[Frontiers in Bioscience E3, 1326-1336, June 1, 2011]

Separation of anti-neoplastic activities by fractionation of a *Pluchea odorata* extract

Sabine Bauer[1], Judith Singhuber[2], Mareike Seelinger[1], Christine Unger[1], Katharina Viola[1], Caroline Vonach[1], Benedikt Giessrigl[1], Sibylle Madlener[1], Nicole Stark[1], Bruno Wallnofer[3], Karl-Heinz Wagner[4], Monika Fritzer-Szekeres[5], Thomas Szekeres[5], Rene Diaz[6], Foster Tut[6], Richard Frisch[6], Bjorn Feistel[7], Brigitte Kopp[2], Georg Krupitza[1], Ruxandra Popescu[2]

[1]Institute of Clinical Pathology, Medical University of Vienna, Waehringer Guertel 18-20, Austria, [2]Department of Pharmacognosy, Faculty of Life Sciences, University of Vienna, Althanstrasse 14, Austria, [3]Department of Botany, Museum of Natural History, Burgring 7, A-1010 Vienna, Austria, [4]Department of Nutritional Sciences, University of Vienna, Althanstrasse 14, Austria, [5]Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Vienna, Waehringer Guertel 18-20, Austria, [6]Institute for Ethnobiology, Playa Diana, San Jose/Peten, Guatemala, [7]Finzelberg GmbH & Co. KG, Koblenzer Strasse 48-54, D-56626 Andernach, Germany

TABLE OF CONTENTS

1. ABSTRACT

Natural products continue to represent the main source for therapeutics, and ethnopharmacological remedies from high biodiversity regions are a rich source for the development of novel drugs. Hence, in our attempt to find new anti-neoplastic activities we focused on ethno-medicinal plants of the Maya, who live in the world's third richest area in vascular plant species. *Pluchea odorata* (Asteraceae) is traditionally used for the treatment of various inflammatory disorders and recently, the *in vitro* anti-cancer activities of different extracts of this plant were described. Here, we present the results of bioassay-guided fractionations of the dichloromethane extract of *P. odorata* that aimed to enrich the active principles. The separation resulted in fractions which showed the dissociation of two distinct anti-neoplastic mechanisms; firstly, a genotoxic effect that was accompanied by tubulin polymerization, cell cycle arrest, and apoptosis (fraction F2/11), and secondly, an effect that interfered with the orchestrated expression of Cyclin D1, Cdc25A, and Cdc2 and that also led to cell cycle arrest and apoptosis (fraction F3/4). Thus, the elimination of generally toxic properties and beyond that the development of active principles of *P. odorata*, which disturb cancer cell cycle progression, are of interest for potential future therapeutic concepts against proliferative diseases.

2. INTRODUCTION

The majority of medicinal drugs used in western medicine are derived from natural products (1, 2). A success story in natural product drug discovery is paclitaxel (Taxol), which is derived from the bark of the Pacific Yew, *Taxus brevifolia* Nutt. (Taxaceae). The antitumor activity of Taxol is based on its ability to stabilize microtubules in tumor cells, triggering mitotic arrest and cell death (3-5). Several Native American tribes have used *Taxus* species for the treatment of non-cancerous diseases (6). Ethno-pharmacological remedies, particularly from high biodiversity regions such as rainforests, can be a rich source for the development of novel drugs (7) and therefore, we investigate traditional healing plants of the Maya who live in a region which is the world's third richest in vascular plant species (8). Over the centuries and millennia, the Maya developed an advanced pharmaceutical knowledge that is still practiced today. In the attempt to find plants with anti-neoplastic activities we select those traditionally used against severe inflammations, because there are several similar signaling pathways, which are commonly up-regulated in both, in inflammatory conditions and in cancer (9). Maya healers prepare decoctions of the Asteraceae *Pluchea odorata* (L.) Cass. (Itza-Maya vernacular name: "Chal Che"), to treat coughs, cold, neuritis, and arthritis and also swelling, bruises,

Table 1. Fractionation of the *Pluchea odorata* CH_2Cl_2 crude extract by VLC

F1-Fractions	Mobile phase (1000 ml)	Yield (g)
F1/1	PE	0.21
F1/2	$CHCl_3$	1.36
F1/3	$CHCl_3$: MeOH (9:1)	**1.46**
F1/4	$CHCl_3$: MeOH (7:3)	0.45
F1/5	$CHCl_3$: MeOH (5:5)	0.09
F1/6	$CHCl_3$: MeOH (3:7)	0.14
F1/7	$CHCl_3$: MeOH (1:9)	0.09
F1/8	MeOH : H_2O (7:3)	0.08
F1/9	MeOH : H_2O (1:1)	0.05
F1/10	H_2O	0.02

Fractionation of the *Pluchea odorata* CH_2Cl_2 crude extract by VLC. The CH_2Cl_2 extract was chromatographed on a silica gel column using the indicated solvents as mobile phase, which resulted in 10 fractions (F1/1 – F1/10).

Table 2. Fractionation of the *Pluchea odorata* F1/3 extract by column chromatography (CC-I)

F2-Fractions	Mobile phase	Yield (mg)	Yield w/o chlorophyll	
			(mg)	(%)
F2/1	$CHCl_3$ (500 ml)	32.5		
F2/2		30.5		
F2/3		12.2		
F2/4		54.2		
F2/5		17.8		
F2/6	$CHCl_3$:MeOH:H_2O (95:1.5:0.1) (600 ml)	26.4		
F2/7		67.2		
F2/8		24.8		
F2/9		48.5		
F2/10		32.9		
F2/11		113.2	25.7	22.7
F2/12	$CHCl_3$:MeOH:H_2O (90:3.5:0.2) (562 ml)	90.7	16.3	17.9
F2/13		170.6	**41.2**	24.2
F2/14		88.6	27.9	31.5
F2/15		26.3	2.4	9.1
F2/16		148.5	21.6	14.5
F2/17		44.1		
F2/18	$CHCl_3$:MeOH:H_2O (85:8.0:0.5) (2000 ml)	64.9		
F2/19		184.6		
F2/20		268.0		
F2/21	MeOH:H_2O (95:5.0) (500 ml)	213.0		

Fractionation of the *Pluchea odorata* F1/3 extract by column chromatography (CC-I). The extract was applied on a silica gel column and eluted with the indicated solvents and 21 main fractions (F2/1 – F2/21) were obtained. Chlorophyll was removed from fractions F2/11-16 and the fraction-yield (mg, %) before and after chlorophyll separation was calculated.

inflammations, and tumors (10). Recently, the anti-cancer activity of extracts of this medicinal herb was described (11). Here, we focused on the dichloromethane extract of *P. odorata* and performed bioassay-guided fractionations to separate and enrich different bioactive principles.

3. MATERIALS AND METHODS

3.1. Plant material

Pluchea odorata (L.) Cass. was collected in Guatemala, Departamento Peten, near the north-western shore of Lago Peten Itza, San Jose, within an area of four year old secondary vegetation ~1 km north of the road from San Jose to La Nueva San Jose (16°59'30" N, 89°54'00" W). Voucher specimens (leg. G. Krupitza & R. O. Frisch, Nr. 1-2009, 08. 04. 2009, Herbarium W) were archived at the Museum of Natural History, Vienna, Austria. The fresh plant material (the aerial plant parts, leaves, caulis and florescence) of *P. odorata* was stored deep-frozen until lyophilization and subsequent extraction.

