An apolar extract of *Critonia morifolia* inhibits c-Myc, cyclin D1, Cdc25A, Cdc25B, Cdc25C and Akt and induces apoptosis

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Abstract. Investigating the bioactivity of traditional medical remedies under the controlled conditions of a laboratory is an option to find additional applications, novel formulations or lead structures for the development of new drugs. The present work analysed the anti-neoplastic activity of increasing polar extracts of the rainforest plant *Critonia morifolia* (Asteraceae) that has been successfully used as traditional remedy to treat various inflammatory conditions in the long-lasting medical tradition of the Central American Maya, which was here also confirmed *in vitro*. The apolar petroleum ether extract exhibited the most potent anti-proliferative and pro-apoptotic effects in HL-60 cells and triggered down-regulation of Cdc25C and cyclin D1 within 30 min followed by the inhibition of c-Myc expression and the onset of caspase-3 activation within 2 h. Subsequent to these very rapid molecular responses Chk2 and H2AX became phosphorylated (γ-H2AX) after 4 h. Analysis of the cell cycle distribution showed an accumulation of cells in the G2-M phase within 8 h and after 24 h in S-phase. This was temporally paralleled by the down-regulation of Cdc25A, Cdc25B, Wee1 and Akt. Therefore, the attenuation of cell cycle progression in the G2-M phase was consistent with the known role of Chk2 for G2-M arrest and with the role of Cdc25B in S-phase progression. These findings suggest the presence of two distinct active principles in the petroleum ether extract of *C. moriflia*. These facilitated the strong apoptotic response evidenced by the rapid activation of caspase-3 that was later enforced by the inhibition of the survival kinase Akt. Importantly, the efficient down-regulation of Akt, which is successfully tested in current clinical trials, is a unique property of *C. morifolia*.

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Introduction

Cancer is one of the leading causes of death worldwide. For 2010, the United States National Institute of Cancer estimates approximately 1.5 million new cases of cancer and more than 500,000 deaths from cancer in the USA (www.cancer.gov). Apart from avoidable risks such as smoking, the most important risk factor for cancer is supposed to be the increase in life expectancy, since most cancers occur in people over the age of 65. However, also younger adults and children are diagnosed with cancer. Thus, there exists a constant need for the development of novel anti-neoplastic agents. Natural products represent a vital resource for therapeutic principles. Like vincristine, more than 60% of all drugs used in Western medicine originate from natural sources including plants, microorganisms and marine organisms (1). Moreover, approximately 80% of the world's population relies on medical plants for their primary health care, especially in less-developed countries. As a result, numerous traditional healing plants successfully passed hundreds and even thousands of years of application in a variety of diseases. The big advantage of natural products is that they comprise a vast diversity of complex structures, whereas synthetic libraries typically show considerably less diversity (2). Although the probability to find new chemotypes from terrestrial plants is low (3) plant derived drugs have frequently shown novel mechanisms of action. Therefore, plant secondary metabolites need

to be investigated in bio-activity guided assays and also crude extracts that are potent *in vitro* should be tested *in vivo* at an early stage of lead compound discovery (4). The screening of natural products encompasses the high probability of duplication, i.e., the isolated active compound might be already known and thus cannot be patented (5). This and the problem of reliable access and supply make drug development from natural sources less attractive to pharmaceutical industries. Therefore, the study of healing- and other plants or organisms has remained with the academic institutions like universities, because they focus less on commercial profits. The traditional medicine of the Maya of Guatemala/Belize is based on the rich biodiversity of the rain forest, and is still practiced effectively to cure a variety of diseases. In the present work the ethno-medicinal plant *Critonia morifolia* (Asteraceae), which is still used as anti-inflammatory remedy, was selected for the investigation of its anti-neoplastic properties, because during inflammation and in cancer cells similar signalling pathways are commonly up-regulated. The genus *Critonia* (Asteraceae) comprises 43 species and is spread from Mexico to Argentina (6). *C. morifolia* grows as herbaceous shrub up to four meters tall and is found in forests and at the edges of forests, riversides and roadsides.

As a remedy the leaves are applied as steam bath in cases of swelling, retention of fluids, rheumatism, arthritis, paralysis, and muscle spasms. Baths of boiled leaves alone or in combination with other healing plants are used to cure skin conditions, wounds, insomnia, flu and aches. Boils, cysts, pus-filled sores and severe skin damages with uncontrolled wound tissue growth (cancer) are treated with leaves that were heated in oil prior to direct application on the swelling (7). Despite of its curative uses as home remedy only limited phytochemical and no pharmacological data were published so far and therefore, the data presented here are unprecedented for this plant.

Materials and methods

Antibodies. Antibodies against: Chk1 (2G1D5, no. 2360), phospho-Ser345 Chk1 (no. 2341), Chk2 (no. 2662), phospho-Thr68 Chk2 (no. 2661), cleaved Asp175 caspase-3 (no. 9661), Wee1 (no. 4936), phospho-Ser462 Wee1 (D47G5, no. 4910), phospho-Ser473 Akt (587F11, no. 4051), Akt (no. 9272) and phospho-Tyr15 Cdk1 (Cdc2, 10A11) were from Cell Signaling (Danvers, MA, USA), PARP-1 (F-2, sc-8007), Cdc25A (F-6, sc-7389), Cdc25B (C-20, sc-326), Cdc25C (C-20, sc-327), Cdk1 (Cdc2, p34, 17, sc-54), cyclin D1 (M-20, sc-718), p21 (C-19, sc-397) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), c-Myc Ab-2 (9E10.3, no. MS-139-P1) fromThermo 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), phosphor-Ser75 Cdc25A (ab47279) from Abcam (Cambridge, UK), and β-actin (AC-15, A5441) was from Sigma (St. Louis, MO, USA). The secondary antibodies peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG were purchased from Dako (Glostrup, Denmark).

