7-Substituted Coumarins Inhibit Proliferation and Migration of Laryngeal Cancer Cells *In Vitro*

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Abstract. *Background: Coumarins are a large group of naturally-occurring compounds with a wide range of biological properties, including anticancer activity. 7-Substituted coumarins (umbelliferone, scoparone, and herniarin) were analyzed for their potential anticancer activity against laryngeal cancer cells (LCC). Materials and Methods: High-performance counter-current chromatography was applied for successful separation of umbelliferone from fruits of Heracleum leskowii. A two-phase solvent system composed of n-heptane-methanol-ethyl acetate-water (1:2:1:2, v/v/v) was successfully used. Cell proliferation was assessed after 48-72 h by means of MTT test, and tumor cell motility by a wound assay model. Measurement of cell death was estimated using enzyme-linked immunosorbent assay (ELISA), and cell-cycle analysis was performed by flow cytometry. Extracellular signal-regulated kinases-1/2 (ERK1/2) and AKT kinase activation status were analyzed by western blotting. Results: Umbelliferone, scoparone, and, to a lesser extent, herniarin reduced viability and migration of RK33 LCC in a dose-dependent manner. Scoparone and herniarin were found to induce apoptosis of LCC. None of the tested compounds influenced the ERK1/2 and AKT kinase activity, nor significantly affected cell-cycle progression in the LCC line studied. Conclusion: Our findings suggest the therapeutic potential of 7-substituted coumarins in the treatment of laryngeal cancer.*

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Coumarins are a large group of naturally-occurring compounds with a wide range of biological properties, including anti-HIV (1), anti-coagulant (2), anti-microbial (3), anti-oxidant, and anti-inflammatory (4), as well as anti-cancer activities (5-8). Results from *in vitro* studies indicated that 7-substituted coumarin derivatives displayed antiproliferative effects in human cancer cell lines *in vitro* (5, 6, 8), and inhibited growth of tumor xenografts (9).

It has been reported that 7-hydroxycoumarin (umbelliferone) is significantly more potent than coumarin (1,2-benzopyrone) for the treatment of some human carcinomas (6, 8). The molecular mechanism of anticancer action of umbelliferone has been linked to decreased expression of the BCL-2 and increase of BAX proteins (5), as well as decreased cyclin-D1 protein expression (6) in lung cancer cells. Umbelliferone was also found to inhibit tyrosine phosphorylation in epidermal growth factor (EGF)-stimulated lung cancer cells by reduction of the tyrosine kinase activity on the EGF receptor (10). The reduced expression of RAS and MYC, proteins associated with cell proliferation, after umbelliferone incubation of *ras*- or *myc*-transfected murine fibroblasts (11), suggests potential involvement of these proteins in the anti-cancer action of this compound. The anti-proliferative effects of the umbelliferone were studied at the level of the cell cycle in asynchronous cells of the NSCLC-N6 (non-small-cell bronchial carcinoma) line with flow cytometry. A blockade of cells in the G_1 phase was demonstrated by a dose-dependent increase in G_1 phase and decrease in S and G_2 and M phases after 72 h of culture in comparison to the control. The effect is due to cell in the G_1 phase arrest, similar to that produced by inducers of terminal differentiation (12). Umbelliferone treatment, administered orally, resulted in inhibition of tumor growth and increased survival time of sarcoma-bearing animals. Additionally, it seems that umbelliferone has immunomodulatory activity

contributing to its direct cytostatic effect (9). However, the exact molecular mechanism responsible for antiproliferative action of umbelliferone in cancer cells is still not fully-resolved. It was also reported that scoparone (6,7-dimethoxycoumarin) inhibits human bladder carcinoma (13), and human T-lymphoid leukemia cell proliferation (14). The cytotoxicity of herniarin, a methoxy analog of umbelliferone, was evaluated in GLC4, a human small cell lung carcinoma cell line and in COLO 32, a human colorectal cancer cell line using the microculture tetrazolium (MTT) assay. Herniarin (7-methoxycoumarin) exhibited very low cytotoxicity against both cell lines after continuous incubation (96 h), with half maximal inhibitory concentration (IC_{50})>200 μM (15).

Here, we investigated the effect of coumarin derivatives: umbelliferone, scoparone and herniarin on laryngeal cancer cells (LCCs).

Materials and Methods

A Spectrum High-performance Counter-current chromatograph apparatus delivered by Dynamic Extractions (Slough, UK) was employed in the present study. The instrument was equipped with two multilayer (PTFE) columns: analytical and semi-preparative (0.8 mm ID, 22 ml volume and 1.6 mm ID, 136 ml volume, respectively). The optimal speed of 1,600 rpm was used. The effluent was monitored at 320 nm by an ECOM Sapphire UV detector. An Alpha 10 pump (ECOM Prague, Czech Republic) was used for pumping both stationary and mobile phases.

An Agilent 1200 HPLC chromatograph coupled with 250 mm×4.6 mm stainless steel column packed with 5 μm C_{18} (Zorbax Eclipse XDB-C18; Agilent Technologies) with a DAD was used for identification of each HPCCC peak fraction.

Methanol used for HPLC was of chromatographic grade (J.T. Baker Inc., the Netherlands). Dichloromethane, *n*-heptane and ethyl acetate used for preparation of the extract from plant material and preparation of two-phase solvent systems were of analytical grade and were purchased from Polish Reagents (POCH, Gliwice, Poland). Water was purified using a Millipore laboratory ultra-pure water system (Simplicity™ system, Millipore, Molsheim, France).