3.2. Extraction and fractionation

Aerial plant parts of *P. odorata* were lyophilised, ground and 192 g were taken for extraction using an accelerated solvent extractor (ASE) (ASE® 200, Dionex, California, USA). The first cycle was performed with PE in order to partly eliminate chlorophyll and lipids. Then, the same plant material was extracted x 3 with CH_2Cl_2. The extraction was performed with a pressure of 150 bar and at 40°C. The CH_2Cl_2 extract was evaporated under reduced pressure to yield 4.0 g dried extract. The crude CH_2Cl_2 extract was subjected to vacuum liquid chromatography (VLC) on a silica gel column, eluting with a stepwise gradient from PE to H_2O (Table 1) to provide ten main fractions (F1/1 – F1/10) which were collected based on similar TLC profiles. Fraction F1/3 (1.46 g) was further chromatographed on a silica gel column (CC-I) using a stepwise gradient from $CHCl_3$ to MeOH : H_2O for elution (Table 2) and led to the collection of 21 main fractions (F2/1 – F2/21). Chlorophyll was separated from fractions F2/11 – F2/16 by redissolving the dried fractions in CH_2Cl_2 (1 g fraction / 150 ml CH_2Cl_2) and adding an equal volume of MeOH : H_2O (1 : 1). Then CH_2Cl_2 was evaporated under reduced pressure to precipitate chlorophyll in the MeOH : H_2O phase. Chlorophyll was removed by filtration and the chlorophyll-free MeOH : H_2O layer was dried under reduced pressure. After the removal of chlorophyll, fraction F2/13 (30 mg) was purified on a silica gel column (CC-II) eluting with $CHCl_3$: MeOH in different ratios (Table 3). Fractions with similar TLC profiles were pooled to give five main fractions (F3/1 – F3/5).

Table 3. Fractionation of the *Pluchea odorata* F2/13 extract by column chromatography (CC-II)

F3-Fractions	Mobile phase	Yield (mg)
F3/1	$CHCl_3$: MeOH (95:0.5) (700 ml)	3.41
F3/2		13.03
F3/3		7.87
F3/4	$CHCl_3$: MeOH (95:0.5) (300 ml)	**3.58**
F3/5	$CHCl_3$: MeOH (90:0.5) (300 ml)	21.32

Fractionation of the *Pluchea odorata* F2/13 extract by column chromatography (CC-II). The extract was chromatographed on a silica gel column using the indicated solvents as mobile phase; the separation resulted in 5 main fractions (F3/1 – F3/5).

3.3. Cell Culture

HL-60 promyelocytic leukaemia cells were purchased from ATCC and grown in RPMI 1640 medium and humidified atmosphere containing 5% CO_2 at 37°C. The medium was supplemented with 10 % heat-inactivated fetal calf serum (FCS), 1 % Glutamax and 1 % Penicillin-Streptomycin. The medium and supplements were obtained from Life Technologies (Carlsbad, CA, USA).

3.4. Proliferation inhibition analysis

HL-60 cells were seeded in T-25 tissue culture flasks or in 24-well plates at a concentration of 1 x 10^5 cells/ml and incubated with increasing concentrations of plant extracts or fractions. Cell counts and IC_{50} values were determined within 24 h using a KX-21 N microcell counter (Sysmex Corporation, Kobe, Japan). All experiments were performed in triplicate. Cell proliferation rates were calculated as described (11-13).

3.5. Cell death analysis

In order to determine the type of cell death, HL-60 cells were seeded in 24-well plates at a concentration of 1 x 10^5 cells/ml and grown for 24 h. Then cells were treated with the indicated concentrations of the extract and fractions for 8 h and 24 h. Hoechst 33258 and propidium iodide were added to the cells at final concentrations of 5 and 2 µg/ml, respectively. After 1 h of incubation at 37°C, cells were examined on a Zeiss Axiovert fluorescence microscope equipped with a DAPI filter. Cells were photographed and analyzed by visual examination to distinguish between apoptosis and necrosis (14-16). For this, cells were judged according to their morphology and the integrity of the plasma membrane on the basis of propidium iodide exclusion. Experiments were performed in triplicate.

3.6. Western blot analysis

HL-60 cells were seeded in T-75 tissue culture flasks at a concentration of 1 x 10^6 cells/ml and incubated with 3 µg/ml fractions (F2/11, F2/13, F3/4, respectively) for 0.5 h, 2 h, 4 h, 8 h and 24 h. At each time point, 2 x 10^6 cells were harvested, placed on ice, centrifuged (1000 rpm, 4 ºC, 4 min), washed twice with cold PBS (pH 7.2), and lysed in 150 µl buffer containing 150 mM NaCl, 50 mM Tris pH 8.0, 1 % Triton X-100, 1mM phenylmethylsulfonylfluorid (PMSF) and Protease Inhibitor Cocktail (Sigma, Schnelldorf, Germany). Debris was removed by centrifugation (12,000 rpm, 4 ºC, 20 min) and equal amounts of total protein were electrophoretically separated by SDS polyacrylamide gels (10 %) and then transferred to PVDF membranes (Hybond P, Amersham, Buckinghamshire, UK) at 100 V and 4ºC for 1 h. To confirm equal sample loading, membranes were stained with Ponceau S (17-19). Customary blotting protocol was employed; primary antibodies were diluted 1:500 in blocking solution and incubated with the membrane at 4 ºC, overnight and the secondary antibodies were diluted 1:2000. Blots were analyzed using an enhanced chemoluminescence technique (ECL detection kit) and detected by exposure of the membranes to Amersham Hyperfilm™ (both Amersham, Buckinghamshire, UK). The antibody against Phospho-Cdc25A (S75) was from Abcam (Cambridge, MA, USA) and against phospho-Cdc25A (S177) from Abgent (San Diego, CA, USA). Anti-gamma-H2AX (pSer139) was purchased from Calbiochem (San Diego, CA, USA) and the antibodies against cleaved caspase-3 (Asp175), Chk2, phospho-Chk2 and phospho-Cdc2 (Tyr15) from Cell Signaling (Danvers, MA, USA). The antibodies against Cdc2 p34 (17), Cdc25A (F-6), Cyclin D1 (M-20), PARP-1 (F-2) and alpha-tubulin (DM1A) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and the antibodies against beta-actin (clone 6-11B-1) and acetylated alpha-tubulin (clone 6-11B-1) 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).

3.7. Statistical analysis

The apoptosis and proliferation experiments were analyzed by t-test using GraphPad Prism version 4 (GraphPad Prim Sofware, Inc., San Diego, CA, USA).

4. RESULTS AND DISCUSSION

4.1. Anti-proliferative activity of *Pluchea odorata* CH_2Cl_2 crude extract and F1 (VLC) fractions

The activity of the obtained CH_2Cl_2 crude extract, which was 2.1 % of the dried plant material input (196 g), was tested in HL-60 leukemia cells. Cells were incubated with increasing concentrations of crude extract (1-15 µg/ml) and the number of cells was counted twice within a time span of 24 h in order to calculate the proliferation rates. The CH_2Cl_2 crude extract significantly decreased the proliferation rate of HL-60 cells; the concentration which inhibited cell proliferation by 50 % (IC_{50}) was ~10 µg/ml (Figure 1). Subsequently, the crude extract was fractionated by VLC resulting in fraction F1/1 – F1/10 (Table 1). All fractions were tested at concentrations of 10µg/ml. The results showed that fraction F1/3, which represented 36.5 % of the CH_2Cl_2 extract input (4.0 g), inhibited proliferation by ~ 60 %, whereas the other fractions had no effect on cell growth (data not shown). Therefore, the gain of activity was not significant and VLC was an insufficient procedure to enrich the active principles. Based on these results, subsequent fractionation of F1/3 by CC-I followed.