Plant material. The aerial parts (leaves, caulis, florescence) of *C. morifolia* [vernacular names: Palo verde, YAAX (Itzá-Maya)] were collected in Guatemala, Departamento Petén, at the north-western shore of Lago Petén Itzá, San José, ~0.5 km NNE Zentrum from San José to La Nueva San José (16 59'16" N, 89 53'45" W). Voucher specimens (leg. G. Krupitza & R.O. Frisch, no. 8-2009 28. 04. 2009, Herbarium W, det. R.O. Frisch) were archived at the Museum of Natural History, Vienna, Austria.

Plant extraction by accelerated solvent extraction (ASE 2000). Lyophilized leaves (22.5 g) of *C. morifolia* were powdered and mixed 2:1 with diatomaceous earth, which acts as a dispersant and drying agent, prior to subjecting the sample to sequential extraction with five solvents of increasing polarity (petroleum ether, dichloromethane, ethyl acetate, methanol and water). The extractions were performed at 150 bar and 40˚C using an ASE 2000 accelerated solvent extractor and a solvent controller (Dionex, Sunnyvale, CA, USA). The scheme of each run of extraction was as follows: 5 min heating, 2 min static at 150 bar and 40˚C, 10 sec flushing and 2 cycles of purging for 60 sec. For each solvent cycle, the solid plant material that remained in the ASE cells after the extractions was flushed with the respective cold solvent (which was pooled accordingly) and dried with nitrogen gas. Then, gained extracts were subjected to rotary evaporation.

Extract yields and stock calculation. The extract weights obtained from serial extraction calculated for 1 mg lyophilized *C. morifolia* leaves are presented in Table I. To determine the anti-proliferative effect of the extracts, HL-60 cells were treated with increasing concentrations (5, 15, 30 and 60 μg/ml). The activity of the apolar extract was rather unstable and therefore, the dried extract was always prepared fresh before each experiment. For all other samples the DMSO concentration was adjusted to 0.5% (final concentration).

Cell culture. HL-60 (human promyelocytic leukemia cell) cells were purchased from American Type Culture Collection (ATCC). The cells were grown in RPMI-1640 medium which was supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% Glutamax and 1% penicillin-streptomycin. Both, medium and supplements were obtained from Life Technologies (Carlsbad, CA, USA). Human umbilical vein endothelial cells (HUVECs) were isolated and cultured in M199 medium supplemented with 20% fetal calf serum (FCS), antibiotics, endothelial cell growth supplement and heparin as previously described (8). The cells were kept at 37˚C in a humidified atmosphere containing 5% CO_2.

SELE (CD62E, E-selectin, ELAM)-induction assay. Each well of a 96-well plate was coated with gelatine by applying 200 μl of 1.0% gelatine for 10 min at room temperature. Outer wells (A1-A12, H1-H12, 1-H1 and A12-H12) contained only 200 μl/well medium and served as an evaporation barrier. HUVECs ($1x10^4$) were seeded in each of the other wells in 200 μl medium and grown for 48 h to optimal confluence. *Critonia* petroleum ether extract at 10, 15 and 20 μg/ml was added to the HUVEC-containing wells in triplicate, and the cells were incubated for 30 min, after which 10 ng/ml TNFα was added per well to stimulate NFκB, and thus SELE. After a further 4-h incubation, the levels of SELE in each of the HUVEC-containing wells were determined by enzyme-linked activity assays (ELISAs) as described below.

Table I. *C. morifolia* extract weights corresponding to lyophilized plant material and applied in proliferation assays.

Extract type	Extract weight (μg) corresponding to 1 mg dried plant	Extract final concentration (μg/ml medium)	Corresponding dried plant weight (μg)
Petroleum ether	59	5	84.7
		15	254.1
		30	508.2
		60	1016.4
Dichloromethane	28	5	178.6
		15	535.8
		30	1071.6
		60	2143.2
Ethyl acetate	14	5	357.1
		15	1071.3
		30	2142.6
		60	4285.2
Methanol	140	5	35.7
		15	107.1
		30	214.2
		60	428.4
Water	184	5	27.2
		15	81.5
		30	163.0
		60	326.0

Cell-surface ELISA SELE: Cells were washed once with PBS and fixed with 100 μl/well 25% glutaraldehyde [40 μl in 10 ml PBS, Sigma-Aldrich (Munich, Germany), stored at -20˚C in aliquots] for 15 min at room temperature. Then, cells were washed 3x with 200 μl per well PBS/0.05% Tween-20, blocked with 200 μl/well 5% BSA/PBS for 1 h, and washed again 3x with 200 μl per well PBS/0.05% Tween-20. Then, anti-SELE-antibody (clone BBA-1, R&D Systems, Minneapolis, MN, USA) diluted 1:5000 in 0.1% BSA/PBS (100 μl per well) was added for 1 h at room temperature and washed thereafter 5x with 200 μl per well PBS/0.05% Tween-20. Subsequently, goat anti mouse-HRP antibody (Sigma-Aldrich, Munich, Germany) diluted 1:10000 in 0.1% BSA/PBS (100 μl per well) was applied and the cells were incubated for a further 1 h in the dark at room temperature and, after decanting, washed five times with 200 μl per well PBS/0.05% Tween-20. The HRP-activity of the cells in each of the wells was estimated using Fast-OPD (o-phenylenediamine dihydrochloride) (Sigma-Aldrich, Munich, Germany) assay and absorbance was measured at OD_{492nm} in a vertical spectrophotometer.

