

# Effects of *Scrophularia* extracts on tumor cell proliferation, death and intravasation through lymphoendothelial cell barriers

BENEDIKT GIESSRIGL<sup>1,2\*</sup>, GÖKHAN YAZICI<sup>3\*</sup>, MATHIAS TEICHMANN<sup>1</sup>, SABINE KOPF<sup>1</sup>,  
SARA GHASSEMI<sup>4</sup>, ATANAS G. ATANASOV<sup>5</sup>, VERENA M. DIRSCH<sup>5</sup>, MICHAEL GRUSCH<sup>4</sup>,  
WALTER JÄGER<sup>2</sup>, ALI ÖZMEN<sup>3</sup> and GEORG KRUPITZA<sup>1</sup>

<sup>1</sup>Institute of Clinical Pathology, Medical University of Vienna, Waehringer Guertel 18-20; <sup>2</sup>Department for Clinical Pharmacy and Diagnostics, Faculty of Life Sciences, University of Vienna, Althanstrasse 14, A-1090 Vienna, Austria;

<sup>3</sup>Institute of Biology, Fen-Edebiyat Fakültesi, Adnan Menderes Üniversitesi, Aydın, Turkey;

<sup>4</sup>Institute of Cancer Research, Department of Medicine I, Medical University of Vienna, Borschkegasse 8a;

<sup>5</sup>Department of Pharmacognosy, University of Vienna, Althanstrasse 14, A-1090 Vienna, Austria

Received November 15, 2011; Accepted January 23, 2012

DOI: 10.3892/ijo.2012.1388

**Abstract.** Different studies describe the anti-inflammatory effects of *Scrophularia* species, a medicinal plant widely used in folk medicine since ancient times. As knowledge regarding the anti-neoplastic properties of this species is rather limited, we investigated the influence of methanol extracts of different *Scrophularia* species on cell proliferation, cell death, and tumour cell intravasation through the lymph endothelial barrier. HL-60 leukaemia cells were treated with methanol extracts of different *Scrophularia* species leading to strong growth inhibition and high cell death rates. The expression of cell cycle regulators, oncogenes and cell death inducers was determined by Western blot analysis. Furthermore the effect of *S. lucida* was studied in an NF- $\kappa$ B reporter assay, and in a novel assay measuring 'circular chemo-repellent-induced defects' (CCID) in lymph endothelial monolayers that were induced by MCF-7 breast cancer spheroids. Methanol extracts of *Scrophularia* species exhibited strong anti-proliferative properties. *S. floribunda* extract inhibited G2/M- and later on S-phase and *S. lucida* inhibited S-phase and in both cases this was associated with the down-regulation of c-Myc expression. Extracts of *S. floribunda* and *S. lucida* led to necrosis and apoptosis, respectively. Furthermore, *S. lucida*, but not *S. floribunda*, effectively attenuated tumour cell intravasation

through lymph endothelial cell monolayers, which correlated with the inhibition of NF- $\kappa$ B. *S. lucida* exhibited promising anti-neoplastic effects and this was most likely due to the down-regulation of various cell cycle regulators, proto-oncogenes and NF- $\kappa$ B and the activation of caspase-3.

## Introduction

While only ~1% of the estimated 300,000 different species of higher plants have a history in food use, up to 10-15% have extensive documentation for application in traditional medicine (1). Natural products have played a significant role in human healthcare for thousands of years, especially in the treatment of infectious diseases (2). Even today, more than 60% of all drugs used are either natural products or directly derived thereof and are used to treat even diseases such as cancer. Among these are very important agents like vinblastine, vincristine, the camptothecin derivatives, topotecan and irinotecan, etoposide, derived from epipodophyllotoxin, and paclitaxel (3-5).

Ethno-medicine does not only explore written sources i.e., traditional Chinese medicine or Ayurveda, but in particular also gives strong attention to the many kinds of folk medicine that was practiced in all parts of the world for centuries.

Very rich plant diversity is found in Turkey, because the Taurus peninsula has seas on three sides and various climatic zones and topographies. The flora of Turkey is rich in medicinal and aromatic plants that have been used to treat different diseases in Turkish and antique folk medicine (6,7). Since ancient times, different *Scrophularia* species have been used as remedies for some medical treatments, including scabies, eczema, psoriasis, inflammatory diseases and tumours (8). There are more than 220 genera of the *Scrophulariaceae* family in which the genus *Scrophularia* is known for the rich presence of sugar esters and iridoid glycosides (9,10), and a few publications describe the anti-inflammatory properties of different *Scrophularia* species (11,12). Oleanonic and ursolonic acids extracted from the root of *S. ningpoensis* Hemsl were found to be cytotoxic against a series of human cancer cell lines (MCF7, K562 and A549) (10).

**Correspondence to:** Dr Georg Krupitza, Institute of Clinical Pathology, Medical University of Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria

E-mail: georg.krupitza@meduniwien.ac.at

Dr Ali Özmen, Biyoloji Bölümü, Fen-Edebiyat Fakültesi, Adnan Menderes Üniversitesi, 09010-Aydın, Turkey

E-mail: aozmen@adu.edu.tr

\*Contributed equally

**Key words:** *Scrophularia lucida*, cell-proliferation, cell-death, oncogenes, intravasation

We have investigated the anti-proliferative and pro-apoptotic potency of the methanol extracts of five different *Scrophularia* species (two endemic to Turkey: *S. libanotica* and *S. pinardii*) and elucidated the corresponding pathways of those two species that showed the strongest anti-neoplastic effect. Furthermore, we discovered a property in *S. lucida*, which correlates with the inhibition of lymph node metastasis of breast cancer cells.

## Materials and methods

**Plant material.** *Scrophularia floribunda*, *S. lucida*, *S. peregrina*, *S. pinardii* and *S. libanotica* subsp. *libanotica* var. *mesogitana* were collected in the south-west of Turkey at a height around 250 m in Aydin and Marmaris, respectively. Flowering times of these plants were identified from books for specifying the collection time. Plants were recognized in the field survey by various plant parts (flower, leaf, stamen, colouring of petals, etc.) and by comparing with previously prepared herbarium samples. Taxonomic determinations were made by Dr Özkan Eren using the serial 'Flora of Turkey and the East Aegean Islands' (Davis, 1965-1988). Voucher specimens (voucher numbers: *S. floribunda* AYDN 432; *S. lucida* AYDN 433), in duplicates were deposited in the herbarium of the Department of Biology, Adnan Menderes University.

**Sample preparation.** Plants were freeze-dried, subsequently milled and extracted with methanol at the ratio of 1:10. Extraction was carried out on a shaker at room temperature overnight. After filtration, methanol was evaporated with a rotary evaporator and extract weight was determined (Table I). For the experiments, the extracts were dissolved in ethanol. For the proliferation- and apoptosis assays the following concentrations as calculated for dried plant material were used: 500 µg/ml, 1, 4 and 10 mg/ml. To exclude an effect of ethanol on cell proliferation and apoptosis, controls were treated with same concentrations of ethanol as used for sample treatment (in general 0.2% EtOH) (13,14).

**Detannification.** For removal of tannins 5 g of the total methanol extract of *S. floribunda* and *S. lucida*, respectively, were dissolved in 60 ml of a methanol/water mixture (10:1). After triple solvent extraction with 60 ml petroleum ether for withdrawal of chlorophyll, waxes and fats, the methanol fraction was diluted with 60 ml of water and subsequently this aqueous solution was extracted three times with 120 ml chloroform. To gain the detannified extract, the collected chloroform fraction was washed three times with 360 ml sodium chloride solution (1%) and after drying with sodium sulphate, the chloroform was evaporated with a rotary evaporator. *S. floribunda* and *S. lucida* total methanol extract yielded 0.24 and 0.11 g per g, respectively.

**Cell culture.** HL-60 promyelocytic leukaemia cells were purchased from ATCC. Cells were grown in RPMI-1640 medium supplemented with 10% heat inactivated foetal calf serum (FCS), 1% L-glutamine and 1% penicillin/streptomycin. Human MCF-7 breast cancer cells were cultivated in MEM medium supplemented with 10% FCS, 1% penicillin/streptomycin, 1% NEAA. Telomerase immortalized human lymph endothelial cells (LECs) were grown in EGM2 MV (Clonetics CC-4147, Allendale, NJ, USA). For CCID formation assays, LECs were stained with cytotracker green. HEK293-NFκB-Luc cells

were cultivated in high glucose DMEM containing phenol red, supplemented with 10% FCS, 1% penicillin/streptomycin and 1% L-glutamine. GFP transfection of HEK293-NFκB-Luc cells using Lipofectamine 2000 was carried out in medium without penicillin/streptomycin.

All cells were grown at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. If not mentioned otherwise, all media and supplements were obtained from Invitrogen Life Technologies (Karlsruhe, Germany).

**3-D co-cultivation of MCF-7 cancer cells with LECs.** MCF-7 mock cells were transferred to 30 ml MEM medium containing 6 ml of a 1.6% methylcellulose solution (0.3% final concentration; cat. no. M-512, 4000 centipoises; Sigma, Munich, Germany). Cell suspension (150 µl) was transferred to each well of a 96-well plate (Greiner Bio-one, Cellstar 650185, Kremsmünster, Austria) to allow spheroid formation within the following two days. Then, MCF-7 spheroids were washed in PBS and transferred to cytotracker-stained LEC monolayers that were seeded into 24-well plates (Costar 3524, Sigma) in 2 ml EGM2 MV medium (15,16).

**Circular chemo-repellent induced defect (CCID) assay.** MCF-7 cell spheroids (3,000 cells/spheroid) were transferred to the 24-well plate containing LEC monolayers. After four hours of incubating the MCF-7 spheroids-LEC monolayer co-cultures, the CCID sizes in the LEC monolayer underneath the MCF-7 spheroids were photographed using an Axiovert (Zeiss, Jena, Germany) fluorescence microscope to visualise cytotracker (green)-stained LECs underneath the spheroids (17). Gap areas were calculated with the Axiovision Re. 4.5 software (Carl Zeiss). MCF-7 spheroids were treated with solvent (ethanol) as negative control. The gap sizes of at least 12 spheroids per experiment were measured.