4.2. Anti-proliferative activity of F2 (CC-I) fractions derived from F1/3

Fraction F1/3 was further subjected to CC-I to provide 21 main fractions (Table 2). The anti-proliferative

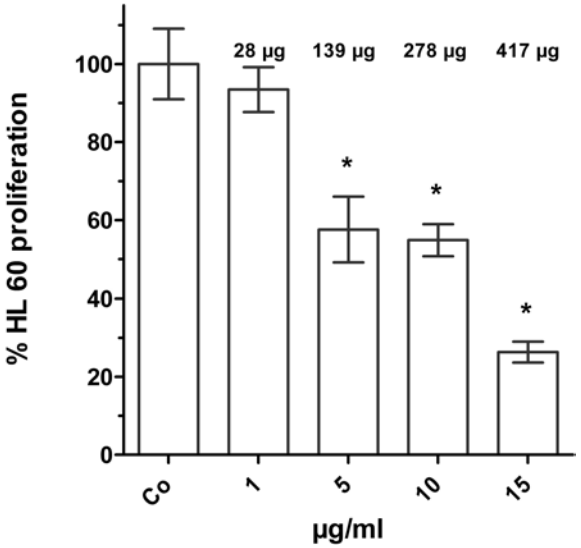

Figure 1. Anti-proliferative effects of *Pluchea odorata* CH_2Cl_2 crude extract. HL60 cells were seeded into T-25 flasks (1 x 10^5 cells/ml), grown for 24 hours, and treated with solvent (0.5 % EtOH) or the specified concentrations of CH_2Cl_2 extract. After 24 hours the proliferation was calculated as percentage of control. Values are mean ± SEM of experiments performed in triplicate. *$p<0.05$ as compared to untreated control.

activity of the fractions (10 µg/ml) was determined in HL-60 cells (Figure 2a). Fractions F2/11 – F2/16 showed effective growth inhibitory activity; fractions F2/11, F2/12, F2/13 and F2/15 inhibited cell growth up to nearly 100 %, F2/16 up to 95 % and F2/14 up to 80 %. Hence, CC-I facilitated the enrichment of the bioactive properties. The TLC profile of the active fractions F2/11 – F2/16 indicated the presence of chlorophyll (data not shown). In order to exclude the possibility that chlorophyll contributed to the anti-proliferative effect, fractions F2/11 – F2/16 were subjected to separation of chlorophyll and then re-evaluated for activity. The results indicated that the anti-proliferative activity was preserved in the chlorophyll-free fractions (Figure 2b). Moreover, the effect of fraction F2/14 was increased after the removal of chlorophyll.

4.3. Anti-proliferative activity of F3 (CC-II) fractions derived from F2/13

Since CC-I fractionation was successful in enriching the anti-proliferative activity, we then selected one of the most active chlorophyll-free fractions for further fractionation. Fraction F2/13 contained the least restrictive amount of material, which was 2.8 % of the F1/3 input (1.46 g). Hence, fraction F2/13 was subjected to a second step CC separation (CC-II). Based on similarities of the TLC profile, five main fractions (F3/1 – F3/5) were obtained (Table 3). In order to determine the anti-proliferative effect, HL-60 cells were treated with the indicated concentrations of fractions (Figure 3). The results suggested fraction F3/4 to be the most potent of the F3 fractions. Fraction F3/4 inhibited proliferation with a calculated IC_{50} of ~0.4 µg/ml; therefore, the increase of the activity compared to the crude extract was ~25-fold.

Additional separations of F3 fractions with reversed phase solid phase extraction resulted in decreased bio-activities (data not shown). Therefore, these fractions seemed to separate different active principles that were additive in F3/4. This evidences that controlled multi-compound preparations of plant extracts, such as F3/4 or i.e. Avemar (20), can be more effective than isolated single compounds. The attempt to identify these active principles would have exceeded the frame of this investigation.

4.4. Western blot analysis of cell cycle and checkpoint regulators

Fractions F2/11, F2/13 and F3/4 showed the highest anti-proliferative activity. Hence, their effect on the expression of cell cycle regulatory proteins was analyzed by Western blotting, because protagonists such as the proto-oncogenes Cyclin D1 and Cdc25A, which are both up-regulated in hyper-proliferative diseases, are goals for new anti-neoplastic therapies (21, 22). The lowest common concentration of fractions F2/11, F2/13 and F3/4, which completely inhibited HL-60 cell proliferation, was 3 µg/ml and therefore, the following analyses were performed with this concentration. Fraction F2/11 suppressed Cyclin D1 expression after 24 h, whereas F2/13 reduced the Cyclin D1 level after 8 h and its derivative fraction F3/4 already after 30 minutes (Figure 4). Temporally the D-family of cyclins appears in early G1 of the cell cycle (23-25) and Cyclin D1 is required for the activation of Cdk4 and Cdk6 (21, 26) and it is also known as the Prad1 proto-oncogene (27).

The intra-S-phase checkpoint prevents the duplication of damaged or broken DNA which would eventually lead to genomic instability. This checkpoint is i.e. regulated by ATM/ATR-Chk2-Chk1-Cdc25A (28). Depending on the type of DNA damage (genotoxic stress), ATM or ATR phosphorylates Chk2 or Chk1, which in turn phosphorylates Cdc25A (29, 30). Thereby, Cdc25A becomes inactivated and causes the inhibition of Cdk2 and Cdc2 (31). Here we demonstrate that Chk2 was phosphorylated at the activating Thr68 site upon treatment with all three tested fractions. F2/11 caused a rapid and transient phosphorylation of Chk2 within 2 h, which returned to constitutive levels after 24 h. In contrast, fraction F2/13 induced phosphorylation of Chk2 after 24 h and fraction F3/4 after 8 h which sustained for 24 h. Therefore, activation of Chk2 by F3/4 was not transient and was caused by a different trigger than by F2/11. Chk2 protein levels remained unchanged upon incubation with F2/13 and F3/4, but the level decreased upon incubation with F2/11 after 24 h (Figure 4). The analysis of Chk2 phosphorylation- and protein levels supported the notion that different active principles are contained in F2/11 compared to F2/13 and its derivative F3/4. The activation of Chk2 by F2/11 was the earliest effect observed in this protein expression study, whereas it was the latest event upon treatment with F2/13 and F3/4. The rapid Chk2 induction indicated that F2/11 caused DNA damage, which was supported by the fact that the phosphorylation of H2AX (gamma-H2AX) was induced even before Chk2-activation (Figure 6) and that the subsequent alterations of gene expression and cellular responses were most likely the consequences of this property. The induction of gamma-