Cytotoxicity testing: For the SELE expression assay the toxicity of the petroleum ether extract was assessed in HUVECs by Calcein AM cytotoxicity assays in 96-well microtitre plates. Portions (20 μl) of the extract concentrations were added in triplicate to the cells, which were then incubated at 37˚C in an atmosphere containing 5% CO_2 for 4 h, after which Calcein AM solution (Molecular Probes, Invitrogen, Karlsruhe, Germany) was added for 1 h according to the manufacturer's instructions. The fluorescence of viable cells was quantified using a Fluoroscan Ascent instrument (Labsystems, Finland) reader and on the basis of triplicate experiments the cytotoxic concentrations were calculated.

Proliferation assay. HL-60 cells were seeded in 24-well plates at a concentration of $1x10^5$ cells/ml allowing logarithmic growth within 48 h. Afterwards cells were incubated with increasing concentrations of plant extracts (5, 15, 30 and 60 μg/ml) for 48 h. After 24 and 48 h the cell number was counted using a KX-21 N microcell counter (Sysmex Corp., Kobe, Japan) and the percent of cell divisions compared to the untreated control were calculated as follows: $[(C_{48\ h\ +\ drug} - C_{24\ h\ +\ drug})/(C_{748\ -\ drug} - C_{24\ h\ -\ drug})]$ x 100 = % cell division, where $C_{48\ h\ +\ drug}$ is the cell number after 48 h of extract treatment, $C_{24\ h\ +\ drug}$ is the cell number after 24 h of extract treatment, $C_{48\ h\ -\ drug}$ and $C_{24\ h\ -\ drug}$ are the cell numbers after 48 and 24 h without extract treatment (9,10).

Apoptosis assay - Hoechst 33258 and propidium iodide double staining. Hoechst 33258 (HO) and propidium iodide (PI) double staining (Sigma, St. Louis, MO) allows the determination of the type of death the cell is undergoing, i.e., apoptosis (early or late)