Plant material. Fruits of *Heracleum leskowii* L. (Apiaceae) were collected in The Medicinal Plant Garden, Department of Pharmacognosy with Medicinal Plant Unit, Medical University, Lublin, Poland, in summer 2009. The plant material was identified by Mrs Krystyna Dąbrowska, Botanical Garden of Maria Curie-Skłodowska University in Lublin, a specialist in botany. Voucher specimen No 26/27-28 was deposited in the herbarium of The Department of Pharmacognosy, Medical University, Lublin.

Fruits were air-dried at room temperature, powdered and a batch (100 g) was extracted with 100 ml of methanol under reflux in 30 min. After filtration, the procedure was repeated twice. The filtrates were combined and concentrated with a rotary evaporator to remove the solvent. The dried crude extract (13 g) was stored in a refrigerator for subsequent HPCCC separation.

Selection and preparation of a two-phase solvent system. Mixtures of *n*-heptane, ethyl acetate, methanol and water (HEMWat) with different volume ratios were tested. In order to find a suitable solvent system, the partition co-efficient *K* was determined by HPLC analysis by comparing the peak area of umbelliferone both in upper and lower phases. The chosen system was prepared and the upper and lower phases were separated and degassed by sonication for 30 min before use.

HPCCC separation procedure. The analytical column (22 ml) was first entirely filled with the upper stationary phase. Then the apparatus was rotated at 1600 rpm and the lower mobile phase was pumped into the column at a flow rate of 1.0 ml/min. After hydrodynamic equilibrium was reached, 30 mg of extract dissolved in 1 ml of two phase solvent system was loaded onto the column through a 1 ml injection valve. When optimal conditions were determined, the method was transferred to a semipreparative column (137 ml volume). The mobile phase was pumped at a flow rate of 6.0 ml/min and 180 mg of extract was dissolved in 6 ml of the two-phase solvent system and was loaded onto the column through a 6 ml injection valve. The solid-phase retention was 70%. The effluent from the column was continuously monitored with a UV detector at 320 nm. Each one-minute fraction was manually collected into test tubes. At the end of the run, the fractions were evaporated under reduced pressure and redissolved in methanol for subsequent HPLC analysis.

Analysis and identification of HPCCC fractions. The crude methanol extract and each fraction obtained from the HPCCC separations were analyzed by HPLC as reported previously (16). The flow rate was 1 ml/min, the column temperature was 25˚C. A stepwise mobile phase gradient was prepared from methanol (A) and water (B). The gradient was: 0-5 min 50-60% A; 5-25 min 60-80% A; 25-30 min isocratic 80% A; 30-40 min 80-100% A.

The identification of isolated umbelliferone was carried out by comparison of retention time and UV-DAD spectra with those obtained by standards under the same conditions.

Cell lines and chemicals. RK33 laryngeal cancer cells (LCCs) were derived from a patient with diagnosed laryngeal squamous cell carcinoma. Cancer tissues were removed from the larynx after total laryngectomy and established as stable cell line, as previously described (17). The RK33 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Foetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml, Sigma-Aldrich, St. Louis, USA). Cultures were kept at 37˚C in a humidified atmosphere of 95% air and 5% CO_2.

Umbelliferone, scoparone and herniarin, as well as phorbol 12-myristate 13-acetate (PMA; purity≥99%), *cis*-Diammineplatinum(II) dichloride (cisplatin, CDDP; purity≥90%), nocodazole (purity ≥99%), dexamethasone (dexamethasone sodium phosphate; purity ≥97%) and insulin-like growth factor (IGF; purity≥97%) were purchased from Sigma Aldrich.

Cell viability assessment. RK33 LCCs were seeded on 96-well microplates (Nunc, Langenselbold, Germany) at a density of 1×10^4 (RK33). The following day, the culture medium was removed and cells were exposed to serial dilutions of herniarin, scoparone, or umbelliferone in fresh culture medium. Cell proliferation was assessed after 48-72 h by means of MTT method in which the yellow tetrazolium salt (MTT) is metabolized by viable cells to purple formazan crystals. RK33 LCCs were incubated for 3 h with MTT solution (5 mg/ml; Sigma). Formazan crystals were solubilized overnight in sodium dodecyl sulfate (SDS) buffer (10% SDS in 0.01 N HCl) and the product was quantified

spectrophotometrically by measuring the absorbance at 570 nm wavelength using an Infinite M200 Pro microplate reader (Tecan, Männedorf, Switzerland).

Cell migration assessment. Tumor cell motility was assessed by a wound assay model as previously described (18). The RK33 LCCs were plated at 1×10^6 cells on 4-cm culture dishes (Nunc). On the following day, the cell monolayer was scratched using a pipet tip (P300), the medium and dislodged cells were aspirated and the plates were rinsed twice with PBS. Fresh culture medium was then applied and the number of cells migrated into the wound area after 24 h was determined in control cultures, and cultures treated with increased concentration of hernairin, umbelliferone, or scoparone (100-500 μM). Plates were then stained using the May-Grünwald-Giemsa method and observations made under a Nikon Eclipse TS100 Microscope (Nikon Precision Inc, Tokyo, Japan). Cells migrated into the wound area were counted on micrographs and results are expressed as the mean cell number migrated in 40 selected fields taken from four micrographs.