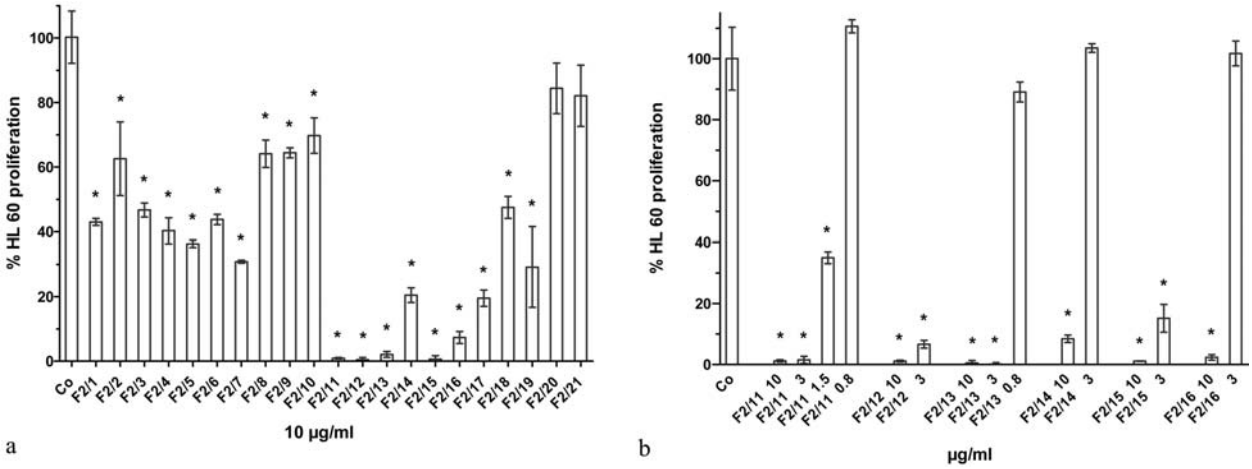

Figure 2. Anti-proliferative effects of *Pluchea odorata* fractions F2/1 – F2/21. HL60 cells were seeded into 24-well plates (1 x 10^5 cells/ml) and grown for 24 hours, and incubated with solvent (0.5 % DMSO) or (a) 10 µg/ml fraction F2/1 – F2/21 and (b) the specified concentrations of chlorophyll-free fraction F2/11 – F2/16. After 24 hours the proliferation was calculated as percentage of control. Values are mean ± SEM of experiments performed in triplicate. *p<0.05 as compared to untreated control.

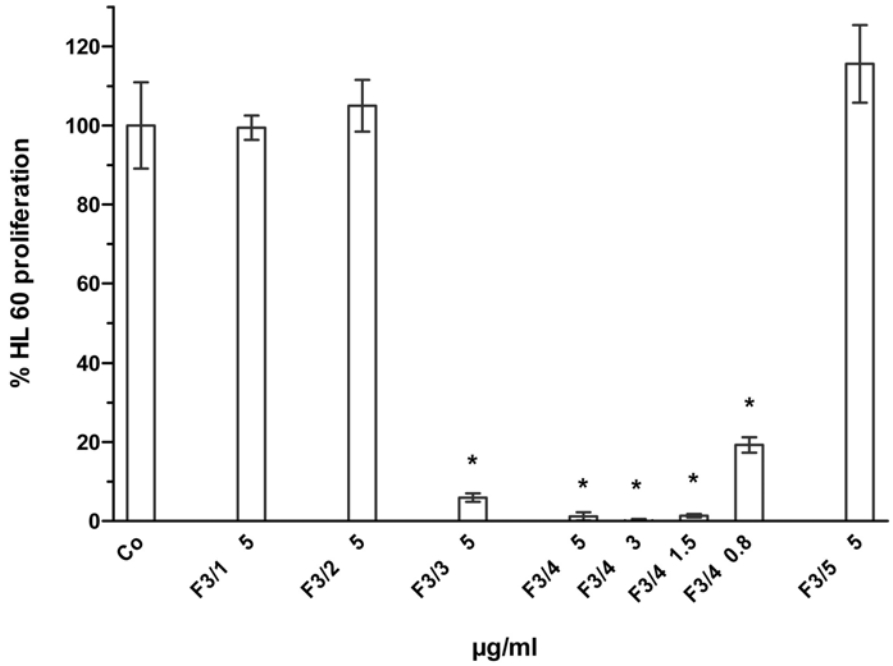

Figure 3. Anti-proliferative effects of *Pluchea odorata* fractions F3/1 – F3/5. Cells were seeded into 24-well plates (1 x 10^5 cells/ml), grown for 24 hours, and incubated with solvent (0.5 % DMSO) or with the specified concentrations of fractions F3/1 – F3/5 for 24 hours. Cell proliferation was calculated as percentage of control. Values are mean ± SEM of experiments performed in triplicate. *p<0.05 as compared to untreated control.

H2AX is among the earliest indicators of DNA strand breaks (32). In contrast, the activation of Chk2 by F3/4 and F2/13 (Figure 4) correlated with the comparatively late activation of caspase 3, respectively (Figure 6) that causes the degradation of DNA as one of several downstream effects. Cdc25A is a direct target of Chk2 and Chk1 and activation of Chk2 can cause the phosphorylation of Ser177 of Cdc25A, Chk1 the phosphorylation of Ser75 of Cdc25A, and both phosphorylations inactivate Cdc25A (33, 34). F2/11 caused an intense phosphorylation of (Ser177)Cdc25A within 4 h and shortly after the activation of Chk2 (Figure 4). Also (Ser75)Cdc25A became phosphorylated, but this was not due to Chk1 because this kinase did not become activated (data not shown). As a consequence, the phosphorylation level of (Tyr15)Cdc2 increased, because inactivated Cdc25A phosphatase did not resume to constitutively de-phosphorylate this Cdc2 site (35). Thus, the kinase activity

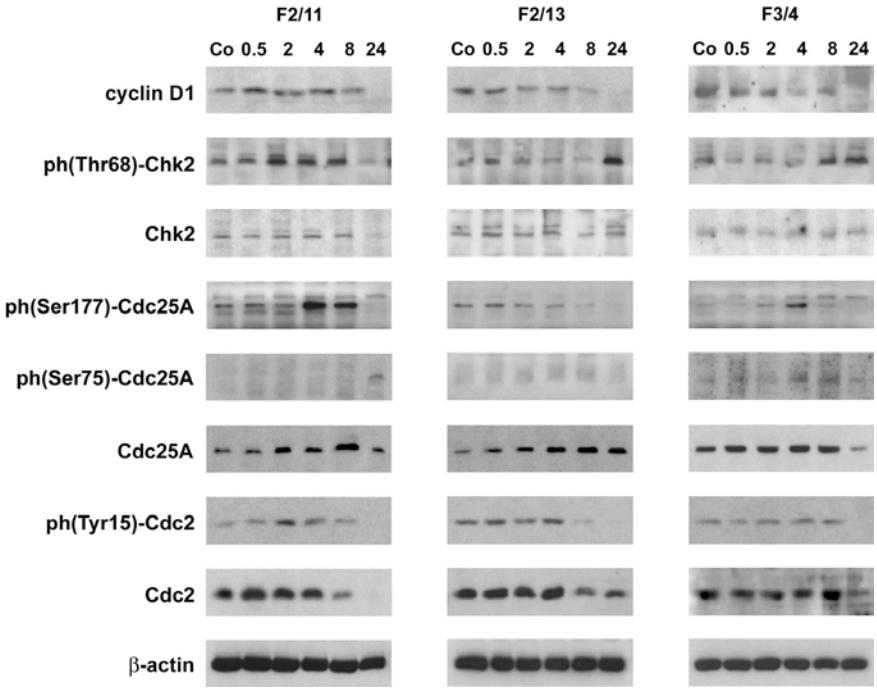

Figure 4. Influence of fraction F2/11, F2/13 and F3/4 on cell cycle and checkpoint regulators**.** Cells were cultivated in T-75 tissue culture flasks (1 x 10^5 cells/ml), grown for 24 hours, and incubated with 3µg/ml fraction F2/11, F2/13 and F3/4 for the specified periods of time. Then, isolated protein samples were subjected to 10 % SDS-PAGE separation and subsequent Western blot analysis using the indicated antibodies. Equal sample loading was controlled by Poinceau S staining and β-actin analysis.

