of compounds exerting the strongest cytotoxic effect. Tested compounds comprised the furostanol glycosides **1**-**4** reported only in *H. caucasicus*, the furostanol derivative (*25S*)-22α,25-epoxyfurost-5-ene-3 β,11 β,26-triol 26-O-β -D-glucopyranoside (**5**), 20-hydroxyecdysone (**6**), and 3β,5β,14β -trihydroxy-19-oxo-bufa-20,22-dienolide 3-*O*-α-L-rhamnopyranoside, known as deglucohellebrin (**7**) (Fig.1).

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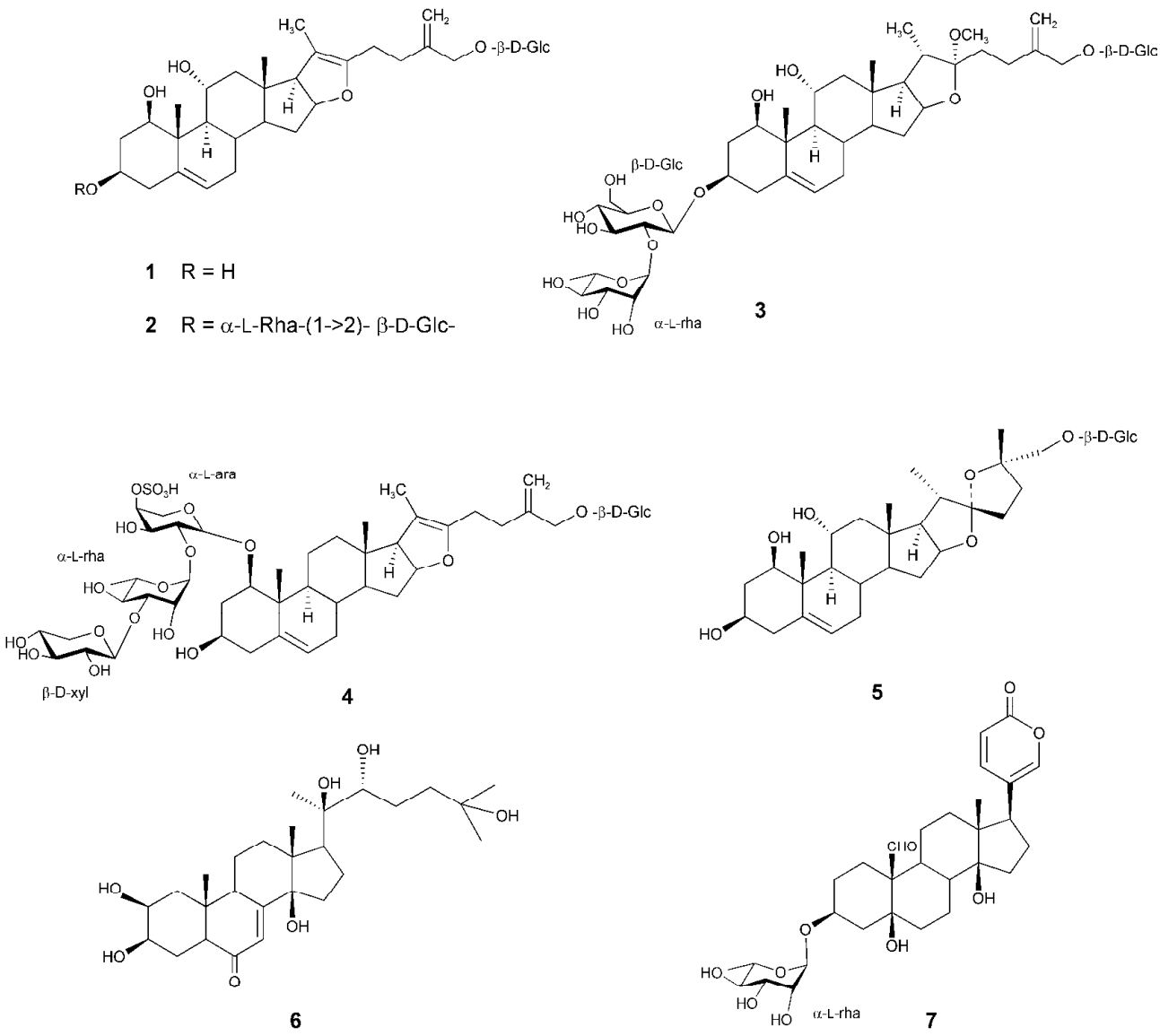

Fig.1: Compounds 1-7 isolated from *H. caucasicus*

2. Methods

2.1. Plant Material

The roots and rhizomes of *H. Caucasicus* A.Br. were collected in Georgia in September 2013. Samples of *H.caucasicus* were identified by Dr.Jemal Aneli, Department of Botany, Institute of Pharmacochemistry, Tbilisi, Georgia.

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2.2. Extraction and isolation

The air-dried roots and rhizomes of *H. caucasicus* (300 g) were defatted with petroleum ether (1 l) and then with chloroform (1 l). After drying, the plant material was extracted with 80% MeOH (1.5 l) three times at 50°C. The collected extracts have been dried under vacuum and the concentrate was partitioned between water and 1-butanol (18 g). Part of the 1-butanolic extract (2.5 g) containing steroidal compounds was subjected to silica gel column chromatography (115 ×2.5 cm, 100/160 μm, Merck) eluting with isocratic system chloroform:methanol:water (26:14:3) yielding 3 combined fractions A (700 mg), B (270 mg), C (330 mg).

Fractions A-C were chromatographed by RP-HPLC (Waters XTerra Prep MSC18 column, 300 ×7.8 mm i.d.) at flow rate 2.0 ml/min using different mixtures of MeOH:H_2O in isocratic conditions.

From fraction A (53% MeOH as eluent) compounds **1** (5.1 mg, t_R = 23.8 min), **5** (4.0 mg, t_R = 17.7 min), **6** (6.1 mg, t_R = 29.6 min), and **7** (1.6 mg, t_R = 7.2 min) were isolated; fraction B (55% MeOH as eluent) yielded compounds **2** (3.0 mg, t_R = 18.0 min) and **3** (3.2 mg, t_R = 20.8 min); from fraction C (50% MeOH as eluent) compound **4** (6.0 mg, t_R = 11.3 min) was isolated.

Characterization of these compounds has been reported in [4].