Assessment of cell death. Measurement of cell death was performed using Cell Death Detection ELISA PLUS kit (Roche Diagnostics, Mannheim, Germany). The assay is based on quantitative sandwich enzyme immunoassay principle using mouse monoclonal antibodies directed against DNA and histones, respectively. This allows the specific determination of mono- and oligonucleosomes in the cytoplasmatic fraction of cell lysates. RK33 LCCs growing on 96-well microplates were subjected to either herniarin (500 μM), umbelliferone (500 μM), scoparone (250 μM), or cisplatin (1 μg/ml) for 24 h, whereupon supernatants were removed and cells lysed with 200 μl of lysis buffer for 30 min on ice. Subsequently, cell lysates were centrifuged at 200 ×*g* for 10 min, and 20 μl of the sample were carefully transferred into a streptavidin-coated 96-well microplate. The immunoreagent (80 μl) containing anti-histone-biotin and anti-DNA-peroxidase mouse monoclonal antibody was added and incubated under gentle shaking (300 rpm) for 2 h at 20˚C. The solution was removed by tapping, each plate well was rinsed three times with 250 μl of incubation buffer, and finally, 100 μl per well of substrate solution (2,2'-azino-bis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt; ATBS) were applied. Plates were then incubated at room temperature for 15 min on a plate shaker (250 rpm) until sufficient color development. Absorbance was measured at 405 nm wavelength using an Infinite M200 Pro microplate reader (Tecan, Männedorf, Switzerland).

Flow cytometric analysis. Experiments were performed using the FACSCalibur™ flow cytometer (BD Biosciences, San Diego, CA, USA), equipped with a 488 nm argon-ion laser. For cell-cycle analysis, cells were fixed in 70% ethanol at –20˚C. After fixation, the cells were stained with propidium iodide (PI) utilizing PI/RNase Staining Buffer (BD Biosciences), according to the manufacturer's instructions. The acquisition rate was at least 60 events per second in low acquisition mode and at least 10,000 events were measured. Cell-cycle analysis was performed by using a non-commercial flow cytometry analyzing software, Cylchred Version 1.0.2 for Windows (source: University of Wales, Cardiff, Wales, UK) and WinMDI 2.9 for Windows (source: facs.scripps.edu/software.html). The cells were acquired and gated by using dot plot FL-2 width (X) in comparison to FL-2 Area (Y)-gate to exclude aggregates and analyzed in histograms displaying FL-2-area (yellow-orange fluorescence: 585 nm).

Western blot analysis. Tumor cells were placed on 6-well plates (Nunc) at a density of 2×10^5 cells/ml. The following day the culture medium was removed and cells were exposed to serial dilutions of herniarin, scoparone, umbelliferone, or to 1 μM PMA, or 50 nM IGF in a fresh culture medium for 3 h. After treatment cells were harvested and lysed in RIPA buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 20 mM NaF, 0.5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor cocktail in PBS pH 7.4), then centrifuged at 3000 ×*g* for 10 min at 4˚C. For Western blot analysis supernatants of RIPA cell lysates were solubilized in 4× Laemmli sample buffer (40% glycerol, 8% SDS, 0.240 M Tris-HCl, pH 6.8, 0.04% bromophenol blue, 5% β-mercaptoethanol), then boiled for 5 min at 100˚C. Cellular protein extracts were run in 10% SDS-PAGE under reducing conditions and transferred by electroblotting onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blocking for 1 h at RT with 5% non-fat dry milk/TBS/0.1% Tween, membranes were probed overnight at 4˚C with primary antibody as follows: anti-phospho-ERK1/2 (Thr202/Tyr204, Cell Signaling, Inc., Danvers, MA, USA), anti-phospho-AKT (Ser 473; Cell Signalling) 1:1000 in 5% BSA/TBS/0,1% Tween, anti-ERK2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-AKT (Santa Cruz Biotechnology) in 1:500 in 5% non-fat dry milk/TBS/0,05% Tween, then incubated with the secondary antibody coupled to horseradish peroxidase (1:1000 in 5% non fat dry milk/TBS/0.1% Tween, Cell Signaling). Detection was performed using enhanced chemiluminescence (ECL; Amersham Biosciences) and serial exposures made on autoradiographic film (Hyperfilm ECL; Amersham Biosciences). For stripping, membranes were incubated with stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) at 50˚C for 20 min, then washed, blocked, and reprobed overnight at 4˚C with relevant antibody, as described above (subsection 5.11.).

Statistical analysis. ANOVA test for multiple comparisons and Student's *t*-test were used. Data are expressed as the mean±SEM.

Results

HPCCC was chosen as a powerful technique for purification of umbelliferone. It is known that selection of a proper two-phase solvent system is a critical part of counter-current analysis. In this study, different solvent systems were tested, among which two were promising. Partition coefficients *K*, expressed as the concentration of umbelliferone in the stationary phase divided by its concentration in the mobile phase, were calculated and are presented in Table I. A mixture of *n*-heptane, ethyl acetate, methanol and water at a ratio of 1:2:1:2 was chosen for further experiments. One-minute fractions were collected and analyzed with an optimal HPLC method. Umbelliferone was detected in fractions 18-20. After injection of 180 mg of crude extract, 1.8 mg of umbelliferone of 99% purity (according to HPLC analysis) was purified. A HPLC chromatogram of the crude methanolic extract of *Heracleum leskowii* is presented in Figure 1 and of isolated umbelliferone together with UV-DAD spectra in Figure 2.