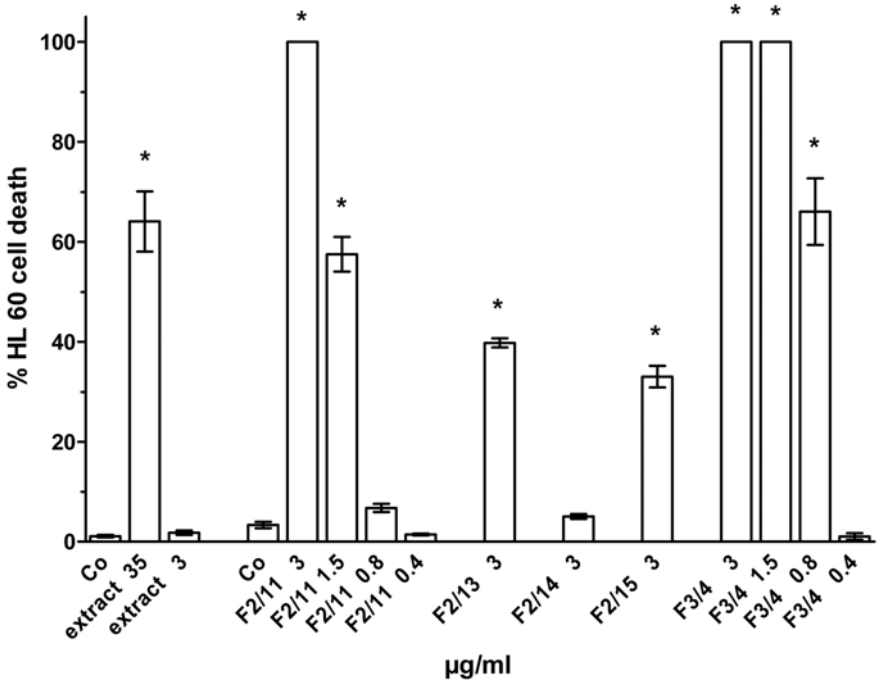

Figure 5. Induction of apoptosis by *Pluchea odorata* CH_2Cl_2 crude extract and fractions F2/11, F2/13, F2/14, F2/15 and F3/4. HL60 cells were seeded into 24-well plates (1 x 10^5 cells/ml) for 24 hours and treated with solvent (0.5% EtOH or DMSO) or the indicated concentrations of CH_2Cl_2 crude extract and fractions F2/11, F2/13, F2/14, F2/15 and F3/4. After 24 hours cells were double stained with Hoechst 33258 and propidium iodide and examined under the microscope with UV light connected to a DAPI filter. Cells with morphological changes indicative for apoptosis were counted and the percentage of cell death was calculated. Values are mean ± SEM of experiments performed in triplicate. *$p<0.05$ as compared to untreated control.

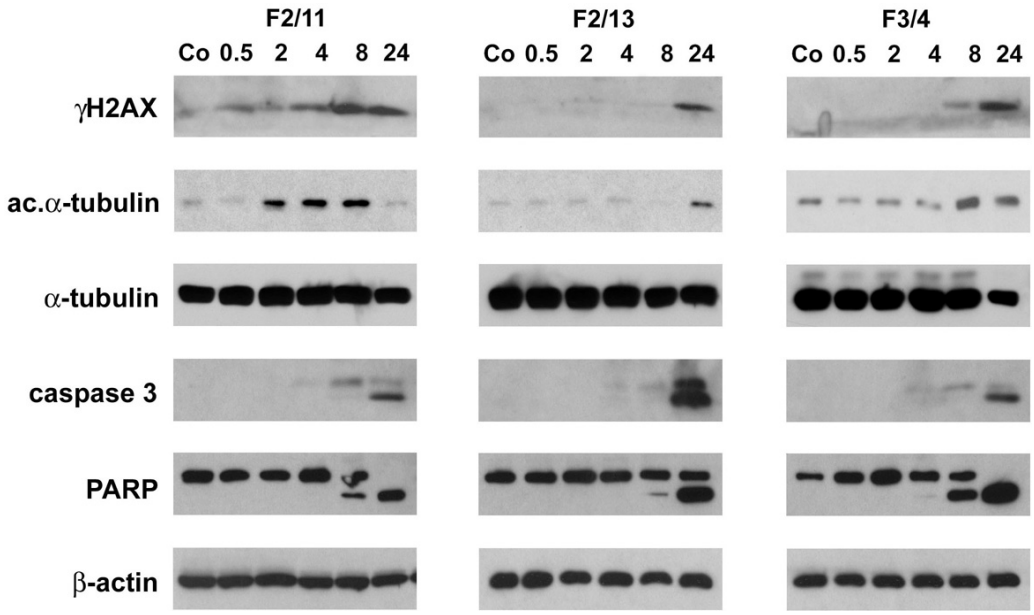

Figure 6. Effect of fraction F2/11, F2/13 and F3/4 on apoptosis-related proteins. Cells were cultivated in T-75 tissue culture flasks (1 x 10^5 cells/ml), grown for 24 hours, and incubated with 3µg/ml fraction F2/11, F2/13 and F3/4 for the specified periods of time. Then, isolated protein samples were subjected to 10 % SDS-PAGE separation and subsequent Western blot analysis using the indicated antibodies. Equal sample loading was controlled by Poinceau S staining and β-actin analysis.

of Wee1 prevailed, which gave rise to a transient accumulation of (Tyr15)Cdc2 phosphorylation upon treatment with F2/11 (36).

In contrast, F2/13 caused the de-phosphorylation of (Ser177)Cdc25A and hence, its activation (37). This was reflected by the de-phosphorylation of (Tyr15)Cdc2 after 8 h. Cdc2 is mandatory for orchestrated G2-M transit. Cdc25A and Cdc25C de-phosphorylate Cdc2, which causes the activation of its kinase domain (30). Cdc2 protein levels were much more stable in cells treated with F2/13 than in those treated with F2/11. In fact, Cdc2 was undetectable upon treatment with F2/11 after 24 h, such as Cyclin D1, and this was most likely causal for cessation of cell proliferation.

F3/4 caused a very transient phosphorylation of (Ser177)Cdc25A and a weak but more sustained phosphorylation of (Ser75)Cdc25A, which correlated with the degradation of this protein after 24 h, and with a slight increase of (Tyr15)Cdc2 phosphorylation levels (Figure 4). We investigated, whether the stress response protein p38/MAPK may have caused phosphorylation of (Ser75)Cdc25A (38). However, constitutive p38 phosphorylation levels were even reduced upon treatment with F3/4 (data not shown). After 24 h the lack of detectable (Tyr15)Cdc2 phosphorylation suggested that this cell cycle protagonist was fully activated. Therefore, suppression of Cyclin D1 together with the activation of Cdc2, as it was observed upon treatment with F3/4 and F 2/13, caused conflicting signals regarding an orchestrated cell cycle progression.

4.5. Fractions F2/11, F2/13 and F3/4 induce apoptosis

Since fractions F2/11, F2/13, and its derivative F3/4 were the most potent inhibitors of proliferation, they were also studied regarding their pro-apoptotic activities. We analyzed apoptosis with a highly sensitive method that identifies very early hallmark phenotypes long before the metabolism collapses and cells actually die (14-16).