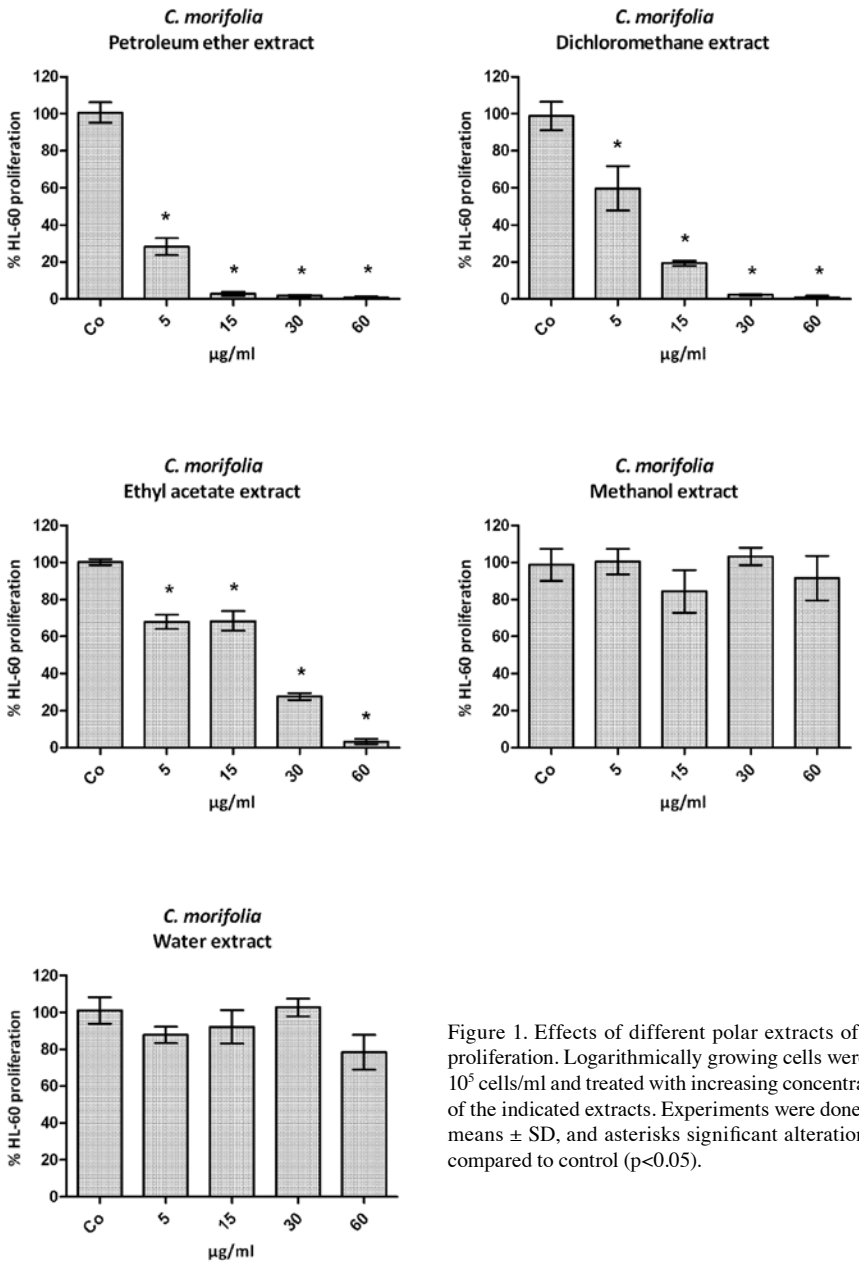

Figure 1. Effects of different polar extracts of *C. morifolia* on HL-60 cell proliferation. Logarithmically growing cells were seeded at a concentration of 10^5 cells/ml and treated with increasing concentrations (5, 15, 30 and 60 μg/ml) of the indicated extracts. Experiments were done in triplicate. Error bars show means ± SD, and asterisks significant alterations of proliferation in percent compared to control ($p<0.05$).

or necrosis (11,12). HL-60 cells were seeded in a 24-well plate at a concentration of $1x10^5$ cells/ml and treated with increasing concentrations of the specified extracts. After a 24-h incubation, 100 μl cell suspension of each well were transferred into separate wells of a 96-well plate and Hoechst 33285 and propidium iodide were added at final concentrations of 5 and 2 μg/ml, respectively. After a 1-h 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.

Cell cycle distribution analyses by fluorescence activated cell sorting (FACS). HL-60 cells were seeded in T-75 tissue culture flasks at a concentration of $2x10^5$ cells/ml and treated with 10 and 15 μg/ml of the petroleum ether extract of *C. morifolia*. After 8- and 24-h incubation at 37˚C cells were harvested, transferred into 15 ml tubes and centrifuged (4˚C, 800 rpm, 5 min) (13,14). The supernatant was discarded and the cell pellet washed with cold PBS (phosphate buffered saline, pH 7.2), centrifuged (4˚C, 800 rpm, 5 min), resuspended in 1 ml cold ethanol (70%), and either fixed for 30 min at 4˚C, or stored at -20˚C prior further handling. After two washing steps with cold PBS, the cell pellet was resuspended in 500 μl cold PBS and transferred into a 5-ml polystyrene round bottom tube. RNAse A and propidium iodide were added to a final concentration of 50 μg/ml and incubated for 1 h at 4˚C. The final cell number was adjusted between 0.5 and $1x10^6$ cells in 500 μl. Cells were analyzed by FACSCalibur flow cytometer (BD Bioscience, San Jose, CA, USA). Cell cycle distribution was calculated with ModFid LT software (Verity Software House, Topsham, ME, USA).

Western blotting. HL-60 were seeded in T-75 tissue culture flasks at a concentration of $1.8x10^5$ cells/ml and incubated with

15 μg/ml petroleum ether extract. Cells were harvested after 0.5, 2, 4, 8 and 24 h. Then, cells were washed twice with cold PBS and centrifuged at 1000 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, Schnelldorf, Germany). The lysate was centrifuged at 12000 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 sulfate) sample buffer and loaded onto a 10% polyacrylamide gel. Proteins were separated by polyacrylamide gel electrophoresis (PAGE) at 120 V. Thereafter proteins were electro-transferred onto a PVDF (polyvinylidene difluoride) membrane (Hybond, Amersham, UK) at 95 V 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). 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 ECL detection kit (Amersham, UK) and membranes were exposed to Amersham Hyperfilm.

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

Results

Apolar C. morifolia extracts inhibit HL-60 cell proliferation. Increasing concentrations of *C. morifolia* extracts were tested in logarithmically growing HL-60 cells. The inhibition of cell proliferation upon treatment with extracts is illustrated in Fig. 1. Petroleum ether extract (5 μg/ml) inhibited growth by >50%. The dichloromethane- and the ethyl acetate extracts showed a dose-dependent response however, both were less active than the petroleum ether extract. When taking the corresponding dried plant weight into account (Table I), the petroleum ether extract and the dichloromethane extract were the most effective ones. The methanol and the water extracts did not show strong effects on HL-60 proliferation. The petroleum ether extract, as the most active one, was studied in more detail by FACS analyses and western blotting to gain further insights into the underlying mechanisms of growth inhibition.

