

Methanol extract of the ethnopharmaceutical remedy *Smilax spinosa* exhibits anti-neoplastic activity

MAREIKE SEELINGER¹, RUXANDRA POPESCU², BENEDIKT GIESSRIGL¹, KANOKWAN JARUKAMJORN^{3,4}, CHRISTINE UNGER¹, BRUNO WALLNÖFER⁵, MONIKA FRITZER-SZEKERES⁶, THOMAS SZEKERES⁶, RENE DIAZ⁷, WALTER JÄGER³, RICHARD FRISCH⁷, BRIGITTE KOPP² and GEORG KRUPITZA¹

¹Institute of Clinical Pathology, Medical University of Vienna, Vienna; ²Department of Pharmacognosy, Faculty of Life Sciences, University of Vienna, Vienna; ³Department of Clinical Pharmacy and Diagnostics, Faculty of Pharmaceutical Sciences, University of Vienna, A-1090 Vienna, Austria; ⁴Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand; ⁵Department of Botany, Museum of Natural History, A-1010 Vienna; ⁶Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Vienna, A-1090 Vienna, Austria; ⁷Institute for Ethno-Biology, Playa Diana, San José/Petén, Guatemala

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Abstract. Plants have been the source of several effective drugs for the treatment of cancer and over 60% of anticancer drugs originate from natural sources. Therefore, extracts of the rhizome of *Smilax spinosa*, an ethnomedicinal plant from Guatemala which is used for the treatment of inflammatory conditions, were investigated regarding their anti-neoplastic activities. By using several solvents the methanol extract was by far the most potent against HL60 cell proliferation (50% inhibition at 60 µg/ml). Furthermore, fractionation of this extract yielded fraction F2, which exhibited enforced pro-apoptotic activity, and activated CYP1A1. Proteins that are relevant for cell cycle progression and apoptosis, as well as proto-oncogenes were investigated by western blotting. This revealed that the methanol extract increased the levels of p21 and this may have caused cell cycle attenuation. The derivative fraction F2 induced apoptosis through the intrinsic pathway, which correlated with the inhibition of Stat3 phosphorylation and concomitant induction of caspase 9, then caspase 8 and caspase 3. In summary, the methanol extract and the derivative fraction F2 of *S. spinosa* showed anti-neoplastic effects in HL-60 cells and CYP1A1 activation in estrogen receptor-positive MCF-7 breast cancer cells but not in estrogen-negative MDA-MB231 breast cancer cells. Based on our data *Smilax spinosa* may be a promising source for novel anticancer agents.

Introduction

In the western industrialised countries one out of three persons develops some type of cancer during their lifetime (1) and >50% of them succumb to the disease (2). For the treatment of many types of cancer, natural products have been the source of effective drugs and today >60% of anticancer drugs originate from natural sources such as plants, marine organisms and micro-organisms (3). An estimation conducted by the WHO reveals that even today 80% of the population of Asia and Africa rely on traditional medicine for primary health care (Traditional Medicine Fact Sheet No. 134, World Health Organization, Dec. 2008, <http://www.who.int/mediacentre/factsheets/fs134/en/>).

Fabricant and Farnsworth (4) describe ethnomedicine as a 'highly diversified approach to drug discovery'. It involves observation, description, and experimental investigation of indigenous remedies for their possible biological or medicinal activity. The drugs (of plants mostly) have been used in traditional medicine for hundreds of years, which is the reason why tolerable toxic effects can be expected in humans. Traditional medicine is practiced by shamans or herbalists who keep the healing skills a secret (5) and hence, little is known about their remedies. Therefore, natural products remain an important source for the discovery of new drugs. However, the primary extracts of natural products consist of complex mixtures and this makes the isolation of the active principles a difficult task. The key compounds may be unstable, or the activity may be based on two or more synergistic constituents that may disappear upon separation. Until 2000 (current data are unavailable) only 6% of higher plant species had been screened for their biologic, mostly anticancer or anti-HIV, activity (4).

Important examples of anticancer drugs derived from an ethnomedicinal plant that are now used in the clinic are the vinca alkaloids vinblastine and vincristine. Both were isolated from the Madagascar periwinkle, *Catharanthus roseus* G. Don (Apocynaceae). Another agent belonging to the ethno-derived

Correspondence to: Professor 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