2.3. Cell cultures

All cell lines employed in this study were obtained from Interlab Cell Line Collection (IST, Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy). Calu-1, human epithelial lung cancer cells, MRC-5, human normal lung fibroblasts, and Caco-2, human colon cancer cells, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 0.2 mM L-glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin (Invitrogen SRL, Milan, Italy). The medium for Caco-2 cells was also supplemented with 1% non-essential amino acids. HepG2, human liver cancer cells, were cultured in Eagle's minimum Essential medium supplemented with 10% (v/v) fetal bovine serum, 0.2 mM L-glutamine, 1% non-essential aminoacids, 50 units/ml

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penicillin and 50 μg/ml streptomycin. Cells were maintained at 37°C in a 5% CO_2, 95% air-humidified atmosphere and passaged twice a week.

2.4. Cell viability assays

To assess cell viability in the presence of *H. caucasicus* butanolic extract, as well as in the presence of isolated compounds, cells were seeded at the density of $5.0x10^4/cm^2$and cultured for 24 h, then treatments were carried out for 24h. Stock solutions of *H. caucasicus* isolated compounds were prepared in dimethylsulfoxide (DMSO) (Sigma-Aldrich, Milan, Italy). The final DMSO concentration in the media was less than 0.1%.Cell viability was analyzed by counting viable cells in a Burker hemocytometer. Cell viability was also determined by a 3-(4,5 dimethylthiazol-2-yl)-2,5diphenyltetrazolium (MTT) assay. The MTT assay is based on the enzymatic conversion of MTT (Sigma-Aldrich) in mitochondria. After incubation with *H. caucasicus* extracts and isolated compounds (or relative vehicles), MTT was added to cell medium (final concentration 0.125 mg/ml) and incubated for 1 h at 37°C. The resulting formazan crystals were dissolved in DMSO and absorbances were measured at wavelength of 570 nm and 690 nm.

2.5. S-phase entry assay

For bromodeoxyuridine (BrdU) (Roche Diagnostics SpA, Monza, Italy) incorporation studies, Calu-1 cells were seeded at the density of $1.5x10^4/cm^2$ on coverslips and, 24 h later, were treated with 1 μM of each isolated *H. Caucasicus* compound or with the vehicle (DMSO) for 21 h. BrdU was added to the medium (final concentration, 100 μM) 3 h before fixing with 3% paraformaldehyde and permeabilizingwith 0.2 % Triton X-100.BrdU incorporation was monitored by treating cells with an anti-BrdU antibody 1:100 (Invitrogen SRL) and a secondary TRITC-conjugated antibody (Invitrogen SRL) 1:100. Finally, cells were stained for 5 min with Hoechst (1 μg/ml in PBS) and coverlips were mounted with Mowiol (Sigma-Aldrich). The number of cells that entered into S-phase was expressed as the ratio between the number of cells incorporating BrdU and the total

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number of cells. Stained cells were observed with an AxioSkop 40 fluorescent microscope (Carl Zeiss MicroImaging, Inc., Jena, Germany). Images were acquired with Axiocam MRc5 and processed with the Axiovision 4.2 software (Carl Zeiss MicroImaging Inc.).

2.6. Microscopic apoptosis detection

To detect cell apoptosis, cells were seeded at the density of $1.5x10^4/cm^2$on glass coverslips and after 24 h they were treated with 1 μM of each isolated *H. caucasicus* compound or with the vehicle (DMSO), for further 24 h, then were stained with Hoechst as described for S-phase entry assay. The appearance of apoptotic condensed chromatin was detected by microscope observation as reported above.

2.7. Caspase-3 activity assay

For caspase-3 assay we utilized as substrate the synthetic tetrapeptide, Asp-Glu-Val-Asp (DEVD), labeled with p-nitroanilide (pNA) (Sigma-Aldrich). DEVD-dependent protease activity is assessed by detection of the free pNA cleaved from the substrate. Briefly, cells treated with or without isolated *H. caucasicus* compounds were lysed in a buffer containing 50 mM HEPES, 0.1% CHAPS, 10 mM dithiothreitol, 100 mM NaCl, 1 mM EDTA and 10% sucrose; cell lysates were incubated for 1 h at 37 °C in a reaction mixture containing DEVD–pNA. Samples were then read on a spectrophotometer at a wavelength of 405 nm. The caspase-3 activation was expressed as percentage of absorption of treated cells versus untreated control cells Etoposide (VP16) was used as positive control at concentration of 60 μg/ml.

2.8. X-box binding protein-1 (XBP1) splicing detection

Calu-1 cells were cultured for 24 h then treated with 10 μM of *H. caucasicus* isolated compounds or DMSO, or alternatively with thapsigargin (THP) 1 μM, for 1h and 4h. After total RNA extraction with the Trizol reagent (Invitrogen SRL), 1 μg of total RNA was used to synthesize the first-strand

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cDNAs by using the QuantiTech Reverse Transcription Kit (QiagenSrl, Milan, Italy).The obtained cDNAs were used to detect the un spliced and the spliced form of human XBP1 by PCR with the following primers (500 nM each): upper, 5'-CCTGGTTGCTGAAGAGGAGG-3'; lower, 5'-CCATGGGGAGATGTTCTGGAG-3'.PCR reactions were run on a MyCycler™ Thermal Cycler System Bio-Rad for 35 cycles with heating at 94°C for 30 sec, followed by annealing at 58°C for 45 sec and polymerization at 72°C for 60 sec. Amplification of XBP1cDNAs was visualized on a 2.5% agarose gel after staining with ethidium bromide.

2.9. Real-time RT-PCR for analysis of glucose regulated protein (GRP)78 mRNA expression

Calu-1 cells were cultured for 24 h then treated with 10 μM of each *H. caucasicus* isolated compound or with DMSO, or alternatively with THP 1 μM, for 4 h and for 20 h. RNA extraction and cDNA synthesis were performed as described above.The human GRP78 cDNA was amplified by real-time PCR with the following primers: upper, 5′-CTGGGTACATTTGATCTGACTGG-3′; lower, 5′-GCATCCTGGTGGCTTTCCAGCCATTC-3′. Each analysis was performed in triplicate with the iQ^{TM} SYBR Green Supermix (Bio-Rad Laboratories, Milan, Italy) and using the iQ^{TM} 5 Multicolor Real Time PCR Detection System (Bio-Rad Laboratories). PCR reaction mixtures contained 250 nM of each primer and 10 μl of SYBR Green Supermix, in a total volume of 20 μl. The PCR program started with 3 min of incubation at 95°C, followed by 40 cycles of 15 sec at 95° C, 15 sec at 60°C, and 20 sec at 72°C. The concentration of mRNA was normalized to the concentration of GAPDH (primers: upper, 5′-TTCAACAGCGACACCCACTG-3′; lower, 5′-CACCCTGTTGCTGTAGCCA-3′).