HL-60 cells were incubated with the indicated concentrations of the respective fractions (F2/11, F2/13, F2/14, F2/15, F3/4), and with 35 µg/ml of *P. odorata* CH_2Cl_2 crude extract for 24 h, to investigate cell death induction. For the CH_2Cl_2 crude extract ,the calculated concentration which induced 50 % apoptosis (A_{IC50}) was ~25µM (Figure 5). Fraction F2/11 and F3/4 were the most potent fractions, inducing 100 % apoptosis. Interestingly, F2/13, which was the precursor of F3/4, induced only 40 % apoptosis, similar to F2/15, and F2/14 was ineffective at the tested concentration. To unravel which of the two fractions was more potent, further dilutions to 1.5 µg/ml, 0.8 µg/ml, and 0.4 µg/ml enabled to calculate the A_{IC50} after 24 h, which was ~1.4 µg/ml for fraction F2/11 and ~0.6 µg/ml for fraction F3/4. In addition, fraction F2/11 und F3/4 were analyzed after 8 h of treatment and the results indicated F3/4 to be twice as active as F2/11 (Table 4). Therefore, in fraction F3/4 the pro-apoptotic activity was 45-fold enriched compared to the crude CH_2Cl_2 extract (Figure 5).

Since in F3/4 the anti-proliferative and pro-apoptotic activities accumulated, we tested, whether an anti-migratory/metastatic property was contained as well and assessed F3/4 in a novel anti-metastasis assay based on the formation of gaps in lymphendothelial cell monolayers generated by MCF-7 breast cancer cell spheroids (39). However, F3/4 did not prevent the formation of gaps (data not shown).

Table 4. Induction of apoptosis by *Pluchea odorata* fractions F2/11, F2/13 and F3/4.

Fractions3 µg/ml	Apoptosis 8h treatment
F2/11	~20 %
F2/13	~5 %
F3/4	~40 %

Induction of apoptosis by *Pluchea odorata* fractions F2/11, F2/13 and F3/4. HL60 cells were seeded into 24-well plates (1 x 10^5 cells/ml) for 24 hours and treated with solvent (0.5% DMSO) or 3 µg/ml fractions F2/11, F2/13 and F3/4. After 8 hours cells were double stained with Hoechst 33258 and propidium iodide and examined under the microscope with UV light connected to a DAPI filter. Cells with morphological changes indicative for apoptosis were counted and the percentage of cell death was calculated. Values are mean ± SEM of experiments performed in triplicate. *$p<0.05$ as compared to untreated control.

4.6. Western blot analysis of apoptosis related proteins

When HL-60 cells were treated with 3 µg/ml of the indicated fractions, the cleavage of caspase 3 to a 19 kDa and a 12 kDa fragment was observed, which is a prerequisite for its activation that was confirmed by signature type cleavage of the downstream target PARP (40). F2/11 caused the induction of gamma-H2AX within 30 minutes (Figure 6) followed by the rapid activation of Chk2 (Figure 4), thereby indicating genotoxicity and the presence of a DNA targeting component in F2/11. In contrast, F2/13 induced gamma-H2AX after 24 h and F3/4 after 8 h, which correlated with caspase 3 activity (Figure 6). In this case, the induction of gamma-H2AX, and also the activation of Chk2 (Figure 4), were most likely the consequence of the activation of Caspase-Activated-DNAse (CAD) through caspase 3 (41).

Fractions F2/11, F2/13, and F3/4 induced the acetylation of alpha-tubulin and therefore, the stabilization of microtubule (42-44). This was reminiscent of the mechanism of taxol that causes mitotic arrest (3, 4). Tilting the fine-tuned equilibrium of polymerized/de-polymerized microtubule is incompatible with normal cell division and this causes cell cycle arrest and apoptosis (5), and therefore, tubulin-targeting drugs are validated anti-cancer therapeutics (45). The effect of fraction F2/11 differed from those of fractions F2/13 and F3/4 in that the acetylation of tubulin was rapid and severe upon treatment with F2/11, whereas fraction F2/13 induced tubulin acetylation only after 24 h and less pronounced. F3/4 induced tubulin acetylation already after 8 h which correlated with the enrichment of bio-activity compared to F2/13 (Figure 6). Therefore, we could separate two very distinct anti-neoplastic properties in fractions that were derived from the *P. odorata* CH_2Cl_2 crude extract. Firstly, a genotoxic property in fraction F2/11, which also triggered strong tubulin polymerization and which was certainly causal for both, cell cycle arrest and apoptosis. Secondly, an even stronger pro-apoptotic property in F3/4, which had more impact on the expression of the oncogenes Cyclin D1 and Cdc25A. The conflicting signals generated by cyclin D1 suppression and Cdc2 activation would specifically affect constantly cycling cancer cells. This was confirmed in experiments utilizing slowly cycling normal human lung fibroblasts, which were affected significantly less by fraction F3/4 than by fraction F2/11 (data not shown).

Previous studies on the genus *Pluchea* showed that the methanol extracts of *P. odorata* exhibited activity against *Giardia lamblia trophozoites* (46), and in the methanol extract of *P. indica* plucheol A and B, which are unique to species of *Pluchea*, were discovered (47). From the chloroform extract of *P. arabia*, godotol A and B were isolated, which exert weak anti-bacterial activity (48). In addition, in the chloroform extract of the aerial parts of *P. sagittalis*, the eudesmane-type sesquiterpenoids cuauthemone was found, which has anti-feedant activity (49), and cuauthemone, pluchin, plucheinol, among other eudesmane-type sesquiterpenoids, were isolated from *P. chingaio* (50). Cuauthemone was furthermore found in *P. odorata* (51), and thus, cuauthemone is a likely constituent of the dichloromethane extract, which was shown to exert anti-inflammatory activity (11, 52). Flavonoids are well known for their anti-oxidant, anti-inflammatory, and anti-neoplastic effects and quercetin and isorhamnetin have been found in the leaves of *P. lanceolata* (53). It is however unlikely, that polar flavonoids were contained in the here described dichloromethane extract of *P. odorata*. In a broad search for eudesmane-type sesquiterpenoids in the Asteraceae family only eudesmane ketones were found in *P. odorata* (54, 55). Whether cuauthemone or other eudesmane ketones may have contributed to the anti-neolpastic effects of the here studied fractions of the *P. odorata* dichloromethane extract remains to be established. The TLC profile after detection with anisaldehyde sulphuric acid reagent proposes the presence of sesquiterpenes in the active fractions.

This study evidenced that the traditional Maya healing plant *P. odorata* used for the treatment of severe and chronic inflammations, has also anti-neoplastic potential. The separation of a genotoxic property in F2/11 from a cell cycle-interfering property in F3/4 is a relevant step to rid off extract components that may cause unspecific and therefore, undesired therapeutic side effects.

5. ACKNOWLEDGEMENTS

We wish to thank Toni Jaeger for preparing the figures. The work was supported by the Funds for Innovative and Interdisciplinary Cancer Research to M.F.-S and G.K and the Hochschuljubilaeumsstiftung der Stadt Wien to G.K.