Table I. *The K value of the umbelliferone in the two most promising solvent systems.*

n-heptane-ethyl acetate-methanol-water solvent system	*K* value
1:2:1:2	1.91
2:3:2:3	0.98

Table II. *Effects of different coumarins on migration of laryngeal cancer cells.*

Concentration (μM)	Herniarin (Mean±SEM)	Scoparone (Mean±SEM)	Umbelliferone (Mean±SEM)
0	102.7±1.16	100±1.15	100±1.45
100	103±2.61	93±1.10*	92.88±1.29***
250	101±1.15	73.87±1.42***	89.9±1.70***
500	60.5±1.15***	68±1.39***	74±0.74***

Significant differences in comparison to control (*p<0.05, **p<0.01, ***p<0.001).

Table III. *Cell-cycle analysis in laryngeal cancer cells.*

	G_1 phase [%] (Mean±SEM)	S phase [%] (Mean±SEM)	G_2 phase [%] (Mean±SEM)
Control	55.8±1.46	19.68±1.04	24.48±1.18
Umbelliferone (500 μM)	60.88±2.51	18.8±1.61	20±2.15
Herniarin (500 μM)	60±1.87	18.8±1.60	20.7±2.30
Scoparone (250 μM)	61±1.17*	18±0.93	20.29±2.94
Dexamethasone (0.5 μM)	74.5±0.92***	12.47±0.92**	14±1.24**
Nocodazole (0.5 μg/ml)	7±0.69***	14±0.47**	78±0.51***

Significant differences in comparison to control (*p<0.05, **p<0.01, ***p<0.001).

Isolated umbelliferone together with other 7-substituted coumarins: herniarin and scoparone were subjected to further experiments. In order to determine whether RK33 LCCs responded to 7-substituted coumarins by growth inhibition, they were treated with increasing concentrations of herniarin, umbelliferone, or scoparone over a period of 48-72 h. Incubation with all coumarin derivatives reduced cell viability compared with non-exposed cells in a concentration- and time-dependent manner (Figure 3). Threshold concentrations of coumarin derivatives required to elicit anti-proliferative effect in RK33 tumor cell line were as low as 2.5 μM for umbelliferone (p<0.01), and 5 μM for scoparone (p<0.01) and herniarin (p<0.05) at the 72 h time point (Figure 3B). When cancer cells were treated for 48 h, the coumarin derivatives revealed a less pronounced effect (Figure 3A). The most potent inhibitory properties were observed for scoparone, followed by umbelliferone, and herniarin. Additionally, control cells were incubated with 0.1% (v/v) DMSO to estimate the potential inhibition of cell growth by this solvent. Of note is that this concentration of DMSO did not influence tumor cell proliferation.

Exposure to coumarin derivatives also triggered apoptotic cell death in RK33 cells as indicated by a dose-dependent increase of immunoreactive cytosolic oligonucleosomal fragments. The onset and degree of apoptosis was compound-dependent. Scoparone caused a 6-fold increase of the enrichment factor of nucleosomes in the cytoplasm at 250 μM in comparison to the vehicle-treated control cells. Herniarin was a less potent apoptosis inducer (3-fold increase of apoptosis at 500 μM), whereas umbelliferone did not induce apoptosis as measured by the cytoplasmic histone-associated DNA fragments enrichment assay. A well-known inducer of apoptosis, cisplatin (1 μM) was used to show the specificity of the assay (Figure 4).

The effect of the coumarin derivatives on cell migration was also analyzed. Cancer cell migration is an important marker of tumor metastatic potential. Exposure to scoparone or umbelliferone for 24 h resulted in diminished migration of larynx cancer cells to the wound area in a dose-dependent manner (Figure 5). In RK33 cultures exposed to umbelliferone, at concentration of 100 μM significantly fewer cells migrated to the wound area (p<0.05), whereas scoparone treatment inhibited LCC migration at higher doses (≥250 μM, p<0.01). Herniarin caused inhibition of RK33 tumor cell migration at a final concentration of 500 μM (p<0.001) (Figure 5, Table II).

FACS analysis (Figure 6) showed almost no changes in the cell-cycle progression. Incubation of the RK33 cells with a high concentration of scoparone slightly increased the resting cell population in G1 phase of the cell cycle (p<0.05), whereas umbelliferone (500 μM) reduced the population of RK33 cells present in the S phase (p<0.05) (Table III). The results demonstrate that the analyzed 7-substituted coumarins exert their anticancer mode of action in a mechanism which is probably not related to slowing down cell cycle progression in RK33 LCCs.

As demonstrated, treatment with 7-substituted coumarins resulted in suppression of cancer cell proliferation and migration (Figures 3 and 5). We therefore investigated pathways involved in cancer cell proliferation which are frequently mutated and hyperactivated in cancer. Blockade of these pathways, including ERK1/2 and AKT kinase cascades, leads to apoptosis and perturbations in the cell cycle progression (19, 20). However, in our experiments we were not able to detect significant changes in ERK1/2 and AKT kinase activity upon treatment with 7-substituted coumarins (Figure 7) as measured by western blotting analysis.