2.10. Western blot analyses to detect GRP78

To analyse GRP78 protein expression, cells were treated for 24 h with 10 μM of each compound, or with DMSO, or with THP, then cell lysates were prepared. Briefly, Calu-1 and MRC-5 cells were

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lysed in a buffer containing 20 mMTris-HCl, pH 7.5, 150 mMNaCl, 1 mM EDTA, 1 mMdithiotreitol, 0.1% sodium dodecyl sulphate, 1% triton X-100, 1 mMortovanadate, and inhibitors cocktail (Sigma-Aldrich). Then 70 μg of total proteins were separated on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Millipore Corp. Bedford, MA, USA)for western blot analyses. Primary antibody (rabbit anti-GRP78antibody (H-129) from Santa Cruz Biotechnology Inc.Heidelberg, Germany) was used at dilution of 1:1000 in PBS containing 1% non-fat dry milk, overnight at 4°C. For normalization, a mouse anti-GAPDH antibody (Santa Cruz) was used. Then blots were incubated with anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories) for 1 hour at room temperature.Immunocomplexeswere revealed using a chemiluminescence detection kit (Euroclone, Milan, Italy) according to the manufacturer's instructions.

2.11. Statistics

All data were expressed as means ± SD of at least 3 independent experiments conducted in triplicates. Statistical analysis was performed by using the Student's t test. In all experiments, differences were considered to be statistically significant at $p<0.05$.

3. Results and discussion

3.1. The butanolic extract from *H. caucasicus* decreases cell viability *in vitro*

The cytotoxic activity of butanolic extract from roots and rhizomes of *H. caucasicus* was characterized employing a cancer cell line of lung origin (Calu-1). The extract on a normal human lung cell line (MRC-5) used as reference was also tested.

Calu-1 cells were treated for 24 hours with increasing concentrations of the butanolic extract (0.002 μg/μl-0.2 μg/μl), then cell viability was analyzed by the MTT assay. We observed that the butanolic extract decreased cell viability of tumor lung cells in a concentration-dependent manner (Fig.2). In particular, at the lowest concentration tested (0.002 μg/μl), it decreased Calu-1 cells viability of

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64%. Instead, at the same concentration, the butanolic extract reduced of only 37% cell viability of MRC-5 cells. Moreover, also in the range of concentration 0.01 µg/µl−0.1 µg/µl, cytotoxicity of the butanolic extract was more pronounced towards Calu-1 cells than towards MRC-5 cells, suggesting that this extract exerted a partial selective cytotoxic effect on a tumor cell line with the respect to a normal one. Only at highest concentration tested, the effect on cell viability was comparable in both cell lines (Fig.2).

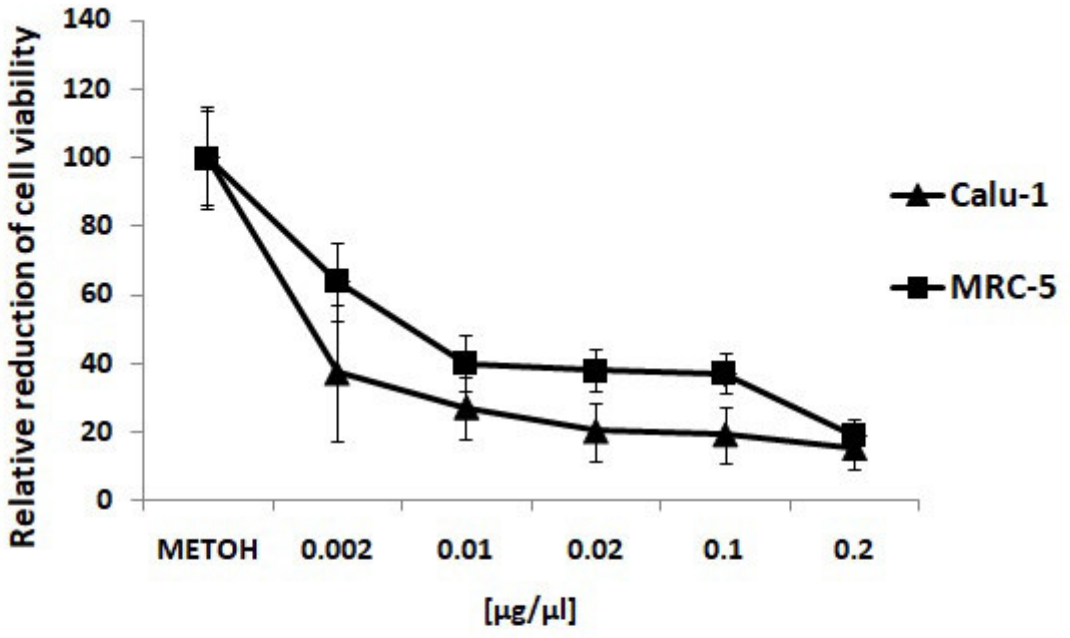

Fig.2: Effect of butanolic extract from *H.caucasicus* on cell viability

Calu-1 and MRC-5 cells were treated for 24 hours with increasing concentrations of butanolic extract (0.002µg/µl-0.2 µg/µl), then cell viability was monitored by an MTT assay. *$p<0.05$ for cells treated with butanolic extract at a concentration ≥ 0.002µg/µl *vs* control cells treated with methanol only.

3.2. Isolated compounds from *H. caucasicus* are cytotoxic for cancer cells

In order to identify which compounds of the butanolic extract were specifically responsible for the observed cytotoxicity, we investigated the effect of 7 isolated compounds from *H. caucasicus* on Calu-1 cells. After treatments for 24 hours with increasing doses (0.1-20 µM) of compounds **1-7**,

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cell viability was measured by the MTT assay.