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chemotherapeutic drugs is paclitaxel isolated from the bark of *Taxus brevifolia* Nutt. This demonstrates that isolated compounds from plants traditionally used as home remedies may lead to the development of novel anticancer agents (3). Owing to their high biodiversity, rain forests are immensely rich sources for new drugs (6). In particular, ancient civilizations collected knowledge over many hundreds of years regarding the natural products that were effective as therapies against several diseases (7). Combination of these ethnopharmaceutical benefits and the advantageous biodiversity through which the medicinal tradition flourished, established the basis for the present work with the objective of finding new potential lead compounds against cancer by investigating a healing plant of the Maya from the Guatemala/Belize lowland rainforest. The rhizome of *Smilax spinosa* Miller (Smilacaceae) is used by the indigenous population as a natural remedy against inflammation. We selected this plant to study potential anti-neoplastic properties, as similar signaling pathways are upregulated during inflammation and in cancer cells (8). To date, only a few pharmacological effects of the *Smilax* species have been investigated in clinical trials (9). For example, *Smilax regelii* (syn. Sarsaparilla) exhibits antimicrobial activities against *Shigella dysenteriae* (10) and a *Smilax glabra* extract had immuno-modulatory activity in rats by decreasing the IL-1-, TNF- and NO-release of macrophages (11). *S. regelii* is mostly applied internally against arthritis, rheumatism (both causing inflammation), psoriasis or dermatitis (skin disorders), impotence, or as a blood purifier (9). It is described to be active against snake bites (12) but an excessive dosage of *S. regelii* has been reported to cause gastrointestinal irritation (9). *Smilax* species are particularly known to contain saponins and plant steroids that can be synthesized into human steroids such as estrogen and testosterone. Also, the majority of *S. regelii*'s activities are reported to be caused by these steroids and saponins (9). The methanol extract of *S. spinosa* renders DPPH, OH, and O₂- radicals innocuous thereby inhibiting lipid peroxidation. Furthermore, it was effective against *Salmonella typhimurium* and *Trypanosoma cruzii*. Therefore, the methanol extract has an anti-oxidative and anti-microbial activity (13). When used against male impotency, *S. spinosa* rhizome and guinweo (a local plant) are soaked in rum and administered twice a day (14). Already in 1536 a *Smilax* root from Mexico was introduced into European medicine to treat syphilis and rheumatism (9). As *S. spinosa* has not yet been investigated for its anti-neoplastic activity, the present study was conducted to analyse its anti-proliferative effects.

Materials and methods

Antibodies. Antibodies against: cleaved Asp175 caspase3 (no. 9661), cleaved caspase 8 (Asp391, 18C8, no. 9496), cleaved caspase 9 (Asp330, no. 9501), phospho-Stat3 (Tyr705)(D3A7, no. 9145), Stat3 (no. 9132) and phospho-Stat5 (Tyr694)(C11C5, 9359) were from Cell Signaling (Danvers, MA, USA). PARP-1 (F-2, sc-8007), Cdc25A (F-6, sc-7389), cyclin D1 (M-20, sc-718), p21 (C-19, sc-397), α -tubulin (DM1A, sc-32293), β -tubulin (H-235, sc-9104), Stat5 (C-17, sc-835), c-Jun (H-79, sc-1694) and Jun B (210, sc-73) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). c-Myc Ab-2 (9E10.3,

Table I. Solvents used for extraction of *S. spinosa* and extract weights.

Solvent	Extract weight (mg) corresponding to 1 g dried rhizome
Petroleum ether	0.7
Dichloromethane	1.1
Ethyl acetate	11.1
Methanol	205.5
Water	147.8

no. MS-139-P1) was from Thermo Fisher Scientific (Fremont, CA, USA), phospho-Ser177 Cdc25A (no. AP3046a) from Abgent (San Diego, CA, USA), phospho-Ser139 H2AX (DR 1017) from Calbiochem (San Diego, CA, USA) and β -actin (AC-15, A5441) as well as anti-acetylated tubulin (clone6-11B-1, T6793) were from Sigma (St. Louis, MO, USA). The secondary antibodies peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG were purchased from Dako (Glostrup, Denmark).

Cell culture. HL-60 (human promyelocytic leukaemia) cells and human MCF-7 and MDA-MB231 breast cancer cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and grown in RPMI-1640 medium (HL-60). The other cell lines were grown in MEM medium, which was supplemented with 10% heat-inactivated foetal calf serum (FCS), 1% Glutamax and 1% penicillin/streptomycin (Life Technologies, Carlsbad, CA, USA), 1% NEAA (Invitrogen, Karlsruhe, Germany). Cells were kept in a humidified atmosphere at 37°C containing 5% CO₂.

Plant material. Parts of the rhizomes of *Smilax spinosa* Miller (vernacular name: 'Kokolmeka roja') were collected in Guatemala, Departamento Petén, at the north-western shore of Lago Petén Itzá, San José, ~1 km north of the road from San José to La Nueva San José (16 59'30" N, 89 54'00" W). Voucher specimens (leg. G. Krupitza and R. O. Frisch, Nr. 4-2009, 19. 04. 2009, Herbarium W, det. B. Wallnöfer (W) 26.1.2010) were archived at the Museum of Natural History, Vienna, Austria.

Extraction. Rhizomes of *S. spinosa* were cut, dried by lyophilisation and then pulverized. Twenty grams of the obtained powder were mixed with 200 ml solvent (1:10; Table I) extracted in an ultra sonic bath for 10 min and afterwards under reflux for 1 h in the water bath. The solution was filtered and the retained plant material (residue) was dried at room temperature before being re-extracted with the next more polar solvent. The liquid extract was evaporated under reduced pressure to give a crude fraction (0.67 mg corresponding to 1 g rhizome; Table I) (15,16). The extract weights obtained from serial extraction of the dried rhizomes of *S. spinosa* with five solvents of increasing polarity are presented in Table I, which illustrates that the weight of the methanol extract corresponds to ~20.6% of the dried rhizome.

Table II. Obtained amounts of sub-fractions derived from 4.1 g of methanol extract.