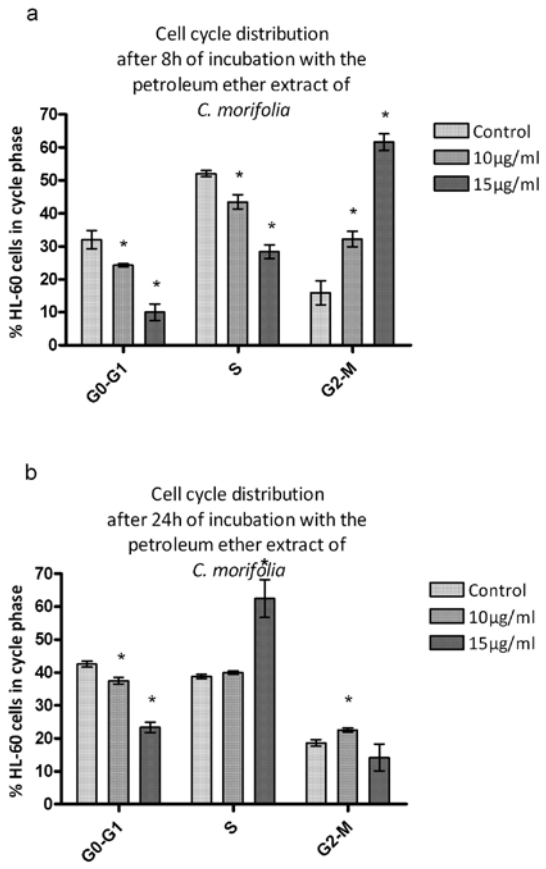

Figure 2. Analysis of cell cycle distribution in HL-60 cells. HL-60 cells ($1.8x10^5$ cells/ml) were incubated with 10 and 15 μg/ml of the petroleum ether extract of *C. morifolia*, harvested after (a) 8 h and (b) 24 h, and subjected to FACS analysis. Error bars indicate means ± SD, and asterisks significant alterations of cell distributions in the respective cell cycle phases compared to control ($p<0.05$). Experiments were performed in triplicate.

The petroleum ether extract induces G2/M and S-phase arrest in HL-60 cells. HL-60 cells were incubated with 10 and 15 μg/ml petroleum ether extract of *C. morifolia* for 8 and 24 h and then subjected to FACS analyses. Interestingly, the extract exhibited distinct effects depending on the duration of treatment. After 8 h, the cell population in G2-M phase increased from 15.9 to 32.3% (10 μg/ml) and up to 61.3% (15 μg/ml; Fig. 2a) upon extract treatment. The dose-dependent accumulation of HL-60 cells in G2-M was caused at the expense of both G0-G1 cells (from 32.0 to 24.2% and 10.0%) and S-phase cells (from 52.1 to 43.5% and 28.4%). By contrast, treatment with 15 μg/ml extract for 24 h resulted in S-phase accumulation of 62.4% of the cells (compared to 38.8% control cells; Fig. 2b) mainly at the expense of cells in G0-G1 phase, which was reduced from 42.6 to 23.3%. The incubation of HL-60 cells with 10 μg/ml extract exhibited only a slight shift of cell population from G0-G1 to G2-M whereas S-phase remained unchanged. Therefore, 15 μg/ml petroleum ether extract was applied in the following experiments.

Modulated expression of cell cycle regulatory proteins by petroleum ether extract treatment. The transient G2-M cell accumulation after 8 h and the subsequent arrest in S-phase caused by the petroleum ether extract were studied in further detail by investigating modulations of key proteins of cell cycle regulation. Prominent regulators of cell cycle transit are the

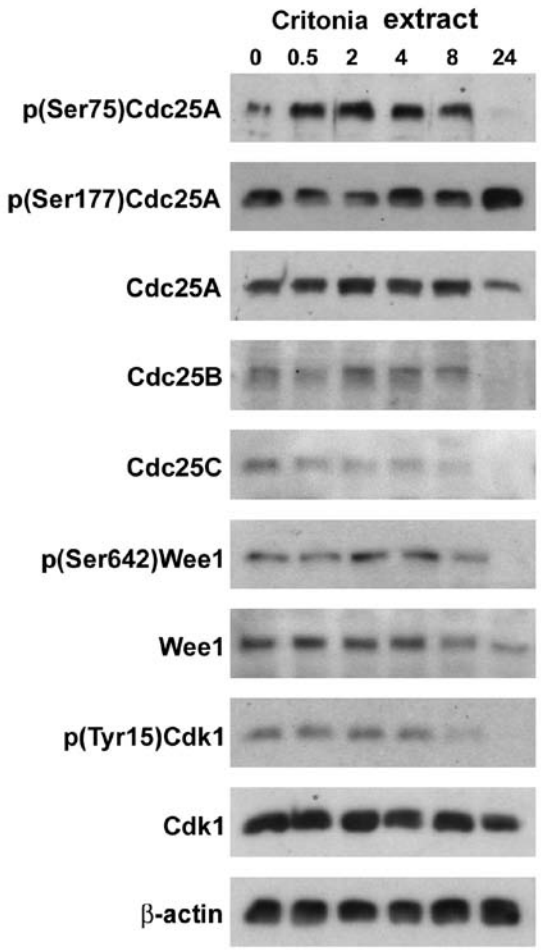

Figure 3. Alteration in the expression of cell cycle regulatory proteins in HL-60 cells. HL-60 cells ($1.8x10^5$ cells/ml) were treated with 15 μg/ml petroleum ether extract of *C. morifolia* and after 0.5, 2, 4, 8 and 24 h cells were harvested, protein isolated and subjected to western blot analysis using the indicated antibodies. Equal sample loading was confirmed by Ponceau S staining and β-actin analysis.