6. REFERENCES

1. Gordon Cragg, David Newman: Antineoplastic agents from natural sources: achievements and future directions, *Expet Opin Investig Drugs* 9, 2783-2797 (2000)

2. Gordon Cragg, David Newman, Stringner Yang: Natural product extracts of plant and marine origin having antileukemia potential. The NCI experience. *J NatProd* 69, 488 - 498 (2006)

3. Raphael Geney, Liang Sun, Paula Pera, Ralph Bernacki, Shujun Xia, Susan Horwitz, Carlos Simmerling, Iwao

Ojima: Use of the tubulin bound paclitaxel conformation for structure-based rational drug design. *Chem Biol* 12, 339 - 348 (2005)

4. Adam Marcus, Jun Zhou, Aurora O'Brate, Ernest Hamel, Jason Wong, Michael Nivens, Adel El-Naggar, Tso-Pang Yao, Fadlo Khuri, & Paraskevi Giannakakou: The synergistic combination of the farnesyl transferase inhibitor lonafarnib and paclitaxel enhances tubulin acetylation and requires a functional tubulin deacetylase. *Cancer Res* 65, 3883 - 3893 (2005)

5. Gianni Piperno, Margaret Fuller: 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)

6. Gordon Cragg, David Newman: Plants as source of anticancer agents. *J Ethnopharmacol* 100, 72 - 79 (2005)

7. Leland Cseke, Ara Kirakosyan, Peter Kaufman, Sara Warber, James Duke, & Harry Brielmann: Natural Products from Plants. *CRC Press*, FL (2006)

8. John Borchardt: Medicine of the Maya Ameridians. *Drug News Perspect* 17, 347 - 351 (2004)

9. Joydeb Kundu, Young-Joon Surh: Inflammation: gearing the journey to cancer. *Mutat Res* 659, 15 - 30 (2008)

10. Rosita Arvigo, Michael Balick: Rainforest Remedies. *Lotus Press*, Twin Lakes (1998)

11. Manuela Gridling, Nicole Stark, Sibylle Madlener, Andreas Lackner, Ruxandra Popescu, Birgit Benedek, Rene Diaz, Foster Tut, Thanh Vo, Daniela Huber, Michaela Gollinger, Phillip Saiko, Ali Oezmen, Wilhelm Mosgoeller, Rainer De Martin, Ruth Eytner, Karl-Heinz Wagner, Michael Grusch, Monika Fritzer-Szekeres, Thomas Szekeres, Brigitte Kopp, Richard Frisch, Georg Krupitza: *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)

12. Sibyle Madlener, Jana Svacinova, Miloslav Kitner, Jiri Kopecky, Ruth Eytner, Andreas Lackner, Thanh Vo, Richard Frisch, Michael Grusch, Rainer De Martin, Karel Dolezal, Miroslav Strnad, Georg Krupitza: *In vitro* anti-inflammatory and anticancer activities of extracts of *Acalypha alopecuroidea* (Euphorbiaceae). *Int J Oncol* 35, 881 - 891 (2009)

13. Nicole Stark, Manulela Gridling, Sibylle Madlener, Sabine Bauer, Andreas Lackner, Ruxandra Popescu, Rene Diaz, Foster Tut, Thanh Vo, Caroline Vonach, Benedikt Giessrigl, Philipp Saiko, Michael Grusch, Monika Fritzer-Szekeres, Thomas Szekeres, Brigitte Kopp, Richard Frisch, Georg Krupitza: A polar extract of the Maya healing plant *Anthurium schlechtendalii* (Aracea) exhibits strong *in vitro* anticancer activity. *Int J Mol Med* 24, 513 - 521 (2009)

14. Georg Rosenberger, Gerhard Fuhrmann, Michael Grusch, Sandra Fassl, Howard Elford, Kees Smid, Godefridus Peters, Thomas Szekeres, Georg Krupitza: The ribonucleotide reductase inhibitor trimidox induces c-myc and apoptosis of human ovarian carcinoma cells. *Life Sci* 67, 3131 - 3142 (2000)

15. Monika Fritzer-Szekeres, Michael Grusch, Cornelia Luxbacher, Susanna Horvath, Georg Krupitza, Howard Elford, Thomas Szekeres: Trimidox, an inhibitor of ribonucleotide reductase, induces apoptosis and activates caspases in HL-60 promyelocytic leukemia cells. *Exp Hematol* 28, 924 - 930 (2000)

16. Michael Grusch, Monika Fritzer-Szekeres, Gerhard Fuhrmann, Georg Rosenberger, Cornelia Luxbacher, Howard Elford, Kees Smid, Godefridus Peters, Thomas Szekeres, Georg Krupitza: Activation of caspases and induction of apoptosis by novel ribonucleotide reductase inhibitors amidox and didox. *Exp Hematol* 29, 623 - 632 (2001)

17. Musa Khan, Benedikt Giessrigl, Caroline Vonach, Sibylle Madlener, Sonja Prinz, Irene Herbaceck, Christine Hoelzl, Sabine Bauer, Katharina Viola, Wolfgang Mikulits, Rizwana Quereshi, Siegfried Knasmueller, Michael Grusch, Brigitte Kopp, Georg Krupitza: Berberine and a *Berberis lycium* extract inactivate Cdc25A and induce alpha-tubulin acetylation that correlate with HL-60 cell cycle inhibition and apoptosis. *Mut Res* 683, 123 - 130 (2010)

18. Ali Oezmen, Sabine Bauer, Manuela Gridling, Judith Singhuber, Stanimira Krasteva, Sibylle Madlener, Than Vo, Nicole Stark, Philipp Saiko, Monika Fritzer-Szekeres, Thomas Szekeres, Tulay Askin-Celik, Liselotte Krenn, Georg Krupitza: *In vitro* anti-neoplastic activity of the ethno-pharmaceutical plant *Hypericum adenotrichum* Spach endemic to Western Turkey. *Oncol Rep* 22, 845 - 852 (2009)

19. Ali Oezmen, Sibylle Madlener, Sabine Bauer, Stanimira Krasteva, Caroline Vonach, Benedikt Giessrigl, Manuela Gridling, Katharina Viola, Nicole Stark, Philipp Saiko, Barbara Michel, Monika Fritzer-Szekeres, Thomas Szekeres, Tulay Askin-Celik, Liselotte Krenn, Georg Krupitza: *In vitro* anti-leukemic activity of the ethno-pharmacological plant *Scutellaria orientalis* ssp. carica endemic to western Turkey. *Phytomed* 17, 55 - 62(2010)

20. Philipp Saiko, Maria Ozsvar-Kozma, Sibylle Madlener, Astrid Bernhaus, Andreas Lackner, Michael Grusch, Zsuzsanna Horvath, Georg Krupitza, Walter Jaeger, Kirsten Ammer, Monika Fritzer-Szekeres, Thomas Szekeres: Avemar, a nontoxic fermented wheat germ extract, induces apoptosis and inhibits ribonucleotide reductase in human HL-60 promyelocytic leukemia cells. *Cancer Lett* 250, 323 - 328 (2007)

21. John Alao: The regulation of cyclin D1 degradation: roles in cancer development and the potential for therapeutic invention. *Mol Cancer* 6, 24 (2007)

22. Dipankar Ray, Hiroaki Kiyokawa: CDC25A phosphatase: a rate-limiting oncogene that determines genomic stability. *Cancer Res* 68, 1251 - 1253 (2008)

23. Yue Xiong, Joan Menninger, David Beach, David Ward: Molecular cloning and chromosomal mapping of CCND genes encoding human D-type cyclins. *Genomics* 13, 575 - 584 (1992)

24. Yue Xiong, Hui Zhang, David Beach: D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. *Cell* 71, 504 - 514 (1992)

25. Toshiya Inaba, Hitoshi Matsushime, Markus Valentine, Martine Roussel, Charles Cherr, Thomas Look: Genomic organization, chromosomal localization, and independent expression of human cyclin D genes. *Genomics* 13, 565 - 574 (1992)

26. Kong Lingfei, Ying Pingzhang, Liu Zhengguo, Gen Jianhua, Zhao Yaowu: A study on p16, pRb, cdk4 and cyclinD1 expression in non-small cell lung cancers. *Cancer Lett* 130, 93 - 101 (1998)