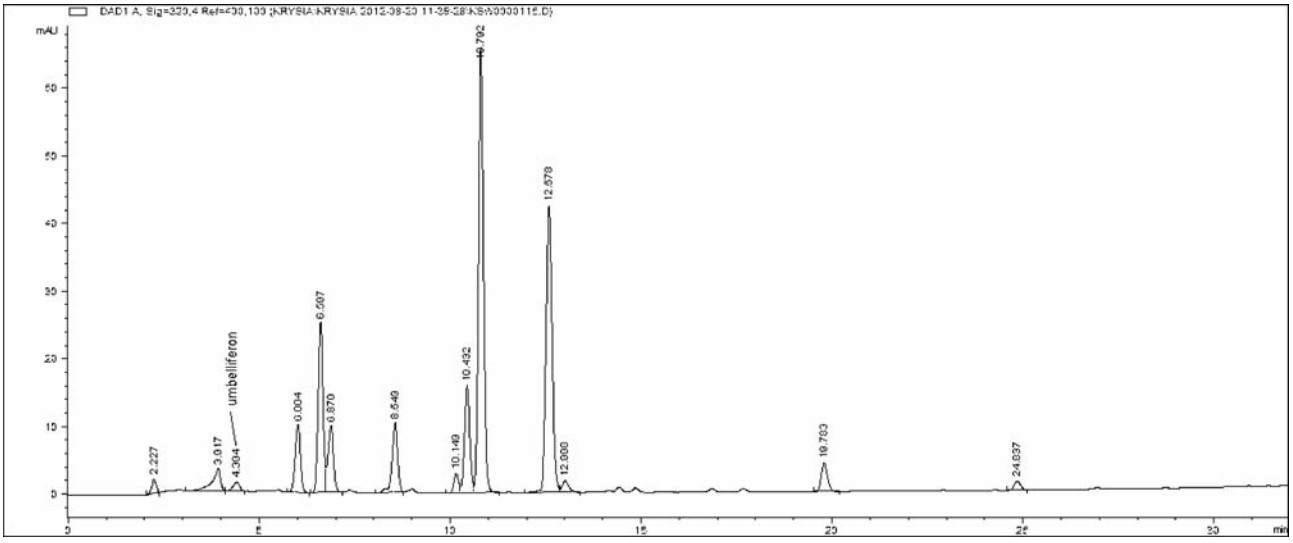

Figure 1. *High-performance liquid chromatogram of crude methanol extract from fruits of Heracleum leskovii.*

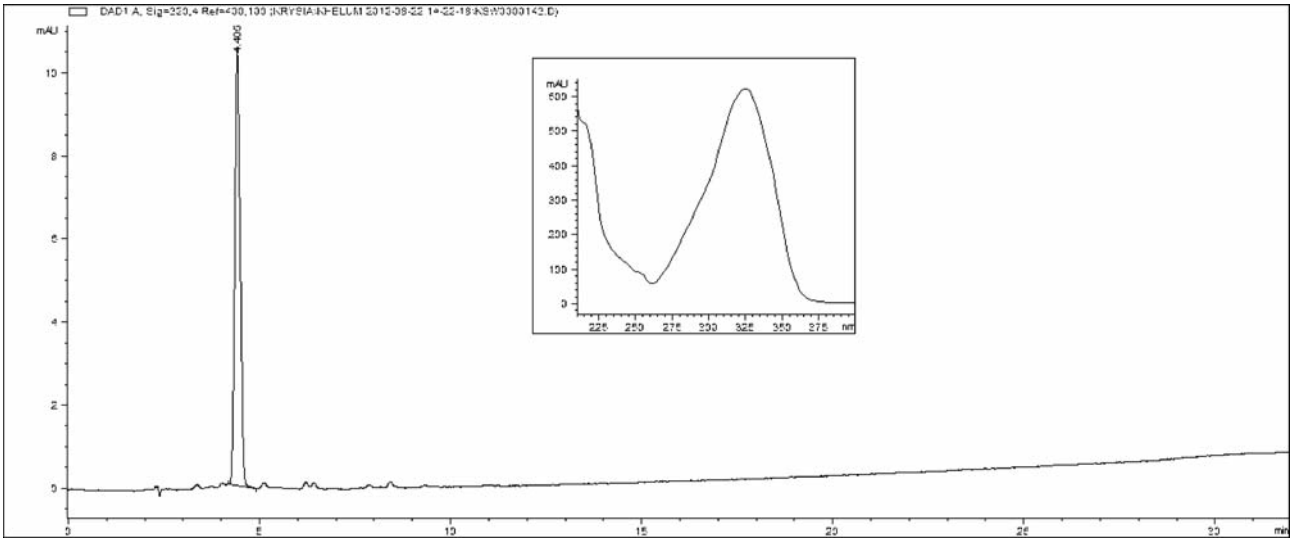

Figure 2. *High-performance liquid chromatogram of umbelliferone together with UV-DAD spectra.*

Discussion

The naturally-occurring coumarins, and their chemically-synthesized derivatives have attracted great attention due to their wide range of biological properties. However, their anticancer properties have only recently been studied in detail (21). The biological activity of coumarin and more complex related derivatives appears to be based on the coumarin nucleus (15). Several 7-substituted coumarins have been reported to have cytostatic and cytotoxic (5, 6, 8) properties against a variety of cancer cells, but not against normal peripheral blood mononuclear cells (PBMC) (22). Here, we analyzed the 7-substituted coumarins for their ability to reduce viability and migration of RK33 LCCs.