**Reagents and antibodies.** Hoechst 33258 and propidium iodide were purchased from Sigma. Amersham ECL-plus Western Blotting Detection System was from GE Healthcare (Buckinghamshire, UK).

**Antibodies:** Mouse monoclonal (ascites fluid) anti-acetylated tubulin clone 6-11B1 cat no. AT6793 and mouse monoclonal (ascites fluid) anti-β-actin clone AC-15 cat no. A5441 were from Sigma. Anti α-tubulin (TU-02) cat no. sc-8035, PARP-1 (F-2) cat no. sc-8007, anti cyclin D1 (M-20) cat no. sc-718, p21 (C-19) cat no. sc-397, cdc25a (F-6) cat no. sc-7389, cdc25b (C-20) cat no. sc-326, cdc25c (C-20) cat no. sc-327, c-jun (C-20) cat no. sc-1694 and jun-B (210) cat no. sc-73 were from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA, USA) phospho-p44/42 MAPK (Erk1/2) (Thr<sup>202</sup>/Tyr<sup>204</sup>) (E10) cat no. 9106, p44/42 MAPK (Erk1/2) (137F5) cat no. 4695, phospho-p38 MAPK (Thr<sup>180</sup>/Tyr<sup>182</sup>) (12F8) cat no. 4631, p38 MAPK cat no. 9212, cleaved caspase-3 (Asp<sup>175</sup>) cat no. 9661, phospho-Wee1 (Ser<sup>642</sup>) (D47G5) cat no. 4910, Wee1 cat no. 4936, phospho-chk2 (Thr<sup>68</sup>) cat no. 2661, chk2 cat no. 2662, myosin light chain 1 cat no. 3672 and phospho-myosin light chain 2 (Ser<sup>19</sup>) cat no. 3671 were purchased from Cell Signalling (Danvers, MA, USA). Anti-c-myc antibody Ab-2 (9E10.3) was from Neomarkers (Fremont, CA, USA) and rabbit polyclonal phospho detect anti-H2AX (pSer<sup>139</sup>) cat no. dr-1017 from Calbiochem (Merck, Darmstadt, Germany). Anti-mouse and anti-rabbit IgG were from Dako (Glostrup, Denmark).

Table I. Extract yields after sample preparation.

Species	Wet weight (g)	Dry weight (g)	Extract weight (g)
<i>S. floribunda</i>	349	87	17
<i>S. lucida</i>	295	87	14
<i>S. peregrine</i>	243	55	11.6
<i>S. pinardii</i>	383	88	17
<i>S. libanotica</i>	280	88	12.5

**Proliferation inhibition analysis.** HL-60 cells were seeded in T-25 Nunc tissue culture flasks at a concentration of  $1 \times 10^5$ /ml and incubated with increasing concentrations of plant extracts (corresponding to 500  $\mu$ g/ml, 1, 4 and 10 mg/ml of the dried plant). Cell counts and  $IC_{50}$  values were determined at 24, 48 and 72 h using a Casy TTC cell counter (Roche, Basel, Switzerland), respectively.

The percent of cell divisions compared to the untreated control were calculated as follows:  $[(C_{72h + drug} - C_{24h + drug}) / (C_{72h - drug} - C_{24h - drug})] \times 100 = \% \text{ cell division}$ , where  $C_{72h + drug}$  is the cell number after 72 h of extract treatment,  $C_{24h + drug}$  is the cell number after 24 h of extract treatment,  $C_{72h - drug}$  and  $C_{24h - drug}$  are the cell numbers after 72 and 24 h without extract treatment (18,19).

**Cell death analysis.** The Hoechst propidium iodide double staining was performed according to the method described by Grusch *et al* (20,21). HL-60 cells ( $1 \times 10^5$ ) were seeded in T-25 Nunc tissue culture flasks and exposed to 20  $\mu$ g/ml detannified extract (corresponding to 0.42 mg/ml of dried *S. floribunda* and 1.10 mg/ml of dried *S. lucida*) for 24 and 48 h. Hoechst 33258 and propidium iodide (Sigma) were added directly to the cells at final concentrations of 5 and 2  $\mu$ g/ml, respectively. After 60 min of incubation at 37°C cells were examined on a Zeiss Axiovert fluorescence microscope (Zeiss) equipped with a DAPI filter. Cells were photographed and analysed by visual examination to distinguish between apoptosis and necrosis (22). Cells were judged according to their morphology and the integrity of their cell membranes by propidium iodide staining.

**FACS analysis.** HL-60 cells ( $1 \times 10^6$  per ml) were seeded in T-25 Nunc tissue culture flasks and incubated with 20  $\mu$ g/ml detannified extract (corresponding to 0.42 mg/ml of dried *S. floribunda* and 1.10 mg/ml of dried *S. lucida*) for 8 and 24 h, respectively. Then, cells were washed with 5 ml cold PBS, centrifuged (800 rpm for 5 min), and resuspended and fixed in 3 ml cold ethanol (70%) for 30 min at 4°C. After two further washing steps with cold PBS, RNase A and propidium iodide were added to a final concentration of 50  $\mu$ g/ml each and incubated at 4°C for 60 min before measurement (23,24). Cells were analysed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and cell cycle distribution was calculated with ModFit LT software (Verity Software House, Topsham, ME, USA).

**NF- $\kappa$ B luciferase assay.** HEK293-NF- $\kappa$ B-Luc cells ( $10 \times 10^6$ ) (Panomics, Fremont, USA) were seeded in 20 ml full growth

DMEM medium in a 15-cm dish. Next day, cells were transfected with the cDNA of green fluorescence protein (GFP). A total of 30  $\mu$ l Lipofectamine 2000 (Invitrogen) and 7.5  $\mu$ g DNA were mixed in 2 ml transfection medium and incubated for 20 min at room temperature followed by adding this mixture to the cells. After incubation for 6 h in a humidified atmosphere containing 5% CO<sub>2</sub>,  $4 \times 10^4$  cells per well were seeded in serum- and phenol red-free DMEM in a 96-transparent-well plate. The next day cells were treated with detannified *S. lucida* extract (corresponding to 0.5, 2 and 4 mg/ml of the dried plant) and 15  $\mu$ M Bay 11-7082 (Sigma Aldrich cat no. B5556) as a specific inhibitor of NF- $\kappa$ B (control). One hour after treatment cells were stimulated with 2 ng/ml human recombinant TNF- $\alpha$  for additional 4 h. Luminescence of the firefly luciferase and fluorescence of the GFP were quantified on a GeniusPro plate reader (Tecan, Grödig, Austria). The luciferase signal derived from the NF- $\kappa$ B reporter was normalized by the GFP-derived fluorescence to account for differences in the cell number or transfection efficiency.

**Western blot analyses.** HL-60 cells ( $0.5 \times 10^6$ ) were seeded into T-75 Nunc tissue culture flasks and incubated with 20  $\mu$ g/ml detannified extract (corresponding to 0.4 mg/ml of dried *S. floribunda* and 1.1 mg/ml of dried *S. lucida*) for 0.5, 2, 4, 8 and 24 h, respectively. At each time-point  $2 \times 10^6$  cells were harvested, washed twice with cold PBS, centrifuged (175 x g) for 5 min and lysed in a buffer containing 150 mM NaCl, 50 mM Tris, 1% Triton-X-100, 1 mM phenylmethylsulfonylfluoride (PMSF) and 2.5% PIC (cat no. P8849 Sigma). After centrifugation (12,000 x g) for 20 min at 4°C the supernatant was stored at -20°C until further analysis. Equal amounts of protein samples were separated by polyacrylamide gel electrophoresis and electrotransferred onto PVDV-membranes (Hybond-P, Amersham) at 4°C overnight. Staining membranes with Ponceau S controlled equal sample loading. After washing with Tris-buffered saline (TBS) pH 7.6, membranes were blocked for 1 h in 5% non-fat dry milk in TBS containing 0.1% Tween-20. Membranes were incubated with the first antibody (in blocking solution, dilution 1:500-1:1000) by gently rocking overnight at 4°C, washed with TBS containing 0.1% Tween-20 and further incubated with the second antibody (peroxidase-conjugated swine anti-rabbit IgG or rabbit anti-mouse IgG, dilution 1:2000-1:5000 in blocking solution) for 1 h. Chemiluminescence was developed by the ECL plus detection kit (GE Healthcare) and detected using a Lumi-Imager F1 Workstation (Roche, Basel, Switzerland).

**Statistical analyses.** All experiments were performed in triplicate and analysed by t-test (GraphPad Prism 5.0 program, GraphPad (San Diego, CA, USA)).