Fraction	Potentially abundant substances	Amount (g)
F1 Petroleum ether	Chlorophyll, wax, resin less polar compounds	0.03
F2 Water-methanol	Tannins, more polar substances	3.65
F3 Chloroform	Chloroform-soluble substances	0.02

Sub-fractionation of the methanol extract. The methanol extract was the most active of the *S. spinosa* extracts, therefore it was further fractionated by dissolving 4.1 g in 60 ml of a water-methanol mixture (9:1). After threefold extraction with 60 ml petroleum ether each for the removal of chlorophyll, waxes and fats, the remaining fraction was diluted with 60 ml of water. Subsequently, this aqueous solution was extracted three times with 120 ml chloroform each. The collected chloroform layers were washed three times with 360 ml sodium chloride solution (1%). After drying with sodium sulphate, the solution was filtered and the chloroform was evaporated under reduced pressure. The weights of the obtained sub-fractions are listed in Table II. Approximately 10% of the starting extract was lost during the fractionation process.

Thin layer chromatography with chloroform: methanol: water (70:22:3.5) revealed that the petroleum ether fraction (F1) exhibited an almost identical banding pattern as the petroleum ether extract (detection under visible light and UV366 with ASR; data not shown), but with some additional bands, which seemed to be responsible for the higher activity of the F1 fraction (data not shown) compared to the petroleum ether extract.

Proliferation and cytotoxicity assays. HL-60 cells were seeded in 24-well plates at a concentration of 1×10^5 cells/ml allowing logarithmic growth within the next 48 h. Cells were then incubated with increasing concentrations of plant extracts (5 μ g/ml, 15 μ g/ml, 30 μ g/ml, 60 μ g/ml) for 48 h. After 24 and 48 h, the cell number was counted using a KX-21 N microcell counter (Sysmex Corporation, Kobe, Japan) and the percent of cell divisions compared to the untreated control were calculated as follows: $[(C48h + drug - C24h + drug)/(C48h - drug - C24h - drug)] \times 100 = \% \text{ cell division}$, whereby C48h + drug or C48h - drug were the cell numbers after 48 h with or without extract treatment, respectively. C24h + drug or C24h - drug were the respective cell numbers after 24 h (17,18).

Apoptosis assay - Hoechst 33258 and propidium iodide double staining. Hoechst 33258 (HO) and propidium iodide (PI) double staining (Sigma, St. Louis, MO) determine the type of death the cell is undergoing, i.e. apoptosis (early or late) or necrosis (19,20). HL-60 cells were seeded in a 24-well plate at a concentration of 1×10^5 cells/ml and treated with increasing concentrations of fractions F1, F2 and F3. After 24 h, 48 h and 72 h of incubation, 100 μ l cell suspension of each well was transferred into separate wells of a 96-well plate and HO and PI were added at final concentrations of 5 μ g/ml and 2 μ g/ml, respectively. After 1 h of incubation at 37°C, stained cells were examined and photographed on a fluorescence microscope

(Axiovert, Zeiss, Jena, Germany) equipped with a DAPI filter. Cell death was evaluated and counted by visual examination of the photographs according to the morphological characteristics revealed by HOPI staining. Experiments were performed in triplicate.

Western blotting. HL-60 were seeded in T-75 tissue culture flasks at a concentration of 1.8×10^5 cells/ml and treated with the indicated concentration of methanol extract or fraction F2. Cells were harvested after 0.5, 2, 4, 8 and 24 h. Then, cells were washed twice with cold PBS and centrifuged at 1,000 rpm for 5 min at 4°C. The cell pellet was lysed in a buffer containing 150 mM NaCl, 50 mM Tris pH 8.0, 1% Triton-X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM Protease Inhibitor Cocktail (PIC), (Sigma, Schnellendorf, Germany). The lysate was centrifuged at 12,000 rpm for 20 min at 4°C. Supernatant was transferred into a 1.5 ml tube and stored at -20°C until further analysis. Equal amounts of protein lysate were mixed with SDS (sodium dodecyl sulphate) sample buffer and loaded onto a 10% polyacrylamide gel. Proteins were separated by polyacrylamide gel electrophoresis (PAGE) at 120 Volt and electro-transferred onto a PVDF (polyvinylidene difluoride) membrane (Hybond, Amersham, Buckinghamshire, UK) at 95 Volt for 80 min. Membranes were allowed to dry for at least 30 min up to 2 h to provide fixing of the proteins to the membrane. Methanol was used to remoisten the membranes. Equal sample loading was checked by staining the membrane with Ponceau S (Sigma, Schnellendorf, Germany). After removing Ponceau S with PBS or TBS (Tris buffered saline, pH 7.6), membranes were blocked in PBS- or TBS-milk (5% non-fat dry milk in PBS containing 0.5% Tween-20 or TBS containing 0.1% Tween-20) for 1 h. Then, membranes were washed with PBS/T (PBS containing 0.5% Tween-20) or TBS/T (TBS containing 0.1% Tween-20), changing the washing solution 4-5 times, for at least 20 min. Next, membranes were incubated with the primary antibody in blocking solution (according to the data sheet TBS-, PBS-milk or TBS-, PBS-BSA) diluted 1:500 - 1:1000, gently shaking at 4°C, overnight. Thereafter, membranes were washed again with PBS/T or TBS/T and incubated with the secondary antibody (peroxidase conjugated anti-rabbit IgG or anti-mouse IgG) diluted 1:2000 in PBS- or TBS-milk at room temperature for 1 h. Chemiluminescence was developed by the ECL detection kit (Amersham, Buckinghamshire, UK) and membranes were exposed to Amersham Hyperfilm.