We found that compounds **1-4** exerted only a little effect on viability of Calu-1 cells, at all concentrations tested (Fig. 3). Instead, compounds **5**, **6** and **7** exerted a strong cytotoxic effect on the same cells (Fig. 3).

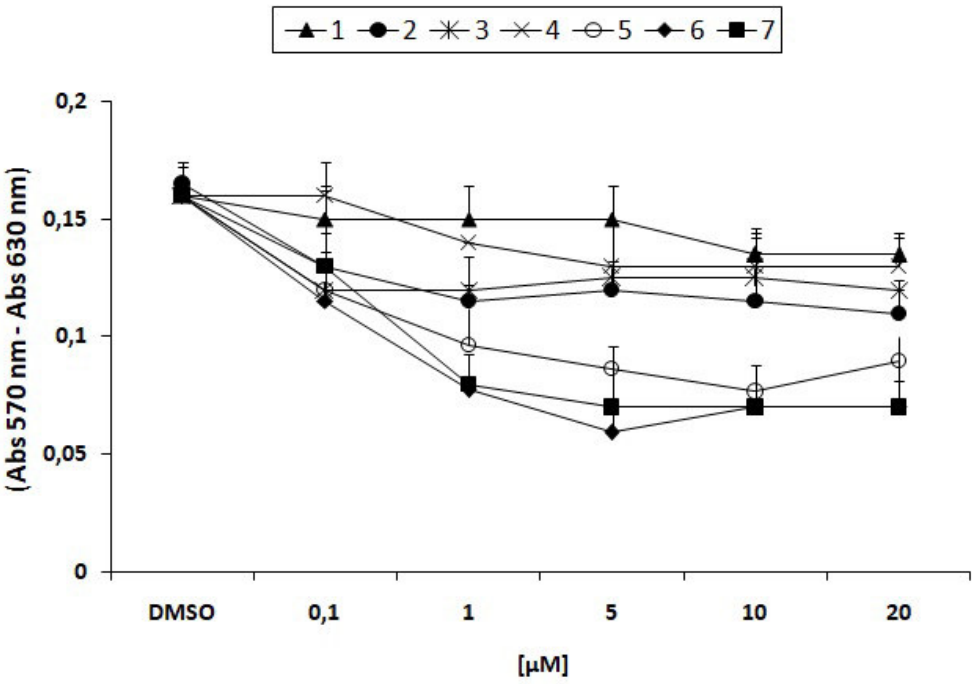

Fig.3: Effect of isolated *H. caucasicus* compounds 1-7 on Calu-1 cells survival.

Calu-1 cells were treated with increasing concentrations of each compound (0.1-20µM), then cell viability was monitored by an MTT assay. *$p<0.05$ for cells treated with compounds **5-7** at a concentration ≥ 1µM *vs* control cells treated with DMSO.

In particular, a noticeable reduction of cell viability was observed at 1 µM (Table 1). At this concentration, compound **5** induced a decrement of cell viability of 40%, whereas compounds **6** and **7** induced the highest reduction (48%) of cell viability.

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Compound	Reduction of cell viability %
1	2 ± 1.4
2	39 ± 0.7
3	25 ± 0.9
4	12.5 ± 1.4
5	40 ± 0.8
6	48 ± 1
7	48 ± 0.5

Table 1: **Effect of compounds 1-7 (1µM) isolated from *H. caucausicus* on Calu-1 cells cell viability**.

Citotoxicity of these compounds was confirmed by counting the number of living Calu-1 cells after 24 h of treatment with 1 µM of compounds **5**, **6** and **7**. Indeed, they reduced the percent of living cells of about 28, 53 and 70%, respectively (Fig. 4A). Interestingly, as already observed for the butanolic extract, these compounds were less efficient in reducing cell viability of MRC-5 cells with respect to Calu-1, as demonstrated by both counting the number of living cells (Fig. 4A) and performing the MTT assay (Fig. 4B).

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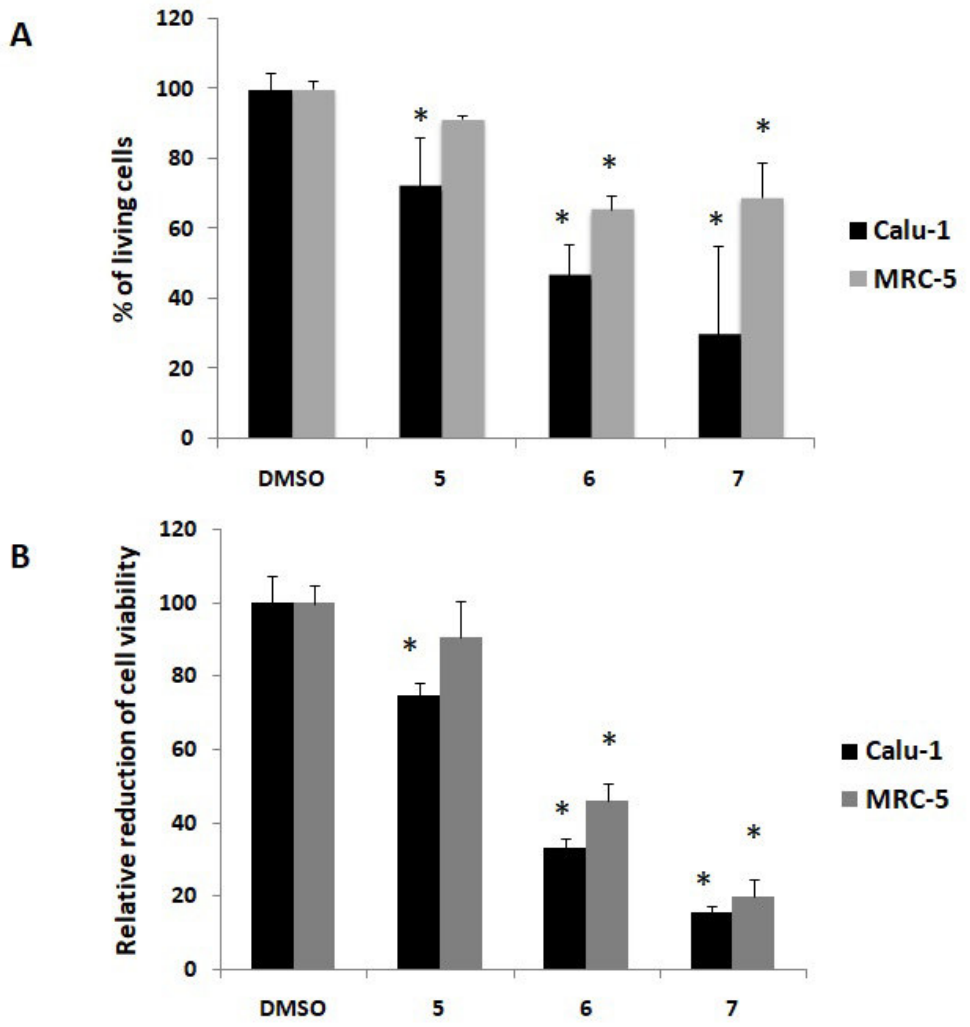

Fig. 4: Comparison of cytotoxic effects of compounds 5-7 on cancer (Calu-1) and normal (MRC-5) cell lines.