## Results

**Anti-proliferative activity.** The methanol extracts of the tested *Scrophularia* species inhibited cell growth of HL-60 promyelocytic leukaemia cells, whereof *S. floribunda* and *S. lucida* showed the strongest inhibition with  $IC_{50}$  values of 0.54 and 0.41 mg/ml, respectively (calculated for dried plant material; Table II and Fig. 1). Methanol extracts contain tannins, which may have caused this effect non-specifically. Therefore, the extracts of those plants exhibiting the strongest activities were purified to

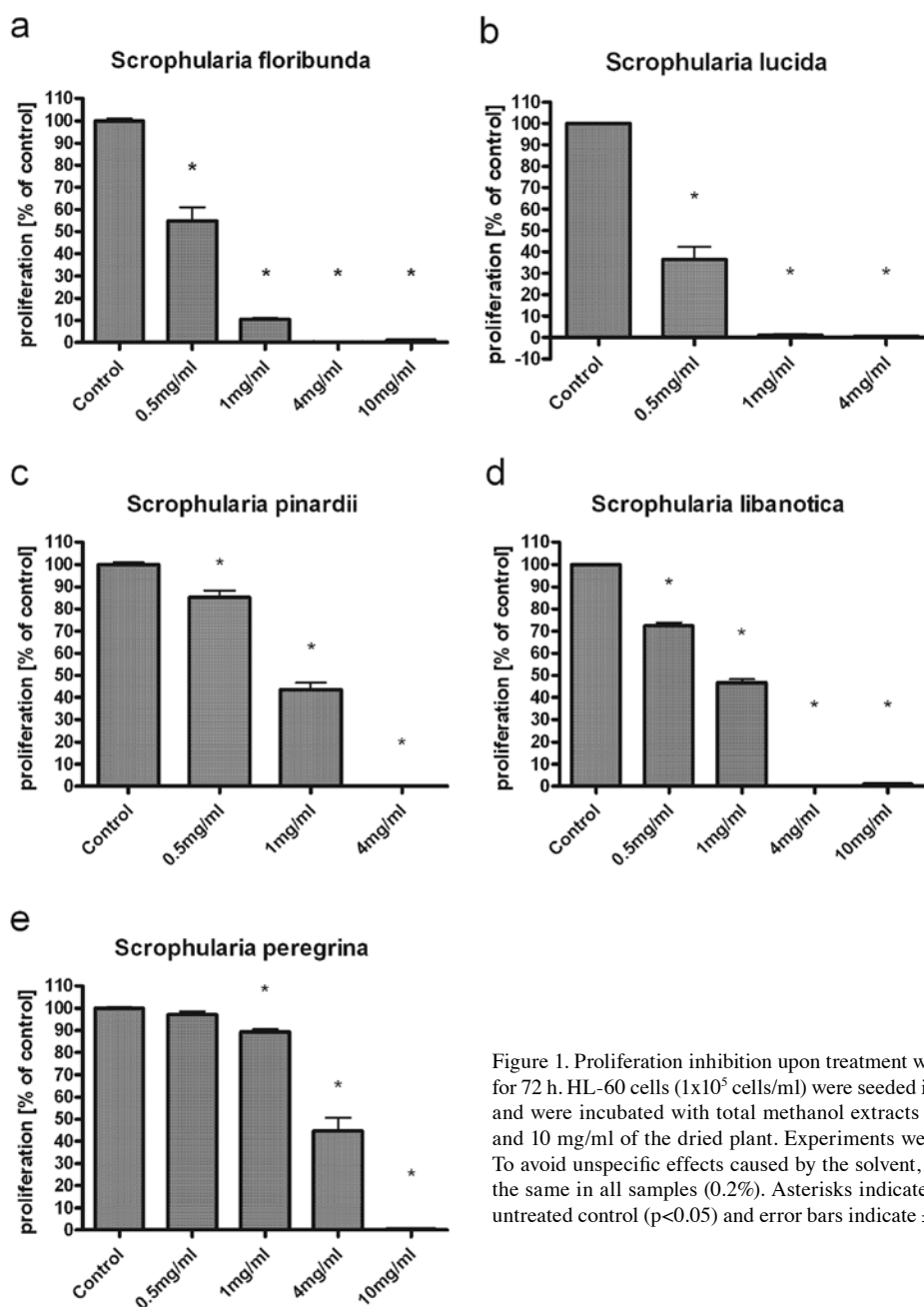


Figure 1. Proliferation inhibition upon treatment with total methanol extracts for 72 h. HL-60 cells ( $1 \times 10^5$  cells/ml) were seeded in T-25 tissue culture flasks and were incubated with total methanol extracts corresponding to 0.5, 1, 4 and 10 mg/ml of the dried plant. Experiments were performed in triplicate. To avoid unspecific effects caused by the solvent, ethanol concentration was the same in all samples (0.2%). Asterisks indicate significance compared to untreated control ( $p < 0.05$ ) and error bars indicate  $\pm$  SD.

Table II.  $IC_{50}$  values in HL-60 cells after 72 h of treatment with the total methanol extracts.

<i>Scrophularia</i> species	
Methanol extract	$IC_{50}$ (mg/ml)
<i>S. floribunda</i>	0.5
<i>S. lucida</i>	0.4
<i>S. peregrina</i>	3.7
<i>S. pinardii</i>	0.9
<i>S. libanotica</i>	0.9

remove chlorophyll and fatty ingredients in a first step and then tannins and other polar substances in a second step. The obtained detannified extracts (dt) were tested again regarding their anti-

proliferative activity and they still showed approximately the same strong growth inhibition ( $IC_{50}$  values of 0.3 and 0.4 mg/ml for *S. floribunda* dt and *S. lucida* dt, respectively, Fig. 2). To compare the two *Scrophularia* species regarding their potency, 20  $\mu$ g/ml of the detannified extracts (corresponding to 1.1 mg/ml *S. lucida* and 0.4 mg/ml *S. floribunda*, respectively) were used for all further experiments.

**Cell cycle distribution.** To investigate the cell cycle distribution, logarithmically growing HL-60 cells were exposed to 20  $\mu$ g/ml detannified methanol extract of *S. lucida* and *S. floribunda* for 8 and 24 h, respectively. Both extracts caused a rapid reduction of G1 cells (Fig. 3). *S. floribunda* treatment induced a strong G2/M arrest after 8 h and a significant accumulation of cells in the S-phase after 24 h. In contrast *S. lucida* did not elicit a G2/M arrest, but a strong accumulation in the S-phase after 8 h

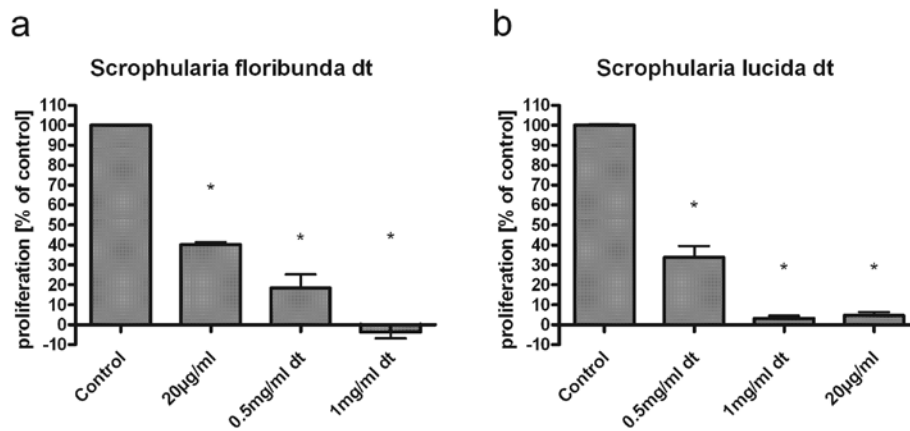


Figure 2. Proliferation inhibition upon treatment with the detannified extracts (corresponding to 0.4, 0.5, 1.0 and 1.1 mg dried plant/ml medium) for 72 h. For *S. floribunda* or *S. lucida* 20 µg dtMeOH extract corresponded to 0.4 or 1.1 mg dried plant weight, respectively. Experiments were performed in triplicate. To avoid unspecific effects caused by the solvent, ethanol concentration was the same in all samples (0.2%). Asterisks indicate significance compared to untreated control ( $p < 0.05$ ) and error bars indicate  $\pm$  SD.

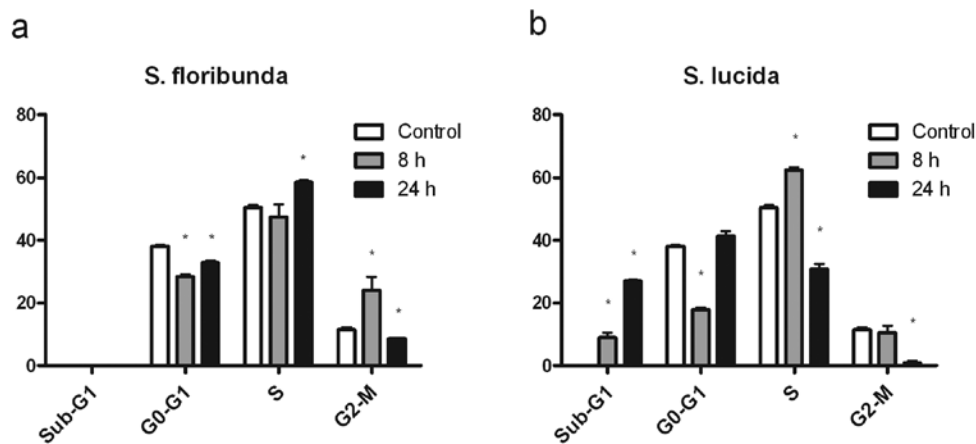


Figure 3. Effects of *Scrophularia* extracts on cell cycle distribution; HL-60 cells ( $1 \times 10^6$  per ml) were seeded in T-25 tissue culture flasks and incubated with 20 µg/ml detannified extract (corresponding to 0.4 mg/ml of dried *S. floribunda* and 1.1 mg/ml of dried *S. lucida*) for 8 and 24 h. Experiments were performed in triplicate. To avoid unspecific effects caused by the solvent, ethanol concentration was the same in all samples (0.2%). Asterisks indicate significance compared to untreated control ( $p < 0.05$ ) and error bars indicate  $\pm$  SD.

and a distinct sub-G1 peak indicating loss of DNA typical for apoptosis.

**Potential mechanisms arresting cell proliferation.** To investigate the underlying mechanisms responsible for the strong proliferation inhibition we analysed the expression profiles of different positive and negative cell cycle regulators (Figs. 4 and 5). *S. floribunda* clearly increased the p21 level after 4 h, while *S. lucida* extract inhibited p21 expression within 4 h. Although p21 is a prominent transcriptional target of p53 another pathway must have triggered the p21 increase since HL-60 cells are p53-deficient (25). As also the activation of the MEK-Erk pathway was shown to up-regulate p21 (26,27), we checked the phosphorylation status of Erk1/2. *S. floribunda* showed a slight increase of the phosphorylation status of Erk1/2 at the 4-h time-point going along with the p21 up-regulation. In contrast *S. lucida* strongly phosphorylated Erk1/2 already after 2 h, followed by a decrease after 8 h and a drop below control level after 24 h. Therefore, p21 must have been regulated independent of Erk1/2. However, both extracts lead to Erk phosphorylation for an unusually long time,

which is known in other contexts to be activated only for some 10-20 min (28).