Ethoxyresorufin-O-deethylase (EROD) assay selective for CYP1A1 activity. MDA-MB-231 and MCF-7 breast cancer cells were grown in phenol red-free DMEM/F12 culture

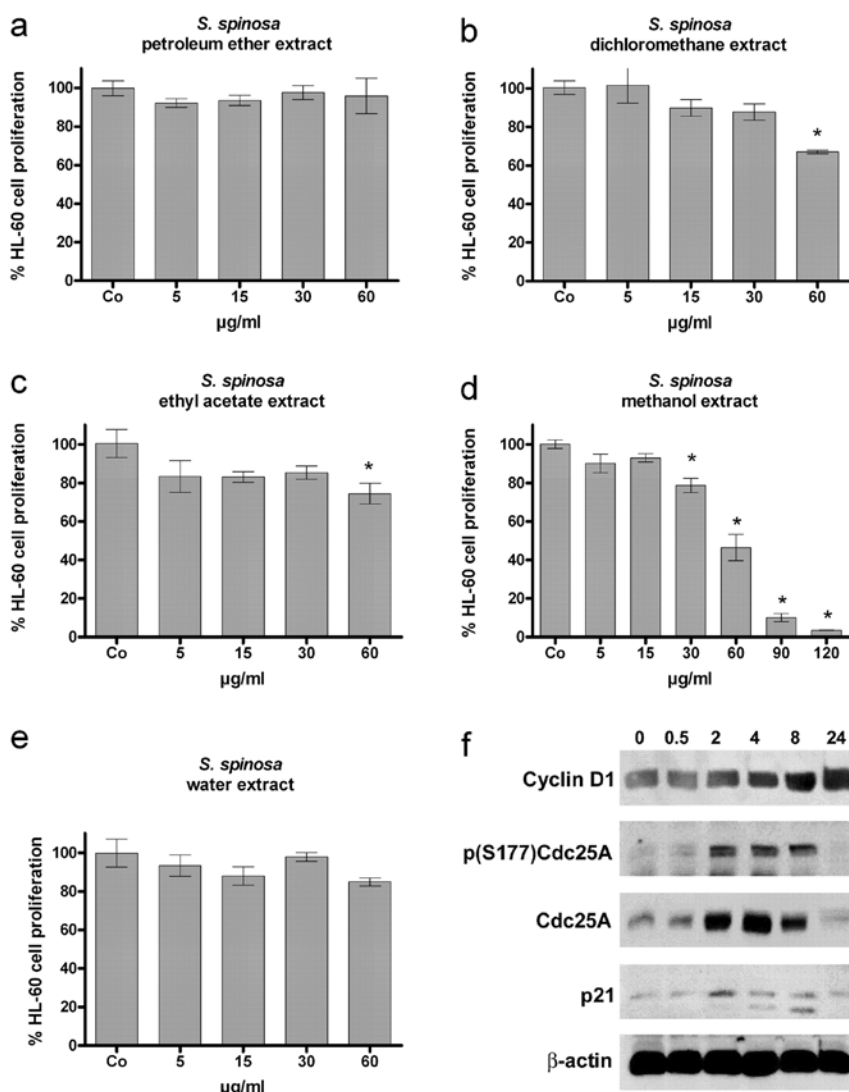


Figure 1. Anti-proliferative effect of increasingly polar extracts; (a) petroleum ether, (b) dichloromethane, (c) ethyl acetate, (d) methanol, and (e) water). HL-60 cells were seeded into 24-well plates (1×10^5 cells/ml), incubated with 5, 15, 30 and 60 $\mu\text{g/ml}$ of each extract (the methanol extract also with 90 and 120 μg) for 48 h. Cells were counted after 24 and 48 h of treatment. The percentage of proliferation between 24 and 48 h was determined in comparison to control. Experiments were performed in triplicate. Asterisks indicate significance compared to untreated control ($p < 0.05$) and error bars indicate the means \pm SEM. (f) Analysis of the expression of cell cycle regulators. HL-60 cells (1×10^6 cells/ml) were incubated with 120 $\mu\text{g/ml}$ of the methanol extract and harvested after 0.5, 2, 4, 8 and 24 h of treatment. Cells were lysed and the obtained protein samples were subjected to SDS-gel electrophoresis and subsequent western blot analysis with the indicated antibodies. Equal sample loading was confirmed by Ponceau S staining and β -actin analysis.

medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS and 1% PS (Invitrogen, Karlsruhe, Germany) under standard conditions at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air. Twenty-four hours prior to treatment, the cells were transferred to DMEM/F12 culture medium supplemented with 2.5% charcoal-stripped FCS (PAN Biotech, Aldenbach, Germany) and 1% PS. The MeOH extract and F2 were dissolved in DMSO and diluted with medium (final DMSO concentration <0.1%) to 30 and 60 $\mu\text{g/ml}$. Experiments under each set of conditions were carried out in triplicate. Blanks contained DMSO in the medium of the test compounds. After 18 h of incubation, ethoxyresorufin (final concentration 5.0 μM , Sigma-Aldrich, Munich, Germany) was added and 0.4 ml aliquots of the medium were sampled after 200 min. Subsequently, the formation of resorufin was analyzed by spectrofluorometry (PerkinElmer LS50B, Waltham, MA,

USA) with an excitation wavelength of 530 nm and an emission wavelength of 585 nm.