Calu-1 and MRC-5 were treated with compounds 5-7 at 1μM for 24 h, then residual living cells were monitored by cell counting (A) and by the MTT assay (B). *$p<0.05$ *vs* control cells treated with DMSO.

Based on these results all subsequent experiments were performed with compounds **5-7**.

To verify whether selected compounds were also active towards other cancer cells, we tested the effect of selected compounds **5-7** on cell viability of other two cancer cell lines, HepG2 (hepatic) and Caco-2 (intestinal). MTT assays carried out under previous conditions showed that all three compounds affected cell viability, however compound **5** was less active compared to **6** and **7**, as

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already observed on Calu-1 cells (Fig.5). Interestingly, Caco-2 cells sensibility was comparable to that of Calu-1 cells, whereas HepG2 cells appeared more resistant than Calu-1 cells, probably depending ona higher general ability of hepatic cells to detoxify toxic compounds.

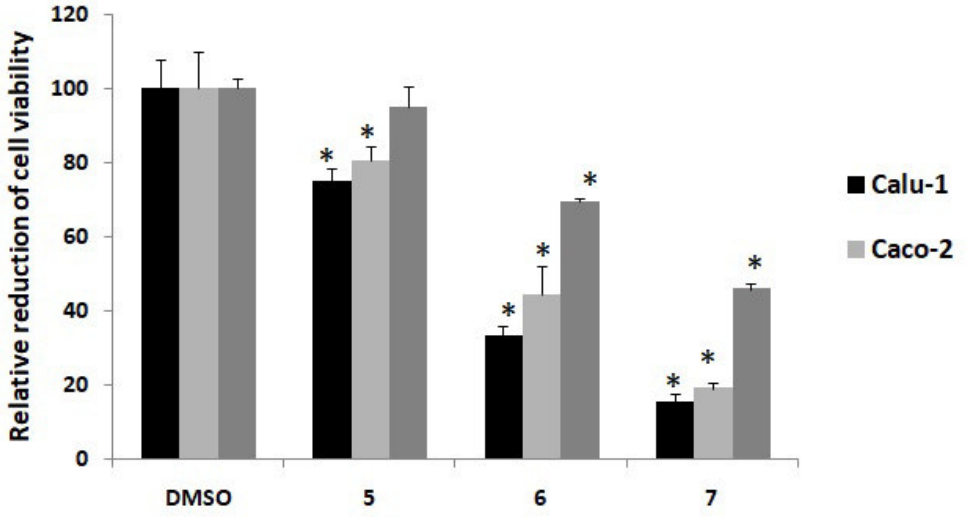

Fig. 5: Effect of compounds 5-7 on tumor cell lines of different origin. Calu-1, Caco-2 and HepG2 were treated with compounds 5-7 at 1μM for 24 h, then cell viability was analyzed by an MTT assay. *p≤ 0.05 *vs* control cells treated with DMSO.

3.3. Effects of compounds 5-7 on cell cycle of Calu-1 cells

Since we observed a reduced number of viable Calu-1 cells in the presence of compounds **5-7**, we investigated whether these compounds could affect cell cycle progression of Calu-1 cells. By an S-phase entry assay, we demonstrated that, by treating cells for 24 h with compounds **5-7** at 1μM, the number of cells incorporating BrdU, i.e. which are in the S-phase of the cell cycle, was sensibly reduced however this reduction resulted significant only for compound **6** (Fig.6).

These results indicated that isolated compounds **5-7** from *H. caucasicus* could partially interfere with cell proliferation, even at low doses.

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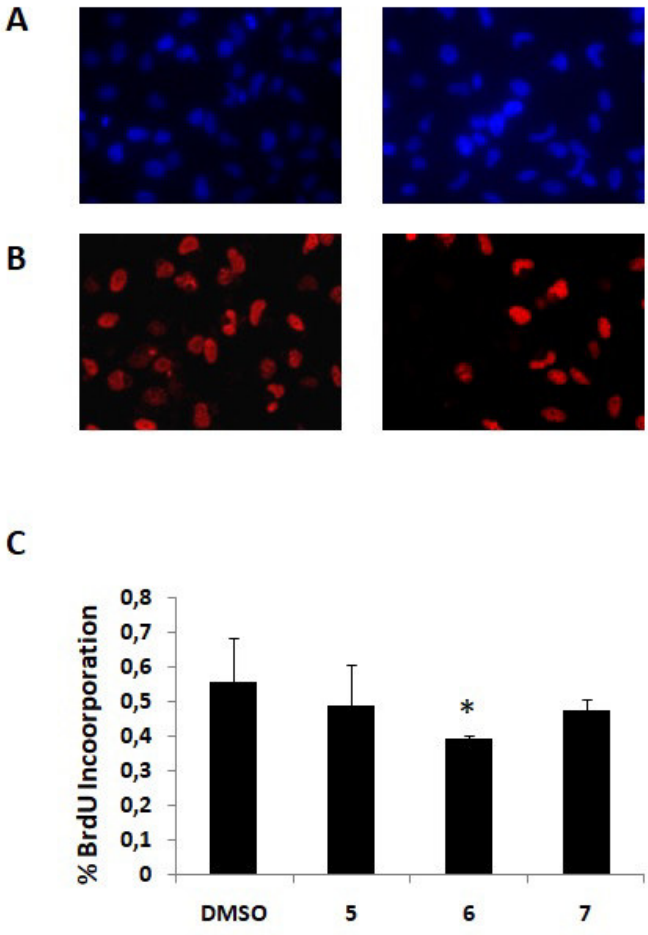

Fig. 6: Effect of compounds 5-7 on BrdU incorporation in Calu-1 cells.