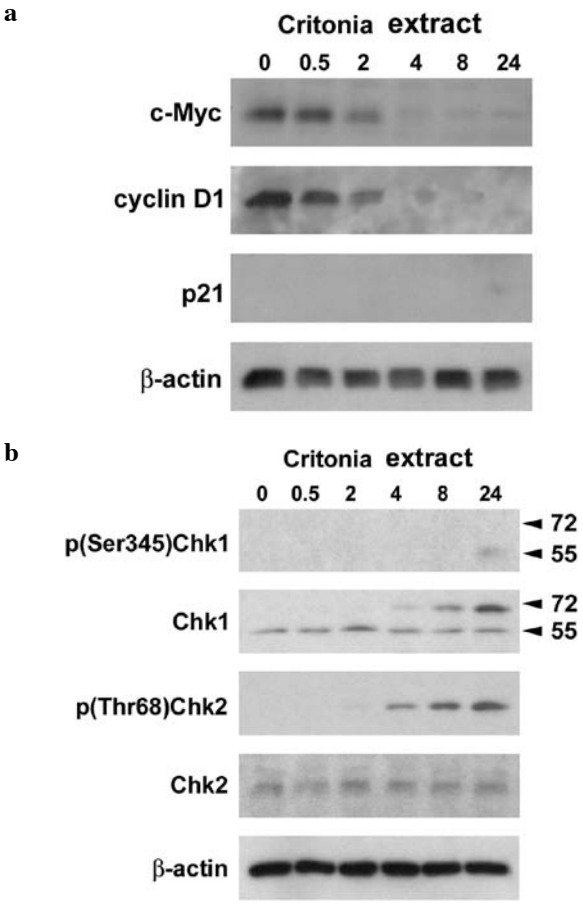

Figure 4. (a) Alteration in the expression of the proto-oncogenes c-Myc and cyclin D1 and the tumor suppressor p21 and (b) activation of checkpoint kinases 1 and 2 in HL-60 cells. HL-60 cells ($1.8x10^5$ cells/ml) were treated with 15 μg/ml petroleum ether extract of *C morifolia* and after 0.5, 2, 4, 8 and 24 h cells were harvested, protein isolated and subjected to western blot analysis using the indicated antibodies. After 4 h of treatment an additional band occurs above Chk1 at 72 kDa. This is presumably due to unidentified posttranslational modifications and it is not clear whether this activates Chk1. Equal sample loading was confirmed by Ponceau S staining and β-actin analysis.

Cdc25 phosphatases (Cdc25A, B and C) through the activation of cyclin-dependent kinases (Cdks) (15). Treatment with 15 μg/ml petroleum ether extract resulted in a transient up-regulation of the phosphatases Cdc25A and Cdc25B followed by a substantial down-regulation (Fig. 3). In contrast, Cdc25C levels continuously decreased and correlated with the G2-M arrest after 8 h. The entry of eukaryotic cells into mitosis is regulated by the phosphorylation level of Tyr15 of Cdk1 (16) through Wee1 and its antagonist Cdc25C. Phosphorylation of Cdk1 was reduced after 8 h and below detection after 24 h, which is indicative for its activation. The reduced phosphorylation level of Cdk1 was not due to Cdc25C activity but to inhibited Wee1 expression and this may have helped to overcome the initial G2-M arrest. Subsequently, the tilting of orchestrated cell cycle signalling and in particular the down-regulation of S-phase specific Cdc25B was most likely responsible for S-phase arrest after 24 h.

The petroleum ether extract inhibits proto-oncogene expression and induces DNA checkpoints. c-Myc has a profound impact on cell proliferation, differentiation and apoptosis and cyclin D1 promotes transition from G1 to S-phase. The up-regulation of the proto-oncogenes c-Myc and cyclin D1 is common for many tumour types and contributes to an abnormal proliferation rate (17,18). Incubation of HL-60 cells with 15 μg/ml petroleum ether extract caused a simultaneous repression of c-Myc- and cyclin D1 levels after 30 min which became undetectable during the time course (Fig. 4a). The down-regulation of cyclin D1 in cooperation with Cdc25B might have arrested HL-60 cells at the G1-S boundary. Furthermore, we investigated whether the p21 tumour suppressor became up-regulated, which is often the case upon treatment with cell cycle inhibitory agents. The main transcription factor for p21 is p53. Although HL-60 cells are p53 deficient the up-regulation of p21 can be achieved through other pathways (19). Nevertheless, the extract did not induce p21 expression.

The effect of the extract on proto-oncogenes and cell cycle regulators was severe and for c-Myc, cyclin D1 and Cdc25C it was also rapid. This tempted us to analyse whether DNA checkpoints became activated as a response to stressed DNA. When the DNA is damaged checkpoint kinases 1 and 2 (Chk1 and Chk2) are main mediators of cell cycle arrest to allow DNA repair prior to mitosis, or apoptosis in case of irreparable damage (20). Extract treatment did not change protein expression levels of