Statistical analysis. For statistical analyses Excel 2003 software and Prism 5 software package (GraphPad, San Diego, CA, USA) were used. The values are expressed as the mean \pm SEM and the Student's t-test was applied to compare differences between control samples and treatment groups. Statistical significance level was set at $p < 0.05$.

Results and Discussion

The lyophilized rhizome of *S. spinosa* was subjected to sequential extraction with five solvents of increasing polarity. The obtained extracts were investigated for their anti-neoplastic potential in HL-60 leukaemia cells, as blood

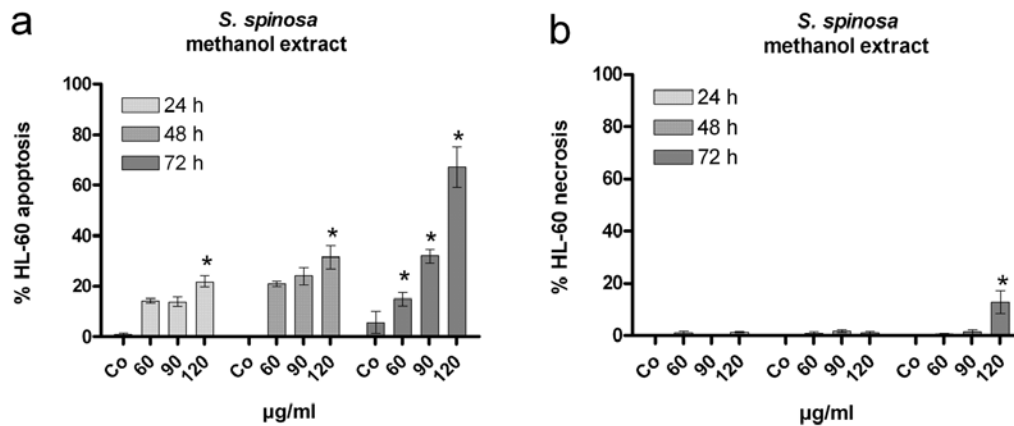


Figure 2. Induction of cell death. HL-60 cells were seeded in 24-well plates (1×10^5 cells/ml) and incubated with 60, 90 and 120 $\mu\text{g/ml}$ methanol extract for 24, 48, and 72 h. Then, cells were double stained with Hoechst 33258 and propidium iodide and examined under the microscope with UV light connected to a DAPI filter. Nuclei with morphological changes which indicated (a) apoptosis or (b) necrosis were counted and the percentage of apoptotic or necrotic cells was calculated. Experiments were performed in triplicate. Asterisks indicate significance compared to untreated control ($p < 0.05$) and error bars indicate the means \pm SEM.

cells are easily accessible and sensitive to pharmacological compounds, thereby providing an appropriate system for an initial testing series.

Inhibition of cell proliferation. To determine the anti-proliferative effects in HL-60 cells, extracts were applied at increasing concentrations (5, 10, 30, 60 $\mu\text{g/ml}$ and partly 90 and 120 $\mu\text{g/ml}$) for 24, 48, and 72 h (Fig. 1a-e). The methanol extract was the most potent and inhibited cell proliferation dose-dependently with an IC_{50} (the concentration inhibiting 50% proliferation) of $\sim 60 \mu\text{g/ml}$. The extract exhibited the highest activity within the first 48 h, which decreased thereafter (data not shown). Although the extract concentration at which proliferation was significantly inhibited was rather high, the corresponding weight of the rhizome was low, as the methanol extract was almost one fifth of the whole lyophilized rhizome substance. This corresponds to 300-500 mg of dried rhizome, or 370-630 mg of fresh rhizome per kg body weight; a person with an average body weight has to consume the alcoholic extract derived from only 20 g of rhizome within two days. Inhibition of cell proliferation was accompanied by a rapid upregulation of Cdc25A expression within 2 h of treatment (Fig. 1f). It seems that the high Cdc25A levels were the result of an increased protein synthesis rather than an inhibited protein degradation, as Ser177 phosphorylation, which tags Cdc25A for recruitment of the proteasome and subsequent proteolysis (21), was sustained throughout 2 and 8 h of treatment, whereas the degradation of the Cdc25A proto-oncogene below control levels was observed after 24 h. The increase in Cdc25A was followed by cyclin D1 upregulation within 4 h. The proto-oncogene cyclin D1 is necessary for the transit from early G1 to beginning of S phase and induction of cyclin D1 expression is indicative for cell cycle activation. p21 inhibits the Cdk2/Cyclin E kinase complex that normally cooperates with cyclin D1 at a later stage to facilitate G1-S transition and then S-phase progression (22). p21 was induced within 2 h of extract treatment and this accurately counteracted the induction of Cdc25A phosphatase, which causes the activation of Cdk2/Cyclin E. (23,24) and therefore, cell cycle progression was blocked. Additionally, p21 binds to the Cdk4/Cyclin D complex. This