Another prominent inducer of cell cycle arrest and apoptosis is cellular stress. p38 MAPK is an important member in a signalling cascade controlling its responses to cellular stress. Phosphorylation of p38 at Thr<sup>180</sup> and Tyr<sup>182</sup> leads to its activation and binding to Jnk or Max modulates transcription (29,30). Both extracts were capable of activating p38 within 2 h indicating that cellular stress was another important factor that may have caused growth arrest.

The activation of Chk2 by *S. floribunda* (Fig. 5) was in time with the phosphorylation of Erk1/2 and the induction of p21. The inhibition of the cell cycle was due to the inactivation of Cdc2, which was reflected by the increased phosphorylation of Tyr<sup>15</sup>. Interestingly, Tyr<sup>15</sup>-Cdc2 phosphorylation correlated with over-expression of Wee1, which specifically phosphorylates this site, but not with Cdc25A and Cdc25C, because these phosphatases responsible for the de-phosphorylation of Tyr<sup>15</sup>-Cdc2 became up-regulated. This was in sharp contrast to the effects on cell cycle regulators elicited by *S. lucida* extract, because Chk2 was

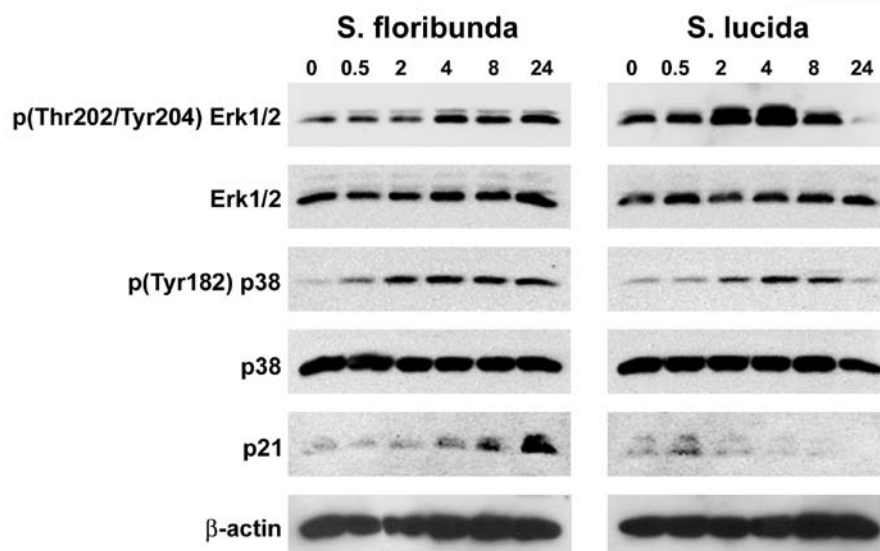


Figure 4. Western blot analyses of different proteins of the MAPK pathway. HL-60 cells/ml ( $1 \times 10^6$ ) were incubated with  $20 \mu\text{g/ml}$  detannified extract and harvested after 0.5, 2, 4, 8 and 24 h of treatment. Cells were lysed and obtained protein samples applied to SDS-PAGE. Western blot analysis was performed with the indicated antibodies. Equal sample loading was confirmed by Ponceau S staining and  $\beta$ -actin analysis.

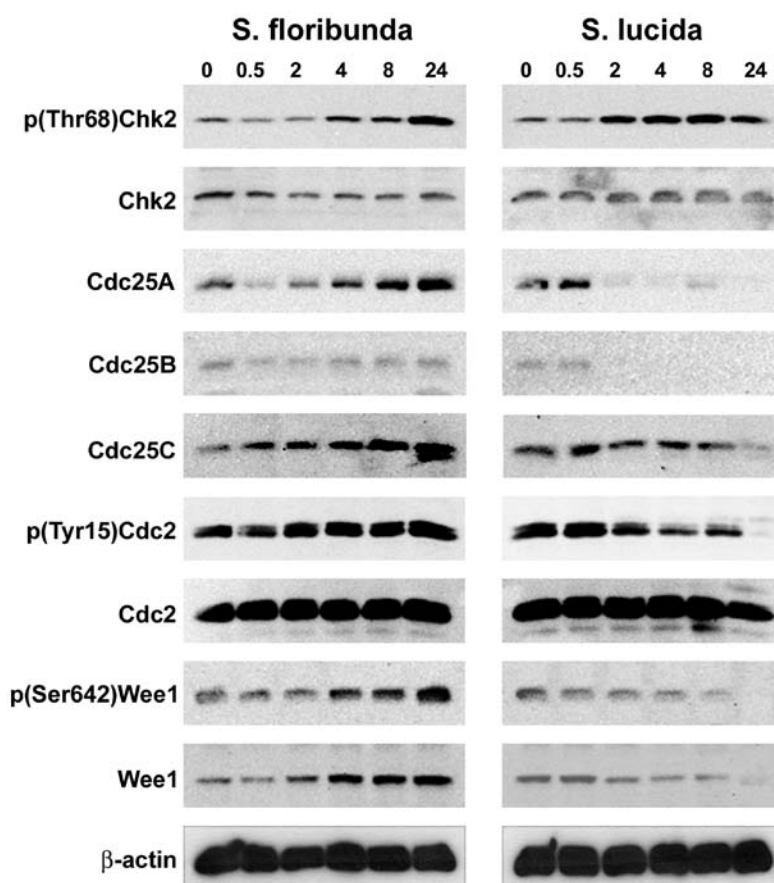


Figure 5. Western blot analyses of cell cycle and check-point regulators. HL-60 cells/ml ( $1 \times 10^6$ ) were incubated with  $20 \mu\text{g/ml}$  detannified extract and harvested after 0.5, 2, 4, 8 and 24 h of treatment. Cells were lysed and obtained protein samples applied to SDS-PAGE. Western blot analysis was performed with the indicated antibodies. Equal sample loading was confirmed by Ponceau S staining and  $\beta$ -actin analysis.

induced much earlier and this correlated with the degradation of the Cdc25 family, which is in accordance with the reported mechanisms of cell cycle inhibition upon DNA check-point activation (31,32). It was expected that this would result in hyper-

phosphorylation and inactivation of the effector kinase Cdc2, but the opposite was the case due to inhibition and down-regulation of Wee1. Therefore, the phosphorylation status of Cdc2 primarily correlates with Wee1, but not with Cdc25A and Cdc25C. Also

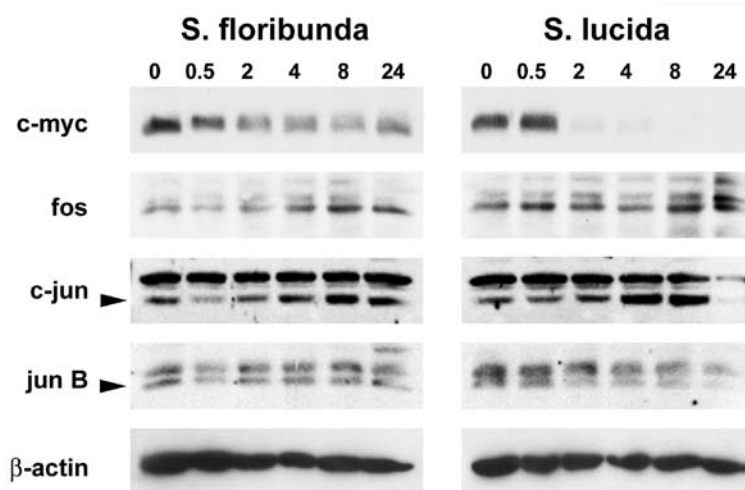


Figure 6. Western blot analyses of different oncogenes. HL-60 cells/ml ( $1 \times 10^6$ ) were incubated with 20  $\mu$ g/ml detannified extract and harvested after 0.5, 2, 4, 8 and 24 h of treatment. Cells were lysed and obtained protein samples applied to SDS-PAGE. Western blot analysis was performed with the indicated antibodies. Equal sample loading was confirmed by Ponceau S staining and  $\beta$ -actin analysis.

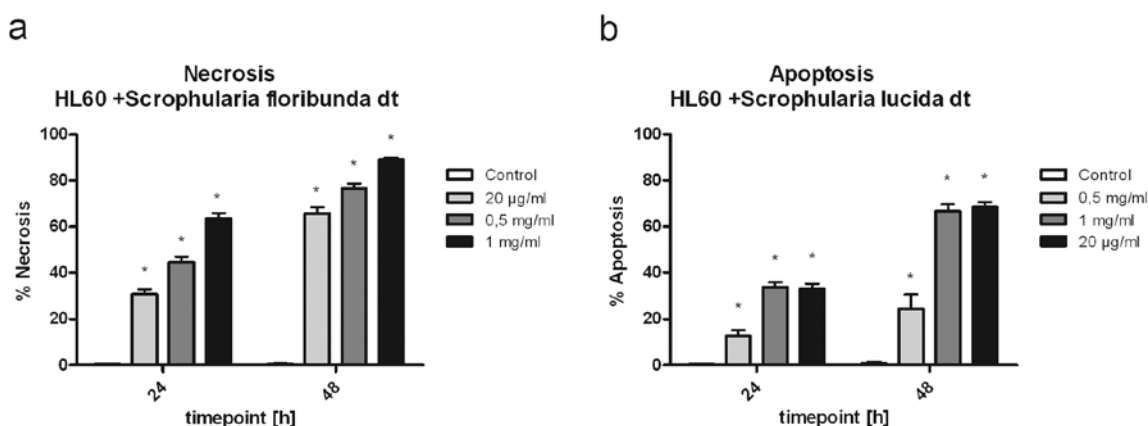


Figure 7. Induction of cell death of HL-60 cells treated with detannified *Scrophularia* extracts. HL-60 cells/ml ( $1 \times 10^5$ ) were seeded in 24-well plates and incubated with 0.5 and 1 mg/ml extract corresponding to dried plant and 20  $\mu$ g/ml to pure extract (corresponding to 1.1 mg/ml *S. lucida* and 0.4 mg/ml *S. floribunda*, respectively). Then, cells were double stained with Hoechst 33258 and propidium iodide and examined under the microscope with UV light connected to a DAPI filter. Nuclei with morphological changes which indicated cell death were counted and the percentages of dead cells were calculated. Experiments were performed in triplicate. Asterisks indicate significance compared to untreated control ( $p < 0.05$ ) and error bars indicate  $\pm$  SD.