(A) Microscopic visualization, x40 with oil, of representative Hoechst-stained (blue) and BrdU-stained (red) nuclei of Calu-1 cells treated with 1 µM of compound 6 for 24 h.(B) Quantification of BrdU incorporation by cells cultured for 24 h in the presence of 1 µM of compounds 5-7. *$p<0.05$ for *vs* control cells treated with DMSO.

3.4. Compound 5-7 from *H. caucasicus* induce apoptosis in Calu-1 cells

Several natural compounds extracted from plants are known to be cytotoxic by inducing cell apoptosis [5-7]. Then, we investigated whether the reduction of cell viability we observed was related to an increased apoptosis. By performing a Hoechst staining we visualized the appearance of several apoptotic nuclei in Calu-1 cells after 24h treatment with compounds **5-7** at 1 µM (concentration at which all compounds induced the highest cytotoxicity) (Fig. 7A). Since caspase-3

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is the major executioner caspase in the apoptotic pathway, we performed a caspase-3 assay to quantify apoptosis activation. The cytotoxic drug etoposide, which targeted topoisomerase II, thus inducing DNA damage, cell cycle arrest and caspase 3-activation, was used as positive control [8]. After treatment with each compound, caspase-3 activity was determined by the DEVD-*p*NA colorimetric assay. As shown in Fig. 7B all compounds stimulated caspase-3 activation; in particular, compound **7** was the most active (about 50% caspase activation), while compounds **5** and 6 activated caspase-3 at comparable lower levels (about 32% and 35 %, respectively). Consistently with previous results, compounds **5-7** were slightly less active on MRC5 cells than on Calu-1 cells. (Fig. 7B)

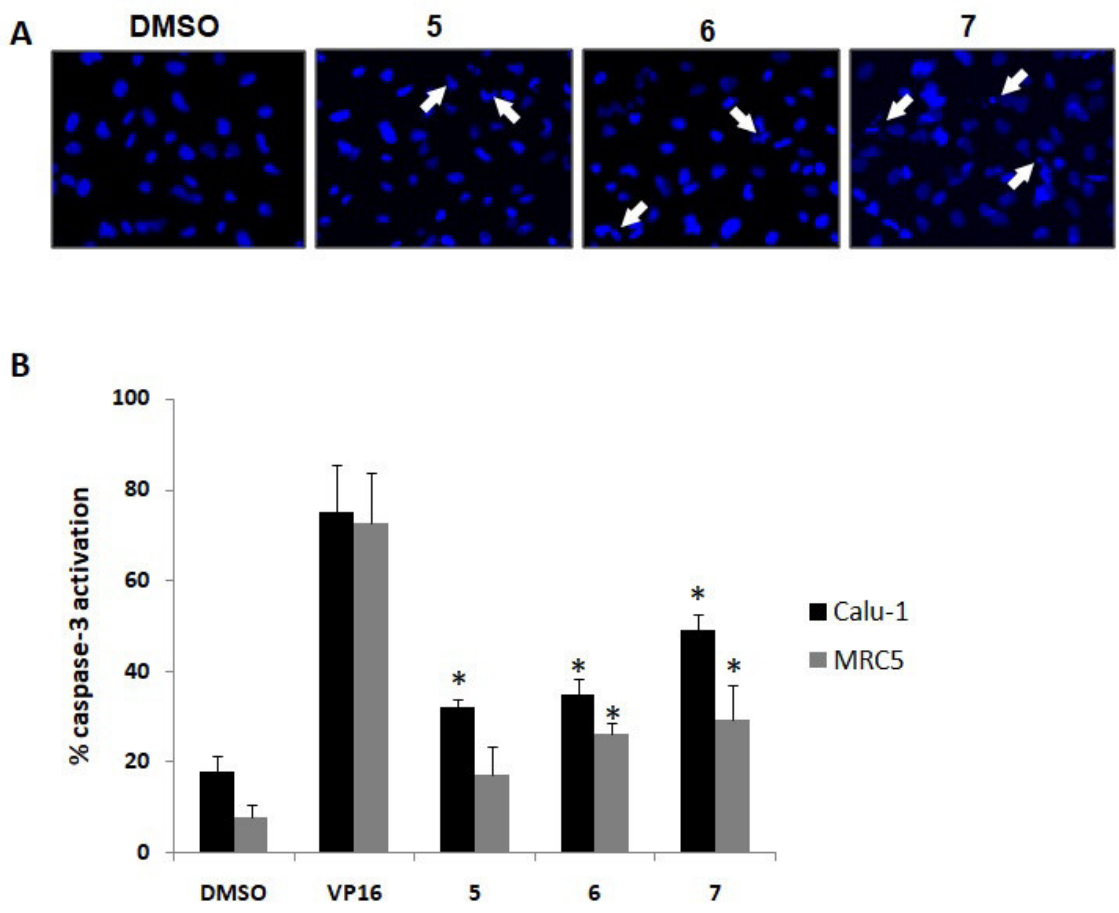

Fig. 7: Effect of compounds 5-7 on apoptosis.

(A) Microscopic visualization, x40 with oil, of representative Hoechst-stained nuclei of Calu-1 cells treated with 1 µM of compounds 5-7 for 24 h. White arrows indicate nuclei in which apoptotic morphology was evident. (B) Calu-1 and MRC-5 cells were treated with compounds 5-7 (10 µM)

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or etoposide (VP16) (60 μg/ml). Cell lysates were used to determine caspase-3 activity by colorimetric assay by using the substrate DEVD-pNA. *$p<0.05$*vs* respective DMSO treated cells.