results in a hypo-phosphorylation and activation of pRb and thereby the suppression of the E2F pathway and cessation of the cell cycle (25). However, when cyclin D1 became induced, p21 levels were already back to control levels. An additional band below 21 kD became visible, which was most likely a degradation product of p21. Expression of p21 transcription is widely regulated by p53. Since HL-60 cells are p53 deficient (26) p21 must have been controlled in a p53-independent manner (27) and it was shown that the proto-oncogene c-Myc negatively regulates p21 (28). The chaotic expression of prominent cell cycle protagonists and proto-oncogenes together with p21 induction undoubtedly affected DNA replication and cell duplication thereby triggering growth arrest.

Induction of apoptosis. Growth arrest due to extra-cellular stressors often elicits apoptosis. Therefore, HL-60 cells were treated with increasing concentrations (60, 90, 120 $\mu\text{g/ml}$) of the methanol extract to analyse cell viability (Fig. 2a). The time- and dose-dependent increase in the number of dead cells showed a morphology which is typical for apoptosis, whereas a small number of necrotic cells were only observed at the highest dose (120 $\mu\text{g/ml}$) after 72 h (Fig. 2b). In an attempt to increase the pro-apoptotic activity the methanol extract was subjected to a fractionation procedure (as described in Materials and methods) obtaining three fractions. Fraction 2 (F2) exhibited the strongest pro-apoptotic effect whereby 40% apoptotic cells were observed upon treatment with 90 $\mu\text{g/ml}$ F2 for 24 h (Fig. 3a) and this was a ~ 2.5 fold increase compared to the primary methanol extract ($< 15\%$ apoptotic cells upon treatment with 90 $\mu\text{g/ml}$ methanol extract for 24 h; Fig. 2a). F2 inhibited cell proliferation less efficiently than the original methanol extract (data not shown). Therefore, a cell cycle inhibitory property was separated from a pro-apoptotic property which was less dependent on the cell cycle. This is of particular significance as tumour cells, which are not cycling, could be targeted by this fraction. To get further insight into the mechanisms of F2, the expression of pro-apoptotic proteins as well as of markers indicating genotoxicity and microfilament stress was investigated. The increased cleavage of caspase 9 was observed after 2 h of incubation, whereas caspase 8 was

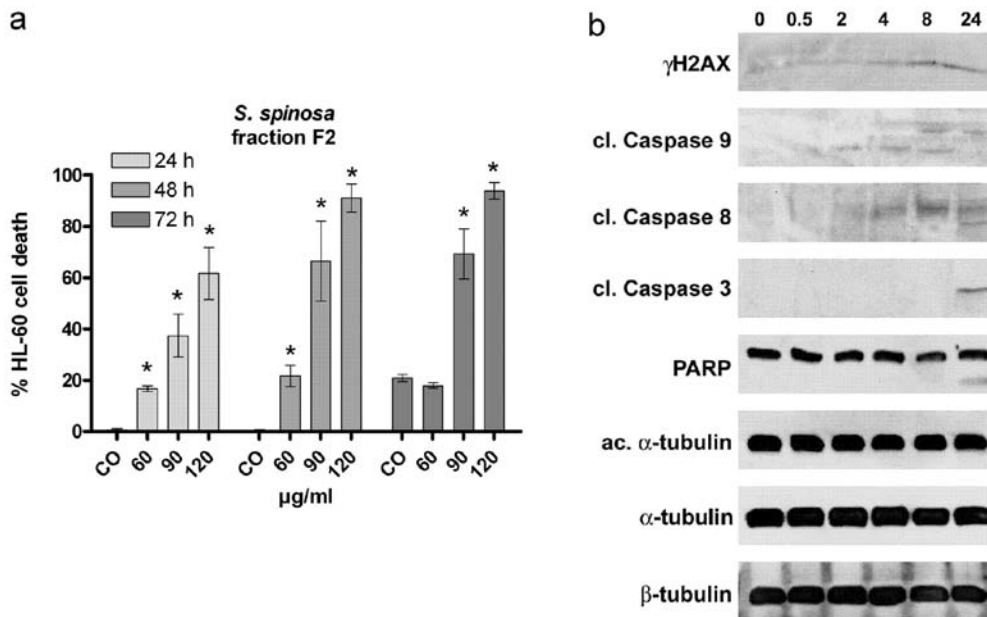


Figure 3. (a) Induction of apoptosis by fraction F2 water-methanol fraction. HL-60 cells were seeded in 24-well plates (1×10^5 cells/ml) and incubated with 60, 90 and 120 $\mu\text{g/ml}$ of each extract for 24, 48, and 72 h. Afterwards cells were double stained with Hoechst 33258 and propidium iodide and examined under the microscope with UV light connected to a DAPI filter. Nuclei with morphological changes which indicated apoptosis were counted and the percentage of apoptotic cells were calculated. Experiments were performed in triplicate. Asterisks indicate significance compared to untreated control ($p < 0.05$) and error bars indicate the means \pm SEM. (b) Analysis of the expression of apoptosis related proteins. HL-60 cells (1×10^6 cells/ml) were incubated with 120 $\mu\text{g/ml}$ of fraction F2 and harvested after 0.5, 2, 4, 8 and 24 h of treatment. Cells were lysed and the obtained protein samples were subjected to SDS-gel electrophoresis and subsequent western blot analysis was conducted with the indicated antibodies. Equal sample loading was confirmed by Ponceau S staining and β -tubulin analysis.