Cdc25B became down-regulated by *S. lucida* but was expressed unchanged upon treatment with *S. floribunda*. This evidenced that *S. floribunda* and *S. lucida* contained distinct 'active principles'. Although potential mechanisms as to how the extract of *S. floribunda* inhibits cell division could be outlined, it was still unclear how the extract of *S. lucida* arrested cell proliferation.

**Down-regulation of oncogenes.** Hence, we investigated the expression of proto-oncogenes, which are involved in tumour cell proliferation. c-Myc, a member of the Myc family of oncogenes, is essential for promoting cell growth by regulating the transcription of target genes required for proliferation and c-Myc was shown to be over-expressed in a wide spectrum of tumours (33). As an over-expression leads to constitutive signals that promote proliferation and angiogenesis of the tumour (34), we checked the expression levels of the c-Myc protein to investigate whether the two *Scrophularia* extracts were capable of down-regulating this oncogene. In fact, treatment of HL-60 cells with the two extracts resulted in c-Myc protein decrease,

in particular with *S. lucida* that showed a dramatic down-regulation within 2 h (Fig. 6). Together with Fos family members, Jun family members form the group of AP-1 proteins which, after dimerisation, bind to responsive elements in the promoter regions of different target genes (35). AP-1 heterodimers are important regulators of genes playing a major role in proliferation, differentiation, invasion and metastasis (36). Therefore, we also checked the expression status of c-Jun, JunB and Fos after incubation with the two *Scrophularia* extracts. While Fos was slightly up-regulated by both extracts, and *S. floribunda* did not affect Jun and JunB, *S. lucida* showed a strong down-regulation of these two oncogenes after 24 h.

**Cell death induction.** Treatment of HL-60 cells with the detannified *S. lucida* and *S. floribunda* extract resulted in high cell death rates (Fig. 7). While incubation with detannified extract of *S. lucida* corresponding to 1 mg/ml of the dried plant induced up to 70% of apoptosis after 48 h, HL-60 cells treated with *S. floribunda* extract showed a para-typical apoptosis phenotype

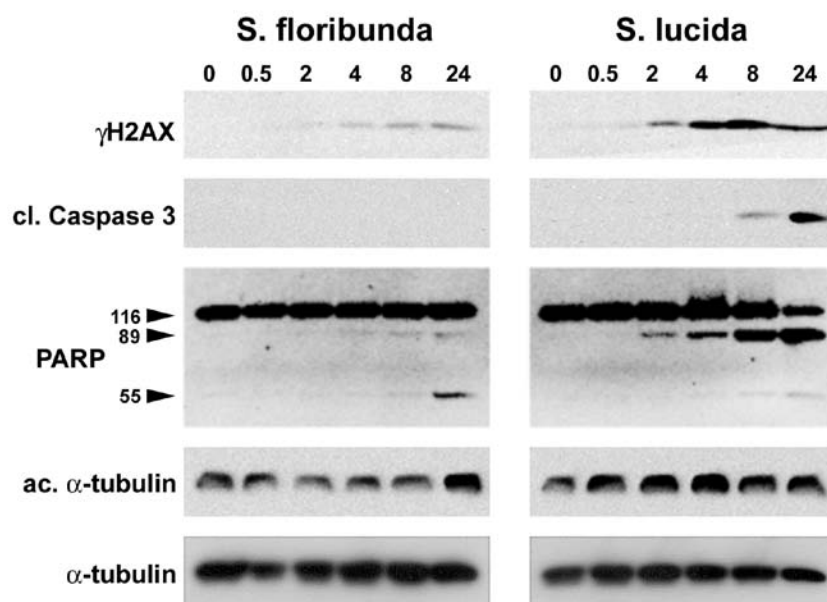


Figure 8. Western blot analyses of apoptosis related proteins. HL-60 cells/ml ( $1 \times 10^6$ ) were incubated with  $20 \mu\text{g/ml}$  detannified extract and harvested after 0.5, 2, 4, 8 and 24 h of treatment. Cells were lysed and obtained protein samples applied to SDS-PAGE. Western blot analysis was performed with the indicated antibodies. Equal sample loading was confirmed by Ponceau S staining and  $\alpha$ -tubulin analysis.

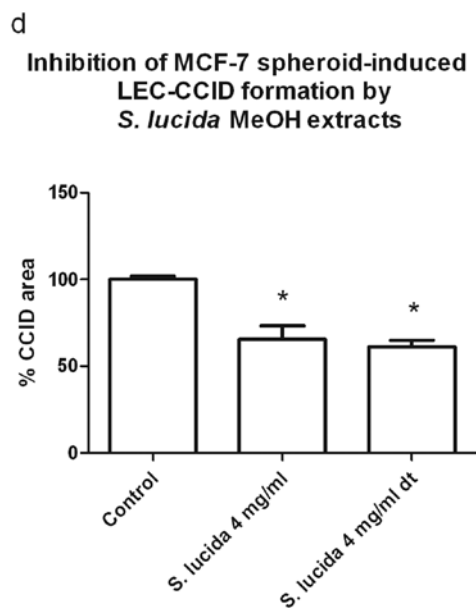
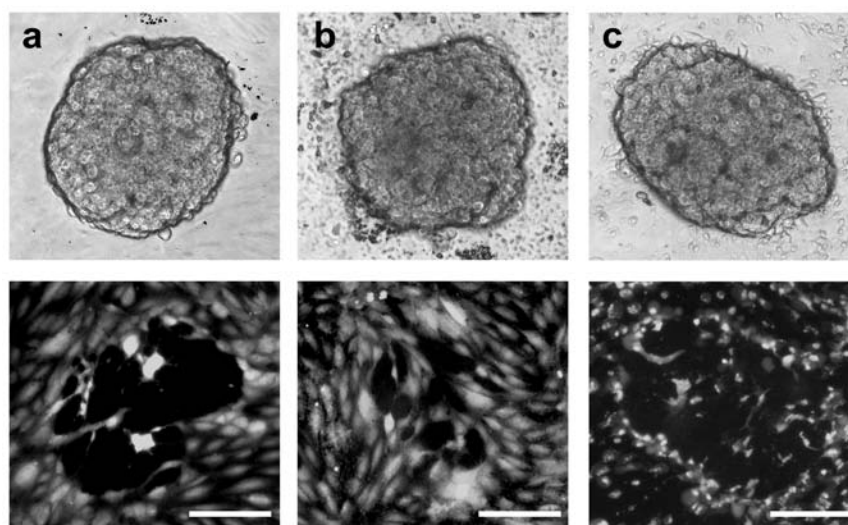


Figure 9. Effect of different *Scrophularia* extracts on MCF-7 spheroid induced gap formation in lymph endothelial cell monolayers. Upper panel, MCF-7 tumour cell spheroids; lower panel, same microscopical frame showing LECs underneath MCF-7 spheroids. The 3D co-cultures were treated either with: (a) solvent (ethanol) or with dtMeOH extracts of (b) *S. lucida* or (c) *S. floribunda* corresponding to 4 mg dried plant weight/ml medium. When the 3D co-cultures were treated with *S. lucida* extract the generated CCIDs in LECs underneath the MCF-7 spheroids were: (d) on average ~40% smaller than those in controls. dt, detannified MeOH extract; scale bars,  $150 \mu\text{m}$ .

with almost instantaneous incorporation of propidium iodide indicating necrosis, which was substantiated in respective Western blot analyses (see below).

**Cell death mechanisms.** FACS analyses (Fig. 3) and HOPI staining (Fig. 7) indicated that *S. lucida* induced apoptosis but not necrosis, while the extract of *S. floribunda* did not show a sub G1 peak. As both compounds led to cell death, we further



investigated the two extracts regarding the mechanisms involved. Caspase-3 plays a critical role in the execution of the apoptotic program and is one of the key enzymes for the cleavage of the 113 kDa nuclear enzyme poly-(ADP-ribose) polymerase (PARP) that is cleaved in fragments of 89 and 24 kDa during apoptosis (37,38).

*S. lucida* caused the specific cleavage of caspase-3 to the active 17 kDa and the proteolytic cleavage of the death substrate PARP into the large 89-kDa fragment demonstrating that caspase-3 was functional and responsible for the pro-apoptotic property of *S. lucida* methanol extract (Fig. 8). In contrast, *S. floribunda* did not show caspase-3 activation and signature type PARP cleavage. Instead of the 89-kDa cleavage product we found a smaller 55-kDa fragment. It was demonstrated that also necrotic cell death of HL-60 cells goes along with degradation of PARP, but different from that observed during apoptosis (39,40). Gobeil *et al* (41) revealed that necrotic treatment of Jurkat T cells did not cause caspase-activation and provoked the appearance of multiple PARP cleavage products mediated by lysosomal proteases. The main fragment was at 55 kDa, which was also found here after treatment of HL-60 cells with *S. floribunda* extract and which correlated with the necrotic phenotype observed by HO/PI double staining (20,21,42).

To investigate whether genotoxicity of the two extracts was responsible for cell death, we analysed the phosphorylation status of the histone H2AX ( $\gamma$ -H2AX), because this core histone variant becomes rapidly phosphorylated in response to DNA double strand breaks. Interestingly both extracts, *S. lucida* and as well *S. floribunda*, caused severe phosphorylation of H2AX after 2- and 4-h incubation, respectively.

Tubulin is the major constituent of microtubules, which facilitates chromosome disjunction during mitosis, and therefore, affecting the tubulin structures is incompatible with functional cell division (43). Alterations of the fine tuned balance of microtubule polymerisation/de-polymerization, such as by taxol are reflected by the acetylation status of  $\alpha$ -tubulin (44). Both methanol extracts increased the acetylation of  $\alpha$ -tubulin demonstrating that cytotoxicity can be attributed to tubulin polymerization.