Laryngeal cancer is the eleventh most common cancer in men worldwide and still a therapeutic problem (23). Despite recent improvements in treatment strategies, survival rates of patients with local and metastatic laryngeal cancer demonstrated a significant decline in the past three decades (24). Thus, discovery or development of

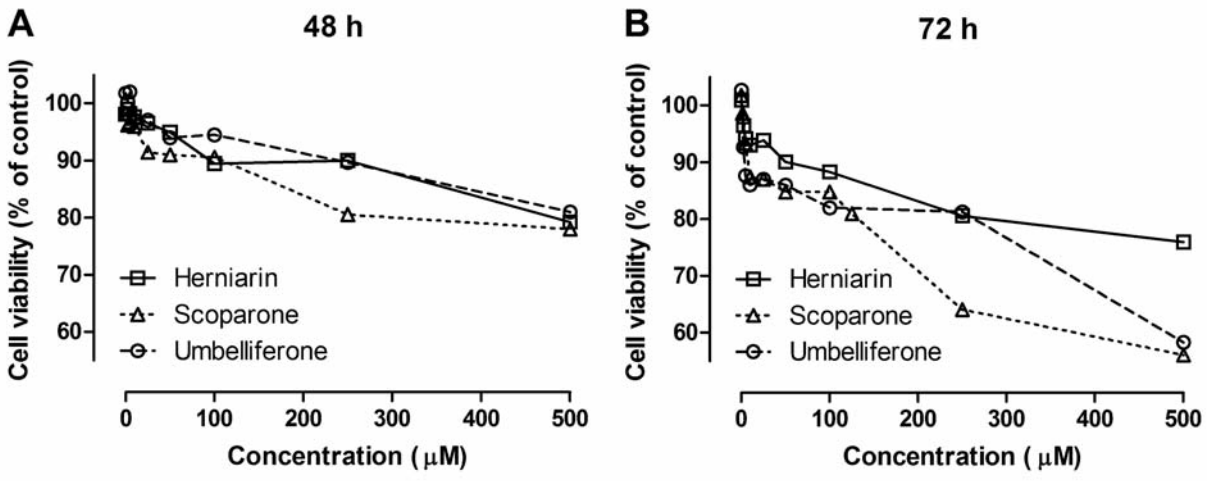

Figure 3. *The coumarin derivatives suppress growth of laryngeal cancer cells. RK33 cells were treated with 2,5–500 μM umbelliferone, scoparone or herniarin. Normalized cell viability, measured by the MTT assay at 48 h (A), and 72 h (B) is presented as mean±SEM at each concentration. Analysis of variance (ANOVA) and Student's t-test revealed a significant effect of treatment with coumarin derivatives on cell viability, compared to vehicle-treated cultures. There is clear concentration dependency of the anti-proliferative action of umbelliferone, scoparone and herniarin (*p<0.05, **p<0.01, ***p<0.001) compared to the control, n=24 per concentration from three independent experiments.*

new alternative therapeutics is needed to improve current treatment modalities. Plants have been used since ancient times in the treatment of various types of human disease. They are unlimited sources of biologically-active compounds, thus finding proper purification methods is a main task for many scientists. HPCCC is an excellent tool since it has many advantages over traditional chromatography. As there is no solid support, even minor compounds present in a complicated mixture of natural substances can be separated. The critical point is finding the correct two-phase solvent systems. A solvent system in which the K value of a particular compound is close to 1 is considered to be the ideal system for separating the compound. Small *K* values result in a loss of peak resolution, while large *K* values tend to produce excessive sample band broadening and long run times (25). Our preliminary experiments were carried out with the two phase solvent system composed of the mixture of heptanes-ethyl acetate-methanol-water at a volume ratio 2:3:2:3. This mixture was recommended in the literature as being a perfectly suited system for separation of umbelliferone (26). Even though the *K* value was very close to 1 and separation of target compound from G.U.E.S.S. mixture (Generally Useful Estimate of Solvent Systems) was excellent, unfortunately it was difficult to separate umbelliferone from other compounds present in the crude methanol extract of *Heracleum leskowii*. In the subsequent studies, another volume ratio was tested (1:2:1:2) for purification of umbelliferone from associated compounds.

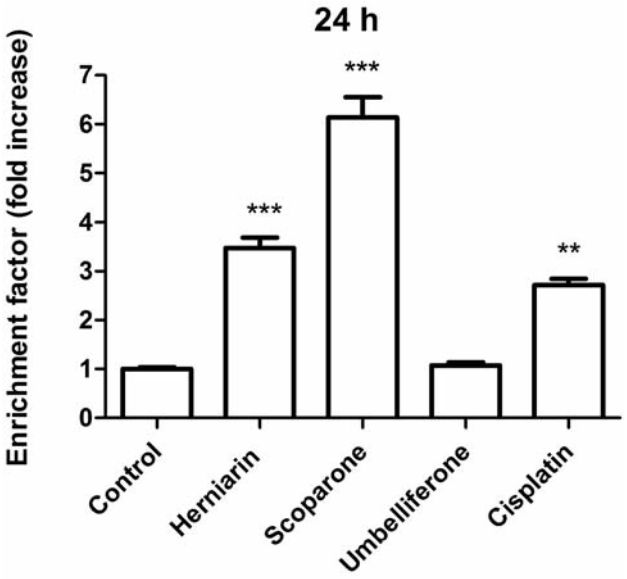

Figure 4. *Scoparone and herniarin induce apoptotic cell death of RK33 cells. Enrichment of oligonucleosome fragments (fold increase) in RK33 cells in the absence (Control) and following 24 h of umbelliferone (500 μM), scoparone (250 μM), herniarin (500 μM), or cisplatin (1 μg/mL) exposure (n=5 per concentration; **p<0.01, ***p<0.001 versus control, Student's t-test).*

Selected coumarin derivatives were tested in order to assess their potential anticancer properties. Our results indicate that umbelliferone, scoparone, and to a lesser extent herniarin, exhibit anticancer activity against LCCs. There are