cleaved after 4 h. Finally the executor, caspase 3, was activated after 24 h. The caspase cascade i.e. the activation of caspase 9 and 8, which both cause the cleavage and activation of caspase 3 and the subsequent induction of apoptosis (2) suggested that F2 induced the intrinsic pathway. The activity of caspase 3 was reflected by the cleavage of its target PARP (120 kD) into a smaller 85 kD fragment (29) (Fig. 3b). The phosphorylation of H2AX (γ H2AX) is a sensitive and commonly used marker for the presence of DNA-double-strand breaks (30). During this experiment, the phosphorylation of H2AX was induced after 8 and 24 h, whereas caspase 9 was activated before phosphorylation of H2AX implicating that the increase of γ H2AX levels was the consequence of caspase 3 activation and the subsequent induction of nucleases causing DNA degradation. Therefore, F2 itself did not induce DNA double strand breaks but this does not exclude the possibility of a genotoxic property of F2 triggering DNA single strand breaks or the generation of DNA adducts.

Several plant compounds were shown to affect the equilibrium of microtubule polymerization. Tilting this fine-tuned equilibrium of polymerized-depolymerized microfilaments is incompatible with normal cell division and causes cell cycle arrest and apoptosis. The acetylation of α -tubulin reflects the polymerization status of the microtubule meshwork (31). However, F2 did not alter tubulin acetylation and therefore did not target the spindle apparatus.

Modulated expression of the proto-oncogenes Stat3, c-Myc and c-Jun. Stat family proteins are transcription factors involved in normal and pathological cellular processes. The Stat3 proto-oncogene product is the most activated Stat protein in

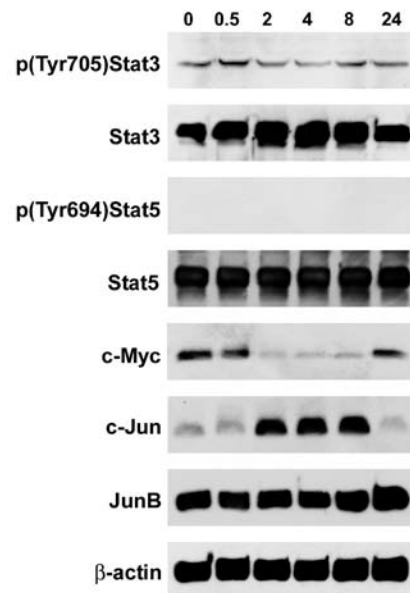


Figure 4. Analysis of proto-oncogene expression. HL-60 cells (1×10^6 cells/ml) were incubated with 120 $\mu\text{g/ml}$ of the methanol extract and harvested after 0.5, 2, 4, 8 and 24 h of treatment. Cells were lysed and the obtained protein samples were subjected to SDS-gel electrophoresis and subsequent western blot analysis with the indicated antibodies. Equal sample loading was confirmed by Ponceau S staining and β -actin analysis.

human cancers (32) accelerating cell proliferation, preventing apoptosis (33) and playing a role in angiogenesis (34). Stat3, which is phosphorylated at Tyr 705, shifts into the nucleus activating target genes (35) and overexpression of Stat3 was found