3.5. Compounds 6 and 7 from *H. caucasicus* down-regulate GRP78 expression

Since bufadienolides, as well as other natural compounds obtained, are able to induce apoptosis by activating a ER-stress response [9-11], we investigated whether apoptosis induced by compounds **5-7** was correlated to ER stress. We first examined the appearance of an ER stress marker, the XBP-1 mRNA spliced form. The translation of this mRNA produces a transcription factor which induces the expression of ER–resident molecular chaperons during ER stress [12]. The RT-PCR analysis revealed that compounds **5-7** from *H.caucasicus* did not induce XBP-1 splicing after 1 and 4 h of treatment; on the contrary, the spliced form appeared by treating cells with the ER-stress inducer THP, which served as positive control (Fig. 8A). To confirm that ER-stress did not occur in the presence of compounds **5-7**, we also examined the expression of GRP78, a more general ER-stress marker. Indeed, GRP78 serves as a gatekeeper to the activation of ER stress transducers[13-14]. The real-time PCR revealed that treatment for 4h with compounds **5-7** at 10 μM did not influence GRP78 mRNA expression, whereas treatment for 20h with compounds **6** and **7** slightly but significantly increased GRP78 expression compared to control (Fig. 8B). To confirm this trend, we analyzed GRP78 protein level by western blot on lysates obtained from Calu-1 cells treated with compounds **5-7** for 24h. Surprisingly, we did not observe an induced GRP78 expression, on the contrary we found that compounds **6** and **7** significantly decreased GRP78 protein expression suggesting that there was a post-trascriptional or post-translational regulation leading to a reduced protein level (Fig. 9A). Finally, we found that compounds **6** and **7** also significantly reduced GRP78 expression in MRC-5 cells (Fig. 9B), suggesting that the mechanism of action of these compounds was similar both in cancer and in normal cells.

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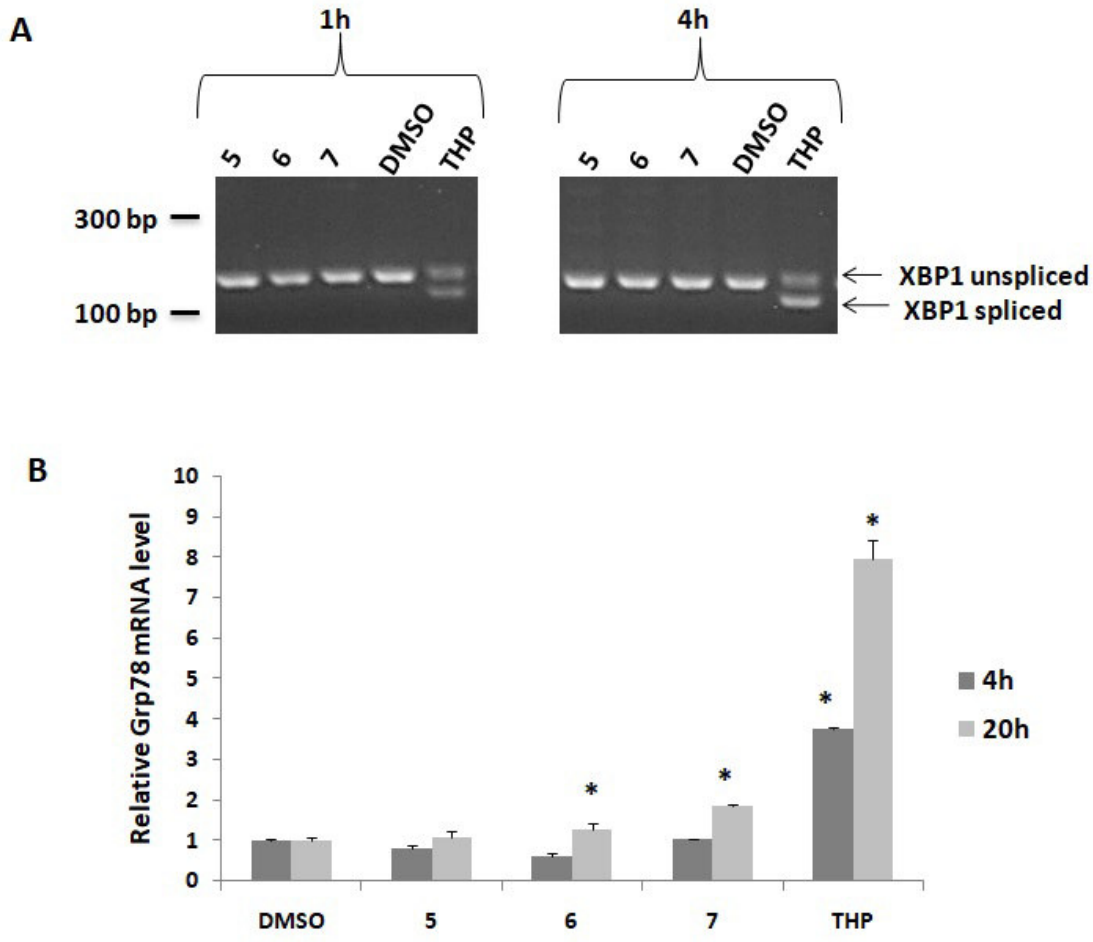

Fig.8: Analysis of XBP-1 splicing and GRP78m RNA level in Calu-1 cells treated with compounds 5-7.

(A) Treatments with 10 µM compounds 5-7 for 1 h and for 4 h did not induce the appearance of the spliced form of XBP-1 on a 2% agarose gel stained with ethidium bromide. THP 1 µM was used as positive control. (B) Analysis of GRP78 mRNA expression level by real-time RT-PCR after 4 h and 20 h of treatment with 10 µM compounds 5-7 or with 1 µM THP. The amount of GRP78 mRNA was normalized to that of GAPDH. *$p<0.05$ *vs.* DMSO treated cells.