**Inhibition of lymph endothelial gap formation induced by co-cultivated tumour cell spheroids.** Tissue invasion and metastasis is one of the hallmarks of cancer described by Hanahan and Weinberg (45,46) and for most tumour types patients are not threatened by the primary tumour but by metastases that destroy the function of infested organs. We tested the extracts of both plants in a recently developed three-dimensional cell culture assay measuring the area of circular chemo-repellent-induced defects (CCIDs) in the lymph endothelial cell (LEC) barrier (Fig. 9) which are induced by exudates [i.e., 12(S)-hydroxyeicosatetraenoic acid] of MCF-7 cancer cell spheroids. CCIDs can be considered as entry gates for tumour cells and are directly responsible for lymph node- and distant metastases (15-17). The extract of *S. floribunda* did not prevent CCID formation but affected the viability of LECs and because of the toxic effect of 1 mg/ml MeOH extract to LECs, the precise effect on CCID formation could not be evaluated. Both extracts of *S. lucida* (MeOH and detannified dtMeOH) significantly inhibited CCID formation in LECs up to 40%. The total MeOH extract showed extremely high fluorescence that disappeared after detannification.

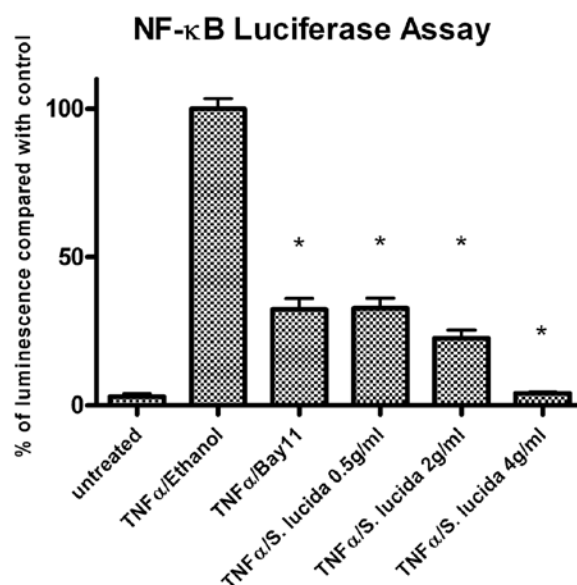


Figure 10. Effect of *S. lucida* extract on the NF- $\kappa$ B transactivation activity. HEK293-NF- $\kappa$ B-Luc cells ( $10 \times 10^6$ ) were transfected with the cDNA of green fluorescence protein (GFP). After incubation for 6 h,  $4 \times 10^4$  cells per well were seeded in serum- and phenol red-free DMEM in a 96-transparent-well plate. On the next day cells were treated with detannified *S. lucida* extract (corresponding to 0.5, 2 and 4 mg dried plant/ml medium), 15  $\mu$ M Bay 11-7082 as a specific inhibitor of NF- $\kappa$ B, or solvent (ethanol). One hour after treatment cells were stimulated with 2 ng/ml human recombinant TNF- $\alpha$  for additional 4 h. Luminescence of the firefly luciferase and fluorescence of the GFP were quantified on a GeniusPro plate reader. The luciferase signal derived from the NF- $\kappa$ B reporter was normalized by the GFP-derived fluorescence to account for differences in cell number or transfection efficiency. Experiments were performed in triplicate. Asterisks indicate significance compared to untreated control ( $p < 0.05$ ) and error bars indicate  $\pm$  SD.

**NF- $\kappa$ B inhibition by *S. lucida* extract.** Besides exudates like 12-S-HETE mentioned above, also NF- $\kappa$ B activation was reported to be associated with tumour cell proliferation, survival, angiogenesis and invasion (47,48). We could show that the inhibition of NF- $\kappa$ B translocation with Bay11-7082, an irreversible inhibitor of I- $\kappa$ B $\alpha$  phosphorylation, blocked MCF-7 spheroid-induced gap formation of LECs in a dose-dependent fashion (16). To check whether the significant inhibition of CCID formation in LECs caused by *S. lucida* extracts may be induced through inhibition of NF- $\kappa$ B activity, we tested the detannified extract in an NF- $\kappa$ B luciferase reporter gene assay. Cells were stimulated with 2 ng/ml TNF- $\alpha$ , and luciferase activity was measured after incubation with the selective NF- $\kappa$ B inhibitor Bay11-7082 and different concentrations of *S. lucida* (corresponding 0.5, 2 and 4 g/ml of the dried plant) and compared with a TNF- $\alpha$ /ethanol treated control. As expected, 15  $\mu$ M of Bay11-7082 (as positive control) inhibited NF- $\kappa$ B activity by nearly 70%. *S. lucida* also decreased the expression of the reporter gene dose-dependently (Fig. 10).

## Discussion

Different species of the *Scrophularia* family are used since ancient times as remedies for some medical conditions including inflammatory diseases and tumours (8,10). While most publications focus on the anti-inflammatory properties (11,12), this work demonstrates for the first time the anti-proliferative and

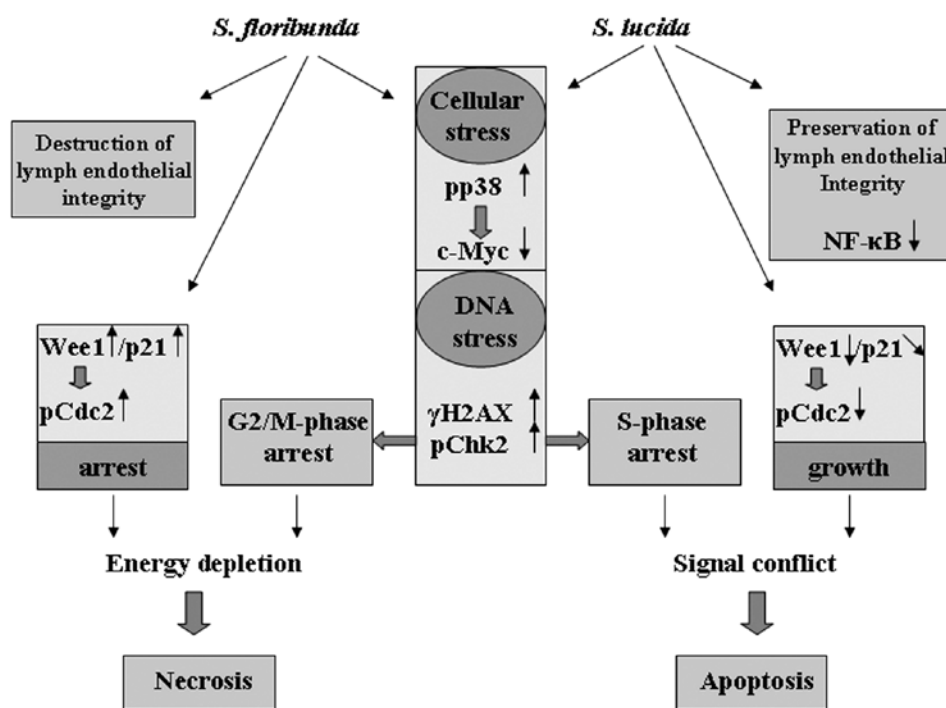


Figure 11. Distinct affection of intracellular signalling and cellular responses upon treatment with the methanol extracts of *S. floribunda* and *S. lucida*. The extracts of the two *Scrophularia* species induce cellular stress as reflected by the activation of p38 and concomitant down-regulation of c-Myc. Subsequent activation of H2AX and Chk2 indicates DNA stress leading to late cell cycle arrest in G2/M upon *S. floribunda* extract treatment. The duplication of DNA consumes the cellular energy pool below a certain threshold level, and together with the stress situation this causes necrotic cell death (21). The high toxicity of this extract kills also LECs and destroys lymph endothelial integrity. In contrast, *S. lucida* extract causes rapid S-phase arrest thereby preserving the cellular energy pool and conflicting 'arrest' signals (pChk2) and 'growth' signals (activation of Cdc2 through dephosphorylation of Tyr 15) induce apoptosis. *S. lucida* extract does not affect lymph endothelial integrity and the inhibition of NF-κB attenuates tumour spheroid-induced CCID formation (16).

pro-apoptotic properties of different *Scrophularia* species, and beyond that we show that *S. lucida* inhibits LEC-CCID formation by co-cultivated MCF-7 cancer cell spheroids (14,17) and inhibited NF-κB activity. Recently we were able to demonstrate that NF-κB activity contributed to LEC-CCID formation through inhibition of VE-cadherin expression and loss of intra-specific LEC adhesion (16).

As of the different tested methanol *Scrophularia* extracts *S. lucida* and *S. floribunda* showed the strongest anti-proliferative properties, these two extracts were chosen to check the underlying mechanisms. Treating HL-60 cells with the detannified *S. floribunda* extract resulted in a strong G2/M arrest after 8 h. This increase of the cell number in G2/M correlated with the phosphorylation status of Cdc2, which is indicative for its inhibition. In contrast, 8-h treatment with *S. lucida* showed a strong accumulation in the S-phase and after 24 h there was a severe G2/M decrease (correlating with Cdc2 activation). The subsequent increase of the sub-G1 peak suggests that the cells are directly running into death from G2/M. Interestingly, the Cdc2 phosphorylation status did not correlate with the expression levels of Cdc25 phosphatases either after treatment with the extract of *S. floribunda* or with that of *S. lucida*, but it correlated with the expression of Wee1. Therefore, *Scrophularia* extracts most likely regulated Cdc2 activity through Wee1 and not Cdc25, demonstrating that Wee1 activity dominates over Cdc25 activity. However, tilting fine tuned Cdc25 activities and expression may trigger cell cycle arrest and finally apoptosis although Cdc2 is active.

The accumulation of HL-60 cells in S-phase after 8-h treatment with *S. lucida* might be caused through degradation of the c-Myc proto-oncogene. c-Myc is associated with a wide range of cancers and is an essential regulator of G1/S transition (49,50). While in normal cells inhibition of c-Myc usually results in a G0/G1 cell cycle arrest (51,52), tumour cells exhibit significant heterogeneity with regard to the positioning of cell cycle arrest in response to c-Myc depletion (53). Cannell *et al* (54) showed that in response to DNA damage c-Myc is translationally repressed by the induction of miR-34c microRNA and that this induction is induced by p38 MAPK/MK2 signalling resulting in S-phase arrest. As c-Myc is over-expressed primarily in cancer cells, its down-regulation may inhibit proliferating cancer cells specifically (55,56).