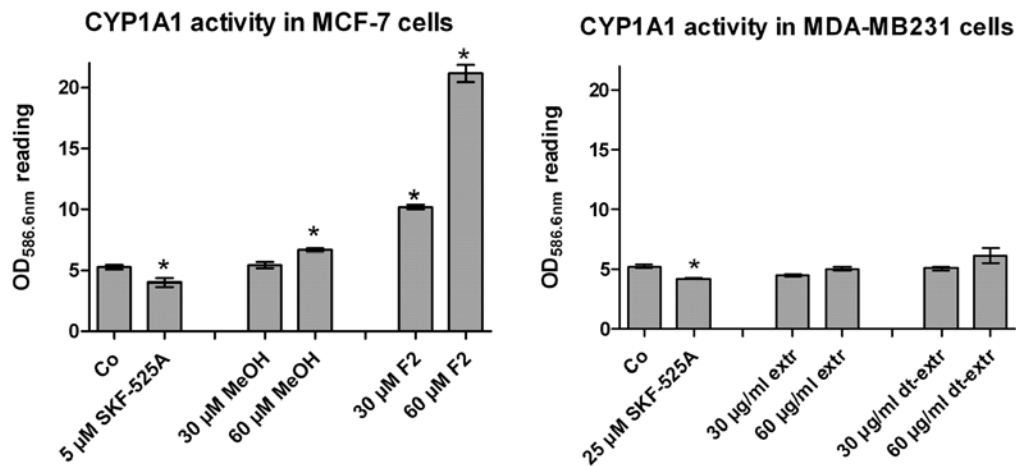


Figure 5. Induction of CYP1A1 activity in breast cancer cells. (a) MCF-7 and (b) MDA-MB231 cells were kept under steroid-free conditions and treated with 30 μ g/ml and 60 μ g/ml *S. spinosa* crude MeOH extract, fraction F2, solvent (DMSO; Co) or 5 μ M and 25 μ M CYP inhibitor SKF-525A. Then, 5 μ M ethoxyresorufin was added and after 200 min the formation of resorufin was analysed, which is specific for CYP1A1 activity. Experiments were performed in triplicate, error bars indicate SEM and the asterisks indicate ($p < 0.05$).

in leukaemia, breast, pancreatic and prostate cancer, as well as in melanoma (36). In F2-treated HL-60 cells the constitutive Tyr 705 phosphorylation of Stat3 was downregulated after 2 h (Fig. 4) which suggests that the anti-apoptotic activity was also decreased temporally correlating with the activation of caspase 9. Also, the Stat5 proto-oncogene induces anti-apoptotic genes such as Bcl-X (37), thereby maintaining cell survival despite drug-induced stresses (38). However, Stat5 was neither constitutively phosphorylated (activated) nor was the protein level decreased by F2. By contrast, the expression of c-Myc was strongly suppressed after 2 h of F2 treatment and was re-expressed (still below control levels) after 24 h. c-Myc causes an abnormal proliferation rate and is overexpressed in many tumour types and influences cell differentiation and apoptosis (39).

c-Jun and JunB belong to the family of Jun transcription factors, which are components of the activating protein-1 (AP1) transcription factor complexes. AP1 heterodimers are important for cell proliferation, differentiation, and activated c-Jun promotes cell cycle progression and neoplastic transformation (40). Markedly, c-Jun was strongly increased between 2 and 8 h of F2 treatment, whereas JunB expression remained unchanged. The strictly inverse expression of c-Jun and c-Myc upon F2 treatment was most likely independent of each other, as it has not been reported that c-Jun and the AP1 complex suppress c-Myc, nor has it been shown that c-Myc negatively regulates c-Jun. However, when in complex with ATF2 and c-Myc, c-Jun binds to the ATF/CRE site of ATF3 and c-Jun/ATF2/c-Myc induce cell proliferation (41). The dramatic disproportional expressions of c-Myc and c-Jun upon F2 treatment excluded the possibility of a transcriptional active c-Myc/ATF2/c-Jun complex and hence, in case of such a scenario, proliferation was most likely compromised.

Induction of CYP1A1 activity in MCF-7 cells. Smilacacea species are reported to contain steroidal compounds with estrogenic and anti-estrogenic effects (42,43). A subset of cytochrome P450 (CYP) enzymes are important regulators of

estrogen and phyto-estrogen metabolism and CYP1A1 plays a role in estrogen receptor (ER) pathway-dependent synergic carcinogenic action of xeno-estrogens (44). Caucasian individuals with polymorphic CYP1A1 (homozygous for A2455G) stand an increased risk for breast cancer (45,46). Therefore, we analysed the activity of CYP1A1 on *S. spinosa* extract treatment in ER positive (MCF-7) and ER negative (MDA-MB231) breast cancer cell lines (Fig. 5). In MCF-7 cells the crude MeOH extract weakly induced CYP1A1, whereas CYP1A1 was severely induced by F2. This effect was not observed in MDA-MB231 cells. CYP1A1 inhibition augments LPS-triggered fever, whereas induction of CYP1A1 controls fever and inhibits inflammation (47,48). This seems to depend on the expression of ER and suggests that this remedy is particularly effective in women and could explain its use against internal haemorrhaging during menstruation or after childbirth (14). CYPs are phase 1 enzymes and detoxify xenobiotics and contribute to drug clearance but they also activate pro-carcinogens. Therefore, it has to be considered that the intake of this remedy can cause unwanted interactions with other drugs or environmental (nutritional) compounds.

Conclusion. Among different extracts of increasing polarity the methanol extract of the rhizome of *S. spinosa* inhibited cell proliferation most significantly, which was associated with the induction of p21. The induction of the proto-oncogenes Cdc25A and cyclin D1 may have counteracted cell cycle arrest, yet they did not prevent it. Further fractionation of the methanol extract increased the apoptotic property (~2.5 fold at 60 μ g/ml), which correlated with the transient inactivation of the proto-oncogene Stat3 and the activation of caspase 9, followed by the induction of caspase 8 and 3. Overexpression of c-Jun did not abrogate, but most likely attenuated apoptosis. Reportedly, the mere overexpression of proto-oncogenes can trigger apoptosis when other co-operating side parameters are limited. The traditional use of 'Kokolmeka roja' for many generations proves that the intake of this remedy is safe and the healing properties prevail over potential adverse effects.

Although it is generally used against inflammatory ailments, in the present study we have shown that the rhizome of *S. spinosa* exhibits significant potential as anti-neoplastic concept and should therefore be tested *in vivo*.

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