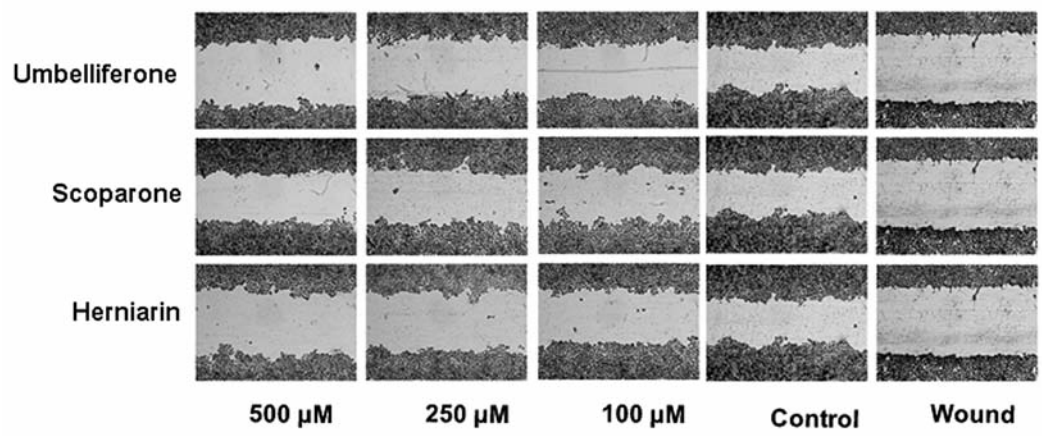

Figure 5. *Effect of coumarin derivatives on migration of tumor cells in wound assay. Scratched monolayers of RK33 cells were incubated for 24 h alone or in the presence of umbelliferone, scoparone or herniarin (100-500 µM). Magnification 40×. Results are expressed as the mean number of cells migrated per field of the wound area±SEM of 50 measurements, as shown in Table I (*p<0.05, ***p<0.001 versus control, Student's t-test).*

no previous reports dealing with the cytostatic or apoptotic actions of coumarin derivatives in LCCs as far as we are aware of. Yet, it has been demonstrated that 7-hydroxycoumarin induced dose-dependent growth inhibition in lung carcinoma cell lines (5, 6) at doses similar to, or higher than those used in our study. However, resistance of epidermoid lung carcinoma (Calu-1) cells to umbelliferone has also been reported (5).

The mechanism responsible for the anticancer activity of umbelliferone has not been fully resolved. It has been demonstrated that exposure to 7-hydroxycoumarin produced cell-cycle arrest in lung tumour cell lines in G_1 phase (6, 8), and S phase (6). We observed only a slight decrease of cell numbers in the S phase after incubation of RK33 cells with umbelliferone. However, the doses used in our experiments were four-times lower than those tested by Lopez-Gonzalez *et al*. in lung cancer cell lines (8), which could explain in part the observed differences. We did not detect apoptosis of RK33 LCCs treated with umbelliferone. Interestingly, in contrast to other types of lung cancer cells, apoptosis was also not observed in squamous lung carcinoma cell lines incubated with umbelliferone (8). Additional studies on squamous cell carcinomas of different origin are required to determine, if this type of histological differentiation (squamous cell carcinoma) is related to a lack of apoptosis after umbelliferone treatment.

We detected a significant induction of apoptosis when RK33 cells were incubated either with scoparone or herniarin. To our knowledge, there are no reports showing an antitumor activity of herniarin. Although the anticancer properties of herniarin were the least effective of the coumarin derivatives tested in our study, it seems that the 7-methoxycoumarin nucleus has promising structural features to produce efficient anti-tumor compounds (21).

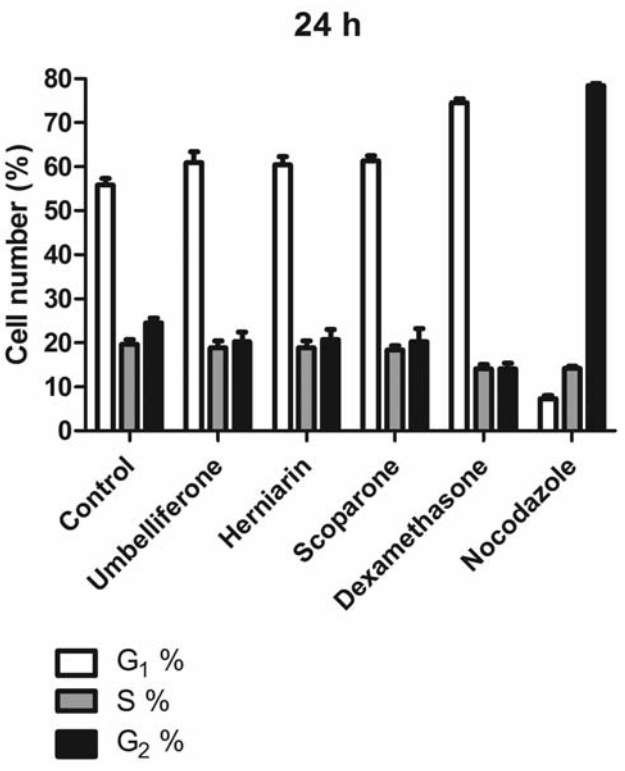

Figure 6. *Flow cytometric analysis of propidium iodide-stained nuclei revealed higher percentages of cells in G_1 and lower percentages of cells in S and G_2 phases of the cell cycle following exposure to umbelliferone (250 µM), scoparone (500 µM), or herniarin (500 µM) compared to controls. To verify functionality of the assay, nocodazole was used to show enrichment of cells in G_2 phase, and dexamethasone to show increase of cell number in G_1 phase of the cell cycle. Table II shows quantitative results of the cell-cycle analysis (% of total±SEM, n=12 measurements from three independent experiments). *p<0.05 versus control, Student's t-test.*