Another important property of a good anti-cancer remedy is its ability to kill cancer cells and beside their anti-proliferative properties both extracts led to cell death in HL-60 cells. Strong phosphorylation of the histone H2AX demonstrates that both extracts are genotoxic. Treatment of HL-60 cells with *S. lucida* resulted in high apoptosis rate after 48 h, driven through caspase-3 activation and subsequent cleavage of PARP into the active 89-kDa fragment. In contrast, *S. floribunda* showed severe necrosis and neither caspase-3 activation nor signature type cleavage of PARP. The main fragment was at 55 kDa and is described as necrotic PARP cleavage product (41). This extremely toxic effect was also observed in the CCID assay, where *S. floribunda* killed the LECs already after 4 h. Due to this generally toxic effect also against normal cells *S. floribunda* has

to be dismissed as an anti-cancer remedy. The rather complex effects of both *Scrophularia* extracts on signalling pathways and cell phenomena are summarised in Fig. 11.

As mentioned previously, most reports highlight anti-inflammatory properties of *Scrophularia* species. Giner *et al* (11) investigated the activity of four glycoterpenoids (two saponins, verbascosaponin A and verbascosaponin, and two iridoids, scropolioside A and scrovalentinoside) isolated from *S. auriculata* ssp. *pseudoauriculata* in different models of acute and chronic inflammation and demonstrated the anti-inflammatory activity in mice against different edema inducers. In another report (12) five phenylpropanoid glycosides isolated from the roots of *S. scordonia* L. have been evaluated as potential inhibitors of some macrophage functions involved in the inflammatory process. They were shown to perform inhibitory effects on enzymes of the arachidonate cascade (COX-1, COX-2) and significant reduction of LPS-induced TNF- $\alpha$  production without relevant effects on the ALOX5 pathway. Treating LEC monolayers with 1  $\mu$ M synthetic 12(S)-HETE, a metabolite of arachidonic acid generated by ALOX12/15, caused the phosphorylation of MLC2 (16) indicating that 12(S)-HETE induced the motility of LECs thereby provoking an early step of metastasis (15,17). This observation is also consistent with an inflammatory process, which is accompanied by the acquisition of a mobile phenotype of the affected cells reflecting 'epithelial to mesenchymal transition' [EMT; (57)]. Interestingly, the extract of *S. lucida* activated the mobility marker MLC2 (data not shown). It was expected that MLC2 would become inhibited, because of the markedly attenuated formation of CCIDs. Therefore, other activities suppressing LEC migration must have prevailed over MLC2 activation and the NF- $\kappa$ B inhibitory property of *S. lucida* is a likely candidate for this effect. As *Scrophularia* species have been used as remedies for different skin diseases, including scabies, eczema and psoriasis (8) the partly pro-migratory property inducing MLC2 phosphorylation could be an explanation for this wound healing effect, which depends on the plasticity of cells.

Furthermore, an ethanol extract prepared from the aerial parts of *S. striata* Boiss significantly and dose-dependently inhibited matrix metalloproteinase (MMP) activity (58). According to the critical role of MMPs in tumour invasion, metastasis and neovascularisation, the inhibition of the degradation of components of the extracellular matrix is a promising approach for the prevention of cancer progression. In order to develop distal metastasis a tumour cell has to encompass different steps: local infiltration into the adjacent tissue, intravasation, survival within the circulatory system, extravasation and subsequent proliferation leading to colonization (57,59). Inhibiting the first steps of this multi-step process should be a major goal of cancer therapy. We could demonstrate that *S. lucida* exhibited significant inhibition of CCID formation and MMP2 and MMP9 play a significant role in this particular assay (15).

In conclusion, we were able to show that the species *S. lucida*, which is a genus widely used as folk remedy, exhibits strong anti-proliferative and killing effects on cancer cells and strong anti-invasive properties. The fractionation of the methanol extract will be a mandatory future approach to identify the compounds responsible for the anti-proliferative and anti-metastatic properties.

## Acknowledgements

We wish to thank Toni Jäger for preparing the figures.

## References

1. Wang MW, Hao X and Chen K: Biological screening of natural products and drug innovation in China. *Philos Trans R Soc Lond B Biol Sci* 362: 1093-1105, 2007.
2. Gonzales GF and Valerio LG Jr: Medicinal plants from Peru: a review of plants as potential agents against cancer. *Anticancer Agents Med Chem* 6: 429-444, 2006.
3. Cragg GM and Newman DJ: Plants as a source of anti-cancer agents. *J Ethnopharmacol* 100: 72-79, 2005.
4. Cragg GM and Newman DJ: Antineoplastic agents from natural sources: achievements and future directions. *Expert Opin Investig Drugs* 9: 2783-2797, 2000.
5. Newman DJ and Cragg GM: Natural products as sources of new drugs over the last 25 years. *J Nat Prod* 70: 461-477, 2007.
6. Baytop T: *Türkiyede Bitkiler ile Tedavi*. Istanbul University Press, Faculty of Pharmacy, p3255, 1999.
7. Vienna Dioscurides, 6th century, National Library, manuscript collection, Vienna, Austria.
8. Galindez J, Diaz-Lanza AM and Fernandez-Metallano L: Biologically active substances from the genus *Scrophularia*. *Pharmaceutical Biol* 40: 45-59, 2002.
9. Çalis I, Gross GA and Sticher O: Phenylpropanoid glycosides isolated from *Scrophularia scopoli*. *Phytochemistry* 26: 2057-2061, 1987.
10. Nguyen AT, Fontaine J, Malonne H, Claeys M, Luhmer M and Duez P: A sugar ester and an iridoid glycoside from *Scrophularia ningpoensis*. *Phytochemistry* 66: 1186-1191, 2005.
11. Giner RM, Villalba ML, Recio MC, Manez S, Cerda-Nicolas M and Rios JL: Anti-inflammatory glycoterpenoids from *Scrophularia auriculata*. *Eur J Pharmacol* 389: 243-252, 2000.
12. Diaz AM, Abad MJ, Fernández L, Silván AM, De Santos J and Bermejo P: Phenylpropanoid glycosides from *Scrophularia scordonia*: in vitro anti-inflammatory activity. *Life Sci* 74: 2515-2526, 2004.
13. Özmen A, Madlener S, Bauer S, Krasteva S, Vonach C, Giessrigl B, Gridling M, Viola K, Stark N, Saiko P, Michel B, Fritzer-Szekeres M, Szekeres T, Askin-Celik T, Krenn L and Krupitza G: In vitro anti-leukemic activity of the ethno-pharmacological plant *Scutellaria orientalis* ssp. *carica* endemic to western Turkey. *Phytomedicine* 17: 55-62, 2010.
14. Özmen A, Bauer S, Gridling M, Singhuber J, Krasteva S, Madlener S, Vo NTP, Stark N, Saiko P, Fritzer-Szekeres M, Szekeres T, Askin-Celik T, Krenn L and Krupitza G: In vitro anti-neoplastic activity of the ethno-pharmaceutical plant *Hypericum adenotrichum* Spach endemic to western Turkey. *Oncol Rep* 22: 845-852, 2009.
15. Kerjaschki D, Bago-Horvath Z, Rudas M, Sexl V, Schneckenthner C, Wolbank S, Bartel G, Krieger S, Kalt R, Hantusch B, Keller T, Nagy-Bojarszky K, Huttary N, Raab I, Lackner K, Krautgasser K, Schachner H, Kaserer K, Rezar S, Madlener S, Vonach C, Davidovits A, Nosaka H, Hämmerle M, Viola K, Dolznig H, Schreiber M, Nader A, Mikulits W, Gnant M, Hirakawa S, Detmar M, Alitalo K, Nijman S, Offner F, Maier TJ, Steinhilber D and Krupitza G: Lipoxigenase mediates invasion of intrametastatic lymphatic vessels and propagates lymph node metastasis of human mammary carcinoma xenografts in mouse. *J Clin Invest* 121: 2000-2012, 2011.
16. Vonach C, Viola K, Giessrigl B, Huttary N, Raab I, Kalt R, Krieger S, Vo NTP, Madlener S, Bauer S, Marian B, Hämmerle M, Kretschy N, Teichmann M, Hantusch B, Sary S, Unger C, Seelinger M, Eger A, Mader R, Jäger W, Schmidt W, Grusch M, Dolznig H, Mikulits W and Krupitza G: NF- $\kappa$ B mediates the 12(S)-HETE-induced endothelial to mesenchymal transition of lymphendothelial cells during the intravasation of breast carcinoma cells. *Br J Cancer* 105: 263-271, 2011.
17. Madlener S, Saiko P, Vonach C, Viola K, Huttary N, Stark N, Popescu R, Gridling M, Vo NTP, Herbacek I, Davidovits A, Giessrigl B, Venkateswarlu S, Geleff S, Jäger W, Grusch M, Kerjaschki D, Mikulits W, Golakoti T, Fritzer-Szekeres M, Szekeres T and Krupitza G: Multifactorial anticancer effects of digalloyl-resveratrol encompass apoptosis, cell-cycle arrest, and inhibition of lymphendothelial gap formation in vitro. *Br J Cancer* 102: 1361-1370, 2010.