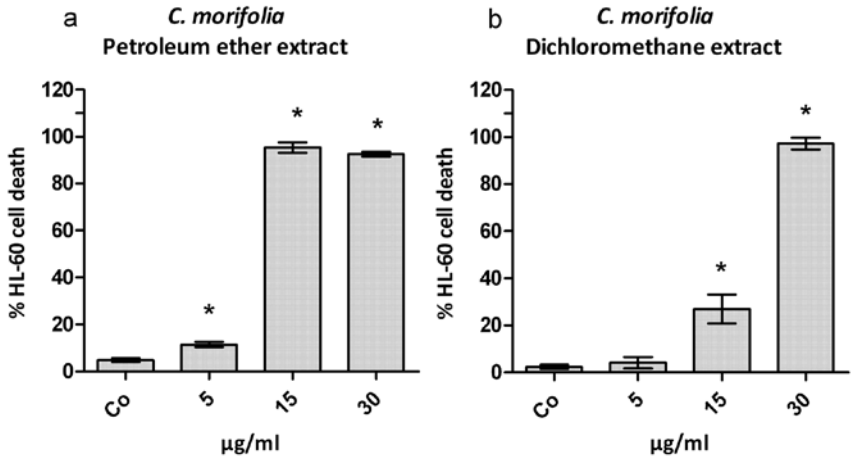

Figure 5. Induction of apoptosis in HL-60 cells. HL-60 cells (10^5 cells/ml) were seeded and allowed to grow for 24 h and then incubated with the indicated concentrations of (a) petroleum ether- and (b) dichloromethane extract for another 24 h. Afterwards cells were stained with propidium iodide and Hoechst 33258 and examined with a fluorescence microscope equipped with a DAPI filter to count apoptotic and viable cells. Experiments were performed in triplicate. Error bars indicate ±SD, and asterisks significant alterations (in percent) compared to control ($p<0.05$).

Chk1 and Chk2 during 24 h but it caused the phosphorylation of Chk2 at the activating Thr68 within 4 h and the phosphorylation level further increased within 24 h (Fig. 4b). This correlated with the accumulation of cells in S-phase at this time-point which is consistent with the role of Chk2 in S-phase arrest (21,22). Activated Chk2 was reported to phosphorylate the Cdc25A phosphatase on Ser177 targeting it for proteasomal degradation (23), as observed after 24 h of drug treatment (Fig. 3). Chk1 typically induces G2-M arrest (24) and extract treatment induced the phosphorylation of Chk1 at the activating Ser345 after 24 h. However, this did not correlate with the observed increase of cells in G2-M after 8 h of treatment.

Apolar C. morifolia extracts induce apoptosis of HL-60 cells. The activation of DNA checkpoints provides time to repair the damage or in case the machinery is overstretched the cell undergoes apoptosis. Therefore, we investigated whether extract- treated cells survive or die. Based on their cell cycle-inhibitory properties the two most potent apolar extracts (petroleum ether- and the dichloromethane extract) were tested regarding their pro-apoptotic potential and HL-60 cells were subjected to increasing extract concentrations for 24 h. Then, propidium iodide and Hoechst 33258 were added to the cells facilitating the distinction between viable, apoptotic and necrotic cells via visual examination. Petroleum ether extract (5 μg/ml) caused a slight induction of apoptosis in HL-60 and 15 μg/ml triggered signs of early apoptosis in >90% of the cells (Fig. 5). This was also achieved with 30 μg/ml of the dichloromethane extract and therefore, this extract was approximately half as active as the petroleum ether extract. Hence, the petroleum ether extract was further studied by western blot analyses. The petroleum ether extract activated caspase-3 indicated by its cleavage into a 17-kDa fragment within 2 h. Caspase-3 is the major executioner of apoptosis and proteolytically degrades target proteins (25) such as poly(ADP-ribose) polymerase (PARP), which is a 116-kDa nuclear enzyme, into a 85-kDa and an (invisible) 31-kDa signature type fragment. Thus, cleavage of PARP serves as a

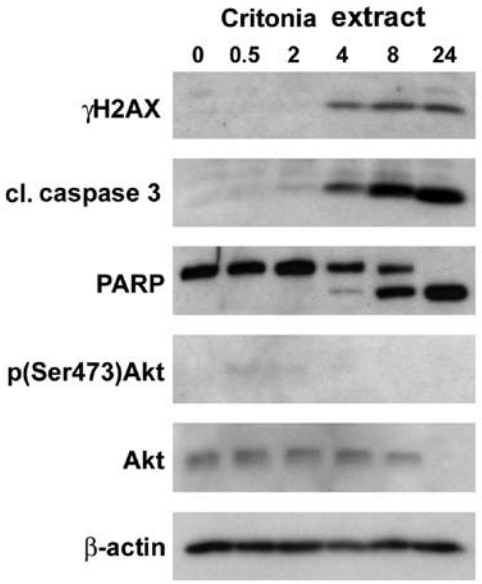

Figure 6. Induction of pro- and anti-apoptotic key proteins in HL-60 cells. HL-60 cells ($1.8x10^5$ cells/ml) were treated with 15 μg/ml petroleum ether extract of *C. morifolia* and after 0.5, 2, 4, 8 and 24 h cells were harvested, proteins isolated and subjected to western blot analysis using the indicated antibodies. Equal sample loading was confirmed by Ponceau S staining and β-actin analysis.

marker of cells undergoing apoptosis which was observed after 4 h of treatment. Also the DNA becomes degraded, because caspase-3 cleaves and activates also the DNAse CAD. This leads to DNA double strand incisions, which are sensed by ATM, triggering the Ser139 phosphorylation of H2AX (which is called γ-H2AX in the phosphorylated form) within minutes (26). Here we detected γ-H2AX after 4 h of treatment, which was subsequent to caspase-3 activation. Furthermore, we studied the expression of Akt, which plays a critical role in cell survival and is successfully tested as target in clinical trials [(27); http://clinicaltrials.gov/)]. This protein kinase

Table II. Selectin E (SELE) expression in TNFα-induced HUVECs.