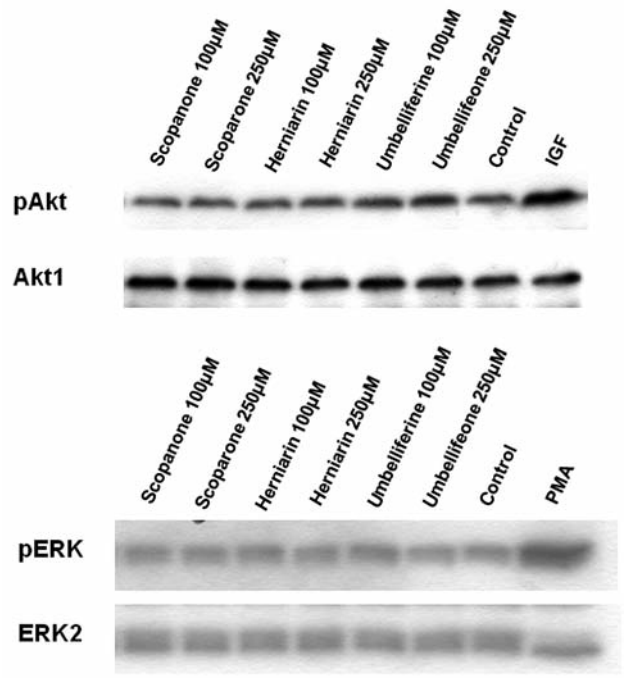

Figure 7. *Coumarin derivatives do not affect AKT or Extracellular signal-regulated kinases1/2 (ERK1/2) activation. RK33 cells were treated with umbelliferone (100, 250 μM), scoparone (100, 250 μM), or herniarin (100, 250 μM) for 3 hours. Control cells were treated with the specific ERK1/2 activator phorbol 12-myristate 13-acetate (PMA), and incubated with 50 nM of insulin-like growth factor (IGF) for three hours as positive controls. The phosphorylation status of AKT, and ERK1/2 are shown. Equal loading was confirmed by immunodetection of total AKT, and ERK2 (lower panels). One representative experiment (out of four) is shown.*

Scoparone was the most effective of the coumarin derivatives displaying anticancer activity in our study. The molecular mechanisms of the anti-tumor action of scoparone have also not been elucidated. Recent studies suggest that scoparone was able to inhibit the NF-κB pathway in PMA-stimulated leukemia cells (27). Since scoparone, umbelliferone and herniarin inhibit proliferation of cancer cells, we tested these compounds regarding their effects on ERK1/2 and AKT kinase, frequently hyperactivated in many types of cancers (20), but we did not detect significant changes for either compound after treatment with coumarin derivatives, which is consistent with other studies showing that umbelliferone did not have a concentration-dependent effect on ERK1/ERK2 phosphorylation in a melanoma cell line up to 48 h of treatment (28). Interestingly, nitro derivatives of umbelliferone displayed inhibition of ERK1/2 kinase activity in melanoma (28), and renal (29) cancer cells. The underlying mechanism changes in the inhibition of intracellular signalling cascades mediated by is currently unknown (30). It has been suggested that coumarins could bind non-specifically to cell surface structures such as receptors, thereby interfering with signal transduction pathways, including GTP-binding proteins (5).

Of great importance in cancer treatment is the prevention of developing local and distant metastases. The metastatic potential of laryngeal cancer is a significant problem to clinicians during treatment and the main reason for unfavorable outcome of this neoplastic disease (23, 24). Thus, there is an urgent need for drugs not only inhibiting tumor proliferation but also preventing formation of metastases.

In our study, to our knowledge, for the first time we show that coumarin derivatives inhibit migration of cancer cells. The most effective was scoparone, followed by umbelliferone and herniarin, as demonstrated by the wound assay. Elinos-Baez *et al*. suggested that coumarin derivatives might be effective in preventing or delaying the formation of metastases based on changed cell morphology after incubation of A427 and Calu-1 lung carcinoma cells with umbelliferone (5). This effect may be related to the modulation of extracellular proteins expression since 4-methylumbelliferone inhibits hyaluronan (HA) synthesis and extracellular HA matrix formation in melanoma and pancreatic cancer cells (31). Increased levels of HA are often associated with the metastatic potential of epithelial cancer (32-34). On the other hand, scoparone, isolated from the extract of the chestnut (*Castanea crenata*, Fagaceae), can prevent cell detachment of skin fibroblasts from culture plates by enhancing the expression of the cell-associated fibronectin and vitronectin (35). Thereby, cell motility could be a potent target for therapeutic intervention with coumarin derivatives. In our experiments, we found that scoparone, umbelliferone and herniarin significantly inhibited migration of RK33 LCCs. Given that a non-toxic concentration of umbelliferone (22) can significantly inhibit migration and proliferation, umbelliferone, as well as scoparone, may be used as a chemopreventive agent. Taken together our findings outline the therapeutic potential of coumarin derivatives for the future treatment of laryngeal cancer.

Conclusion

Umbelliferone, scoparone, and, to a lesser extent, herniarin reduced viability and migration of RK33 LCCs in a dose-dependent manner. Scoparone and herniarin were also found to induce apoptosis of LCCs.

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