18. Maier S, Strasser S, Saiko P, Leisser C, Sasgary S, Grusch M, Madlener S, Bader Y, Hartmann J, Schott H, Mader RM, Szekeres T, Fritzer-Szekeres M and Krupitza G: Analysis of mechanisms contributing to AraC-mediated chemoresistance and re-establishment of drug sensitivity by the novel heterodinucleoside phosphate 5-FdUrd-araC. *Apoptosis* 11: 427-440, 2006.
19. Strasser S, Maier S, Leisser C, Saiko P, Madlener S, Bader Y, Bernhaus A, Gueorgieva M, Richter S, Mader RM, Wesierska-Gadek J, Schott H, Szekeres T, Fritzer-Szekeres M and Krupitza G: 5-FdUrd-araC heterodinucleoside re-establishes sensitivity in 5-FdUrd- and AraC-resistant MCF-7 breast cancer cells overexpressing ErbB2. *Differentiation* 74: 488-498, 2006.
20. Grusch M, Fritzer-Szekeres M, Fuhrmann G, Rosenberger G, Luxbacher C, Elford HL, Smid K, Peters GJ, Szekeres T and Krupitza G: Activation of caspases and induction of apoptosis by amidox and didox. *Exp Haematol* 29: 623-632, 2001.
21. Grusch M, Polgar D, Gfatter S, Leuher K, Huettenbrenner S, Leisser C, Fuhrmann G, Kassie F, Steinkellner H, Smid K, Peters GJ, Jayaram H, Klepal T, Szekeres T, Knasmüller S and Krupitza G: Maintenance of ATP favours apoptosis over necrosis triggered by benzamide riboside. *Cell Death Differ* 9: 169-178, 2002.
22. Hüttenbrenner S, Maier S, Leisser C, Polgar D, Strasser S, Grusch M and Krupitza G: The evolution of cell death programs as prerequisites of multicellularity. *Rev Mutat Res* 543: 235-249, 2003.
23. Madlener S, Svacinová J, Kitner M, Kopecky J, Eytner R, Lackner A, Vo NTP, Frisch R, Grusch M, de Martin R, Dolezal K, Strnad M and Krupitza G: In vitro anti-inflammatory and anticancer activities of extracts of *Acalypha alopecuroides* (Euphorbiaceae). *Int J Oncol* 35: 881-891, 2009.
24. Gridling M, Stark N, Madlener S, Lackner A, Popescu R, Benedek B, Diaz R, Tut FM, Vo NTP, Huber D, Gollinger M, Saiko P, Özmen A, Mosgoeller W, de Martin R, Eytner R, Wagner KH, Grusch M, Fritzer-Szekeres M, Szekeres T, Kopp B, Frisch R and Krupitza G: In vitro anti-cancer activity of two ethno-pharmacological healing plants from Guatemala *Pluchea odorata* and *Phlebodium decumanum*. *Int J Oncol* 34: 1117-1128, 2009.
25. Biroccio A, del Bufalo D, Ricca A, D'Angelo C, D'Orazi G, Sacchi A, Soddu S and Zupi G: Increase of BCNU sensitivity by wt-p53 gene therapy in glioblastoma lines depends on the administration schedule. *Gene Ther* 6: 1064-1072, 1999.
26. Park KS, Jeon SH, Oh JW and Choi KY: P21<sup>Cip1/WAF1</sup> activation is an important factor for the ERK pathway dependent anti-proliferation of colorectal cancer cells. *Exp Mol Med* 36: 557-562, 2004.
27. Facchinetti MM, De Siervi A, Toskos D and Senderowicz AM: UCN-01-induced cell cycle arrest requires the transcriptional induction of p21(waf1/cip1) by activation of mitogen-activated protein/extracellular signal-regulated kinase/extracellular signal regulated kinase pathway. *Cancer Res* 64: 3629-3637, 2004.
28. Ebner HL, Blatzer M, Nawaz M and Krumschnabel G: Activation and nuclear translocation of Erk in response to ligand-dependent and -independent stimuli in liver and gill cells from rainbow trout. *J Exp Biol* 210: 1036-1045, 2007.
29. Lee CM, Onésime D, Reddy CD, Dhanasekaran N and Reddy EPJLP: A scaffolding protein that tethers JNK/p38<sup>MAPK</sup> signaling modules and transcription factors. *Proc Natl Acad Sci USA* 99: 14189-14194, 2002.
30. Zervos AS, Faccio L, Gatto JP, Kyriakis JM and Brent R: Mxi2, a mitogen-activated protein kinase that recognizes and phosphorylates Max protein. *Proc Natl Acad Sci USA* 92: 10531-10534, 1995.
31. Donzelli M and Draetta GF: Regulating mammalian checkpoints through Cdc25 inactivation. *EMBO Rep* 4: 671-677, 2003.
32. Falck J, Mailand N, Syljuasen RG, Bartek J and Lukas J: The ATM-Chk2-Cdc25A checkpoint pathway guards against radio-resistant DNA synthesis. *Nature* 410: 842-847, 2001.
33. Baudino TA and Cleveland JL: The Max network gone mad. *Mol Cell Biol* 21: 691-702, 2001.
34. Dang CV: C-Myc target genes involved in cell growth, apoptosis and metabolism. *Mol Cell Biol* 19: 1-11, 1999.
35. Milde-Langosch K: The Fos family of transcription factors and their role in tumorigenesis. *Eur J Cancer* 41: 2449-2461, 2005.
36. Tulchinsky E: Fos family members: regulation, structure and role in oncogenic transformation. *Histol Histopathol* 15: 921-928, 2000.
37. Fernandes-Alnemri T, Litwack G and Alnemri ES: CPP32, a novel human apoptotic protein with homology to *Caenorhabditis elegans* cell death protein Ced-3 and mammalian interleukin-1 beta-converting enzyme. *J Biol Chem* 269: 30761-30764, 1994.
38. Germain M, Affar EB, d'Amours D, Dixit VM, Salvesen GS and Poirier GG: Cleavage of poly(ADP-ribose) polymerase during apoptosis. *J Biol Chem* 274: 28379-28384, 1999.
39. Shah GM, Shah RG and Poirier GG: Different cleavage pattern for poly(ADP-Ribose) polymerase during necrosis and apoptosis in HL-60 cells. *Biochem Biophys Res Commun* 229: 838-844, 1996.
40. Casiano CA, Ochs RL and Tan EM: Distinct cleavage products of nuclear proteins in apoptosis and necrosis revealed by autoantibody probes. *Cell Death Differ* 5: 183-190, 1998.
41. Gobell S, Boucher CC, Nadeau D and Poirier GG: Characterization of the necrotic cleavage of poly(ADP-ribose) polymerase (PARP-1): implication of lysosomal proteases. *Cell Death Differ* 8: 588-594, 2001.
42. Fritzer-Szekeres M, Grusch M, Luxbacher C, Horvath Z, Krupitza G, Elford HL and Szekeres T: Trimidox, an inhibitor of ribonucleotide reductase, induces apoptosis and activates caspases in HL-60 promyelocytic leukemia cells. *Exp Hematol* 28: 924-930, 2000.
43. Piperno G and Fuller M: Monoclonal antibodies specific for an acetylated form of alpha-tubulin recognize the antigen in cilia and flagella from a variety of organisms. *J Cell Biol* 101: 2085-2094, 1985.
44. Xiao H, Verdier-Pinard P, Fernandez-Fuentes N, Burd B, Angeletti R, Fiser A, Horwitz SB and Orr GA: Insights into the mechanism of microtubule stabilization by taxol. *Proc Natl Acad Sci USA* 103: 10166-10173, 2006.
45. Hanahan D and Weinberg RA: The hallmarks of cancer. *Cell* 100: 57-70, 2000.
46. Hanahan D and Weinberg RA: Hallmarks of cancer: the next generation. *Cell* 144: 646-674, 2011.
47. Brown M, Cohen J, Arun D, Chen Z and van Waes C: NF-kappa B in carcinoma therapy and prevention. *Expert Opin Targets* 12: 1109-1122, 2008.
48. Flister MJ, Wilber A, Hall KL, Iwata C, Miyazono K, Nisato RE, Pepper MS, Zawieja DC and Ran S: Inflammation induces lymph-angiogenesis through up-regulation of VEGFR-3 mediated by NF-kappaB and Prol. *Blood* 115: 418-429, 2010.
49. Adhikary S and Eilers M: Transcriptional regulation and transformation by Myc proteins. *Nat Rev Mol Cell Biol* 6: 635-645, 2005.
50. Gauweryk CE and Croce CM: Chromosomal translocations in leukaemia. *Semin Cancer Biol* 4: 333-340, 1993.
51. De Alboran IM, O'Hagan RC, Gartner F, Malynn B, Davidson L and Rickert R: Analysis of c-myc function in normal cells via conditional gene-target mutation. *Immunity* 14: 45-55, 2001.
52. Prathapam T, Tegen S, Oskarsson T, Trumpff A and Martin GS: Activated Src abrogates the myc requirement for the G0/G1 transition but not for the G1/S transition. *Proc Natl Acad Sci USA* 103: 2695-2700, 2006.
53. Wang H, Mannava S, Grachtchouk V, Zhuang D, Soengas MS, Gudkov AV, Prochownik EV and Nikiforov MA: c-Myc depletion inhibits proliferation of human tumor cells at various stages of cell cycle. *Oncogene* 27: 1905-1915, 2008.
54. Cannell IG, Kong YW, Johnston SJ, Chen ML, Collins HM, Dobbyn HC, Elia A, Kress TR, Dickens M, Clemens MJ, Heery DM, Gaestel M, Eilers M, Willis AE and Bushell M: p38 MAPK/MK2-mediated induction of miR-34c following DNA damage prevents Myc-dependent DNA replication. *Proc Natl Acad Sci USA* 107: 5375-5380, 2010.
55. Krupitza G, Harant H, Dittrich E, Szekeres T, Huber H and Dittrich C: Sodium butyrate inhibits c-myc splicing and interferes with signal transduction in ovarian carcinoma cells. *Carcinogenesis* 16: 1199-1205, 1995.
56. Krupitza G, Grill S, Harant H, Hulla W, Szekeres T, Huber H and Dittrich C: Genes related to growth and invasiveness are repressed by sodium butyrate in ovarian carcinoma cells. *Br J Cancer* 73: 433-438, 1996.
57. Van Zijl F, Krupitza G and Mikulits W: Initial steps of metastasis: cell invasion and endothelial transmigration. *Mutat Res* 728: 23-34, 2011.
58. Hajiaghazadeh R, Monsef-Esfahani HR, Khorramizadeh MR, Saadat F, Shahverdi AR and Attar F: Inhibitory effect of aerial parts of *Scrophularia striata* on matrix metalloproteinases expression. *Phytother Res* 21: 1127-1129, 2007.
59. Eger A and Mikulits W: Models of epithelial-mesenchymal transition. *Drug Disc Today; Disease models* 2: 57-63, 2005.