Analysis		Control	TNFα	TNFα and **10 μg/ml *Cr*-E**	TNFα and **15 μg/ml *Cr*-E**	TNFα and **20 μg/ml *Cr*-E**
Inflammatory reaction	SELE (OD)	**0.056** SD 0.001	**0.136** SD 0.003	**0.123** SD 0.013	**0.106** SD 0.001	**0.106** SD 0.024
Cytotoxicity	Calcein AM (OD)	49.9 SD 5.8	47.3 SD 8.7	46.5 SD 6.1	43.7 SD 5.3	41.5 SD 7.7

HUVECs/well ($1x10^4$) were seeded into 96-well plates and grown to confluence. Indicated concentrations of *C. morifolia* petroleum ether extract (*Cr*-E) were added 1 h prior to application of 10 ng/ml TNFα for another 4 h. Then cells were fixed and SELE levels analysed by ELISA. In parallel, extracts were analysed by Calcein AM assay to monitor non-specific extract toxicity.

is an important downstream effector of the PI3K- and the mTOR pathway preventing apoptosis when activated by phosphorylation at the serine 473 residue (28,29). In petroleum ether extract-treated HL-60 cells Akt did not become phosphorylated at Ser473 (Fig. 6) and the protein became down-regulated within 8 h and was undetectable after 24 h. This certainly supported the apoptotic process initiated by caspase-3 activation and therefore the petroleum ether extract of *C. morifolia* contains a unique property blocking Akt expression.

Analysis of inflammatory response. C. morifolia was chosen because of its anti-inflammatory applications in the Maya traditional medicine. Here we show that the petroleum ether extract dose-dependently suppressed TNFα-induced SELE induction in HUVEC's (Table II), which supports the empirical observations of herbalists. Interestingly, the cytotoxic methanol extract of another anti-inflammatory remedy prepared of the rhizome of *Smilax spinosa* did not inhibit SELE induction (data not shown).

Discussion

Based on the traditional uses in Mayan medicine, an ethnomedical plant from Central America, *C. morifolia*, was investigated regarding its anti-carcinogenic activity. Conditions treated by extracts of the aerial parts of this plant range from parasitic ailments to various types of inflammation. Here, the latter was confirmed as SELE induction was inhibited. Oily preparations of *C. morifolia* leaves, which are enriched in apolar plant constituents are directly applied on severe skin damages with uncontrolled wound tissue growth (which is called cancer by the local population) (7). Despite its manifold curative usage in folk medicine, only little pharmacological research has been published on *C. morifolia*. Various sesquiterpene lactones and pyrrolizidine alkaloids (6), including a unique one named morifoline, were isolated (30) and we show that apolar, yet unidentified compounds of *C. morifolia* caused the degradation of cell cycle regulators and the down-regulation of the proto-oncogenes cyclin D1 and c-Myc. Cyclin D1 and c-Myc play an important role in the development of various cancer types (17,18). D-cyclins bind to Cdk4 and Cdk6, thereby promoting the transition from G1 into S-phase (31,32). In addition, cyclin D1 regulates and associates with transcription factors in a Cdk-independent manner. Hence, the repression of cyclin D1 would suggest a cell cycle inhibition in G1 phase, which is in contrast with both the G2-M and the S-phase arrest observed upon extract treatment after 8 and 24 h, respectively. Therefore, the extract blocked also another cell cycle regulatory mechanism which prevailed over the expected effect of cyclin D1 suppression.

Cdc25 dual specificity phosphatases are another mechanism regulating cell cycle transition. Whereas Cdc25A controls both the transit from G1 to S phase as well as that through G2-M phase (33,34), Cdc25B seems to promote the entry from S phase into G2-M (35,36). Cdc25C enables G2-M transit that can be compensated by Cdc25A (37), and recent studies suggest an undefined level of functional redundancy among Cdc25 phosphatases (34). The G2-M arrest observed after 8 h of treatment correlated with the down-regulation of Cdc25C, which (such as Cdc25A) regulates G2-M transition via activation of Cdk1 by de-phosphorylating Tyr15 (15,16). Antagonizing the de-phosporylation, Wee1 kinase inhibits entry into mitosis by the phosphorylation of Cdk1 at the tyrosine 15 site (38). Wee1 phosphorylation at serine 642 leads to the nuclear-cytoplasmatic translocation and thus, to its inactivation since Wee1 becomes separated from its target. Noteworthy, after more than 8 h also Wee1 was repressed and hence, Cdk1 was maintained in an activated state and this may have enabled progression into S-phase. In S-phase, cells were arrested a second time, because meanwhile Cdc25B became degraded and Chk2 strongly activated (39). The activity of Chk1 and Chk2 was reflected by the increased phosphorylation levels of Ser75 and Ser177 of Cdc25A, respectively (23,40). The temporally separated effects on cell cycle inhibition were probably due to distinct anti-neoplastic compounds present in the petroleum ether extract.

Careful interpretation of the temporal succession of the observed effects (inhibition of cell cycle progression, repression of proto-oncogenes, and induction of apoptosis) suggested that the extract activity did not involve genotoxicity, because genotoxic events should have activated Chks first and then caspase-3 activation may follow. However, caspase-3 was induced before the activation of Chk2 implicating that the extract was not damaging the DNA directly.

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