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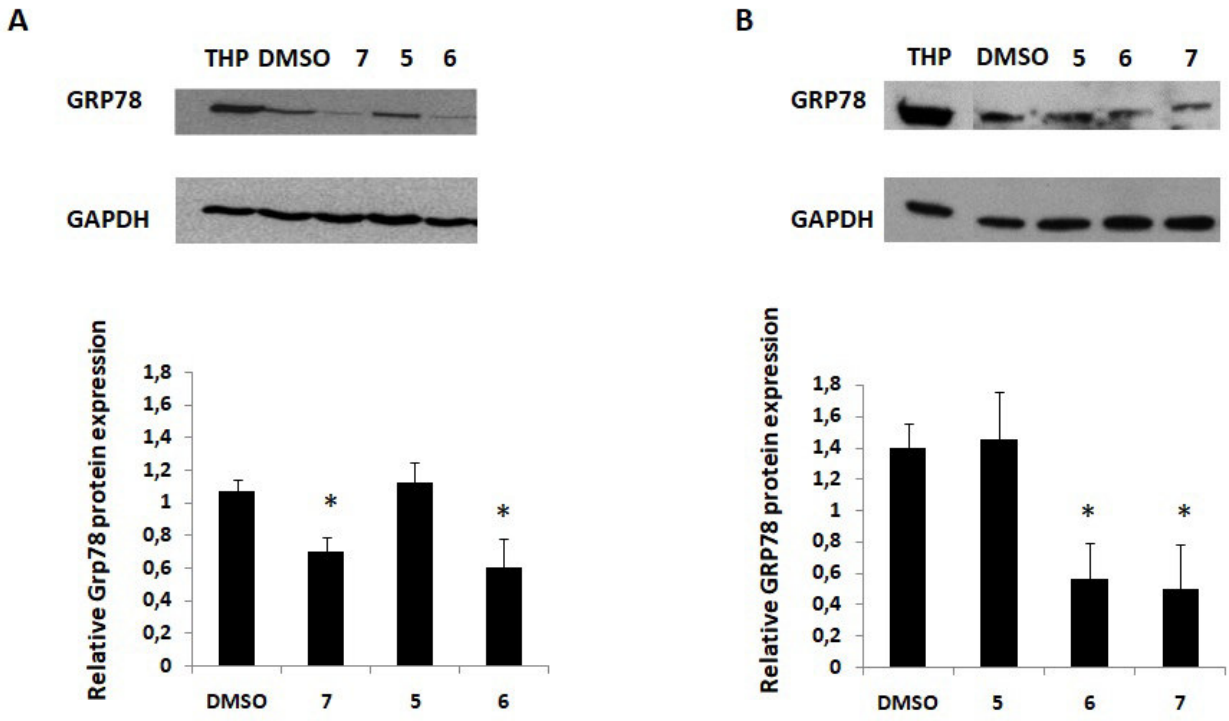

Fig.9: Effects of compounds 5-7 on GRP78 protein expression.

Representative western blots showing GRP78 protein after 24 h of treatment with compounds 5-7 (10 μM) in Calu-1 cells (A) and in MRC-5 cells (B). In the densitometric analysis, the amount of GRP78 is normalized to that of GAPDH. The positive control THP induces a 3.5-fold and a 5-fold GRP78 protein expression in Calu-1 cells and MRC-5 cells, respectively.. *$p<0.05$ *vs.* DMSO-treated cells.

4. Discussion

In the present study we examined in depth the biological activity of compounds occurring in thebutanolic extract of the underground parts of *H. caucasicus* in order to explore the possibility of their potential use as cytotoxic agents.

Preliminarily, we confirmed and extended previously reported data showing that crude extracts and enriched non-polar fraction from underground parts of *H. caucasicus* were cytotoxic towards both cancer and normal cells [3]. Our finding were also in line with recently described cytotoxic activity

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of extracts from *H. niger* [2,15]. Then, we evaluated cytotoxic effects induced by isolated compounds. Since compounds **1-4** did not have a relevant effect on cell viability measured by the MTT assay, we focused attention on compounds **5-7**, which strongly affected cell viability of cancer cells. Among these three compounds, compounds **6** and **7** displayed the highest cytotoxic activity, acting in a slightly different way; indeed, compound **6** was more effective in reducing the S-phase entry, whereas compound **7** was a stronger inducer of cell apoptosis.

Compound **6**, 20-hydroxyecdysone, belongs to the class of ecdysteroids, steroidal hormones widely occurring in insects, deriving their name (ecdy-) from the process of molting in insects, called ecdysis. Ecdysone and 20-hydroxyecdysone regulate larval molts, onset of puparium formation, and metamorphosis. They also occur in plants and are well studied as plant and insect growth factors [16]. Ecdysteroids show some biological effects in mammals when orally ingested, and have been reported as exerting activities similar to anabolic steroids without the androgenic effect [17]. Moreover, 20-Hydroxyecdysone has been reported to exert protection against cerebral ischemia injury by inhibiting ROS/RNS production and modulating oxidative stress-induced signal transduction pathways [16].

Compound **7** is a bufadienolide, an important group of polyhydroxy C-24 steroids, characterized by the presence of a six-membered lactone (α-pyrone) ring located at position C-17β of the perhydrophenanthrene nucleus. Both plants and animals are promising source of bufadienolides. The plants belonging to the Crassulaceae,Hyacinthaceae, Iridaceae, Melianthaceae, Ranunculaceae and Santalaceae families are rich sources of bufadienolides, which show conservity in the lactone scaffold and diversity in the steroidal skeleton. The animal sources of bufadienolides include *Bufo* (toad), *Photinus* (fireflies) and *Rhabdophis* (snake) genera [18]. Bufadienolide-containing preparations from frog and toad skin have been (and still are) used for treatment of congestive heart failure (CHF) in the traditional medicine of the Far East [19]. From a pharmacological point of view, bufadienolides can act as endogenous steroidal hormone, displaying a large range of activities related to Na+/K+-ATPase enzyme blockage: antiangiogenic, anti-hypertensive, immunosupressor,

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antiendometriosis, positive inotropic action and a possible association with mood control and ethanol addiction [18]. Nowadays, bufadienolides including bufotalin, bufalin and resibufogenin have been found to exert potent cytotoxic and growth-inhibitory activity against various human cancer cells including lung cancer cells [20-21]. A lot of natural bufadienolides and their derivatives exhibited moderate to strong activity against human HL-6, SF-295, MDA-MB-435, and HCT-8 cancer cell strains without hemolysis.

Interestingly, both compounds **6** and **7** induced a down-regulation of GRP78 expression. It has been reported that GRP78 has important anti-apoptotic functions and that several cancer cells display higher levels of GRP78 as an adaptation to chronic stress in the tumor microenvironment [22-23] As a consequence, reducing GRP78 expression represents a new approach to support cancer treatment [24-25]. In this scenario, compounds **6** and **7** of *H. caucasicus*, could represent good candidates to cooperate with other proapoptotic agents, thanks to their ability to reduce GRP78 anti-apoptotic functions.

Conclusion

Our work is the first report on the ability of plant compounds belonging to ecdysteroid and bufadienolide classes to decrease GRP78 protein expression. This finding highlights a further mechanism to explain the proapoptotic activity of these compounds and open the way to further studies aimed to develop new anti-cancer and chemopreventive drug from plants.

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