27. Carol Rosenberg, Emily Wong, Elizabeth Petty, Allen Bale, Yoshihide Tsujimoto, Nancy Harris, Andrew Arnold: PRAD1, a candidate BCL1 oncogene: mapping and expression in centrocytic lymphoma. *Proc Natl Acad Sci U S A* 88, 9638 - 9642 (1991)

28. Syed Meeran, Santosh Katiyar: Cell cycle control as a basis for cancer chemoprevention through dietary agents. *Front Biosci* 13, 2191 - 2202 (2008)

29. Christina Karlsson-Rosenthal, Jonathan Millar: Cdc25: mechanisms of checkpoint inhibition and recovery. *Trends Cell Biol* 16, 285 - 292 (2006)

30. James Mailer: Mitotic control. *Curr Opin Cell Biol* 3, 269 - 275 (1991)

31. Jennifer Pietenpol, Zoe Stewart: Cell cycle checkpoint signaling: Cell cycle arrest versus apoptosis. *Toxicology* 181 - 182 475 - 481 (2002)

32. Matthew Wasco, Robert Pu, Limin Yu, Lyndon Su, Linglei Ma: Expression of gamma-H2AX in melanocytic lesions. *Hum Pathol* 39, 1614 - 1620 (2008)

33. Zhan Xiao, Zehan Chen, Angelo Gunasekera, Thomas Sowin, Saul Rosenberg, Steve Fesik, Haiying Zhang: Chk1 mediates S and G2 arrests through Cdc25A degradation in response to DNA-damaging agents. *J Biol Chem* 278, 21767 - 21773 (2003)

34. Jacob Falck, Niels Mailand, Randi Syljuasen, Jiri Bartek, Jiri Lukas: The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. *Nature* 410, 842-847 (2001)

35. Arne Lindqvist, Veronica Rodriguez-Bravo, Rene Medema: The decision to enter mitosis: feedback and redundancy in the mitotic entry network. *J Cell Biol* 185, 193 - 202 (2009)

36. Douglas Kellogg: Wee1-dependent mechanisms required for coordination of cell growth and cell division. *J Cell Sci* 116, 4883 - 4890 (2003)

37. Sibylle Madlener, Margit Rosner, Sigurd Krieger, Benedikt Giessrigl, Manuela Gridling, Thanh Vo, Christina Leisser, Andreas Lackner, Ingrid Raab, Michael Grusch, Markus. Hengstschlaeger, Helmut Dolznig, Georg Krupitza: Short 42 degrees C heat shock induces phosphorylation and degradation of Cdc25A which depends on p38MAPK, Chk2 and 14.3.3. *Hum Mol Genet* 18, 1990 - 2000 (2009)

38. Annette Khaled, Dmitry Bulavin, Christina Kittipatarin, Wen Li, Michelle Alvarez, Kyungjae Kim, Howard Young, Albert Fornace, Scott Durum: Cytokine-driven cell cycling is mediated through Cdc25A. *J Cell Biol* 169, 755 - 763 (2005)

39. Sibylle Madlener, Philipp Saiko, Caroline Vonach, Katharina Viola, Nicole Huttary, Nicole Stark, Ruxandra Popescu, Manuela Gridling, Than Vo, Irene Herbacek, Agnes Davidovits, Benedikt Giessrigl, Somepalli Venkateswarlu, Silvana Geleff, Walter Jaeger, Michael Grusch, Dontscho Kerjaschki, Wolfgang Mikulits, Trimurtulu Golakoti, Monika Fritzer-Szekeres, Thomas Szekeres, Georg Krupitza: Multifactorial anticancer effects of digalloyl-resveratrol encompass apoptosis, cell-cycle arrest, and inhibition of lymphendothelial gap formation *in vitro*. *Brit J Cancer* 102, 1361 - 1370 (2010)

40. Teresa Fernandes-Alnemri, Gerald Litwack, Emad Alnemri: 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)

41. Brian Larsen, Shravanti Rampalli, Leanne Burns, Steve Brunette, Jeffrey Dilworth, Lynn Megeney: Caspase 3/caspase-activated DNase promote cell differentiation by inducing DNA strand breaks. *Proc Natl Acad Sci U S A* 107, 4230 - 4235 (2010)

42. Gianni Piperno, Michel LeDizet, Xiao Chang: Microtubules containing acetylated alpha-tubulin in mammalian cells in culture. *J Cell Biol* 2, 289 - 302 (1987)

43. Paula Wilson, Arthur Forer: Effects of nanomolar taxol on crane-fly spermatocyte spindles indicate that acetylation of kinetochore microtubules can be used as a marker of poleward tubulin flux. *Cell Motil Cytoskeleton* 37, 20 - 32 (1997)

44. Akihisa Matsuyama, Tadahiro Shimazu, Yuko Sumida, Akiko Saito, Yasuhiro Yoshimatsu, Daphne Seigneurin-Berny, Hiroyuki Osada, Yasuhiko Komatsu, Norikazu Nishino, Saadi Khochbin, Sueharu Horinouchi, Minoru Yoshida: *In vivo* destabilization of dynamic microtubules by HDAC6-mediated deacetylation. *EMBO J* 21, 6820 - 6831 (2002)

45. Mary Jordan, Leslie Wilson: Microtubules as a target for anticancer drugs (Review). *Nat Rev Cancer* 4, 253 - 265 (2004)

46. Sergio Peraza-Sanchez, Seydi Poot-Katun, Luis Torres-Tapia, Filogonio May-Pat, Paulino Sima-Polanco, Roberto Cedillo-Rivera: Screening of native plants of Yucatan for anti-*Giardia lamblia* activity. *Pharm Biol* 43 594 - 598 (2005)

47. Taketo Uchiyama, Toshio Miyase, Akira Ueno, Khan Usmanghani: Terpene and lignan glycosides from *Pluchea indica*. *Phytochemistry* 30, 655 - 657 (1991)

48. Majekodunmi Fatope, Rani Nair, Ruchi Marwah, Haifaa Al-Nadhiri: New sesquiterpenes from *Pluchea arabica*. *J Nat Prod* 67, 1925-1928 (2004)

49. Nancy Vera, Rosana Misico, Manuel Sierra, Yoshinori Asakawa, Alicia Bardon: Eudesmanes from *Pluchea sagittalis*. Their antifeedant activity on *Spodoptera frugiperda*. *Phytochemistry* 69, 1689 - 1694 (2008)

50. Volker Zlabel, William Watson: Plucheinol and (3alphaH)-Pluicheinol. *Acta Crystallographica* B38, 584 - 588(1982)

51. Koji Nakanishi, Rosalie Crouch, Iwao Miura, Xorge Dominguez, Acosta Zamudio, Roberto Villarreal: Structure of a sesquiterpene, cuauhtemone, and its derivative. Application of partially relaxed Fourier transform carbon-13 nuclear magnetic resonance. *J Am Chem Soc* 96, 609 – 611 (1974)

52. Francisco Perez-Garcia, Esther Marin, Salvador Canigueral, Tomas Adzet: Anti-inflammatory action of *Pluchea sagittalis*: involvement of an antioxidant mechanism. *Life Sci* 59, 2033 - 2040 (1996)

53. Amrik Chawla, Balbir Kaith, Sukhdev Handa, Dinesh Kulshreshtha & Rikhab Srimal: Chemical investigation and anti-inflammatory activity of *Pluchea lanceolata*. *Fitoterapia* 62, 441 - 444 (1991)

54. Quan-Xiang Wu, Yan-Ping Shi, Zhong-Jian Jia: Eudesmane sesquiterpenoids from the Asteraceae family. *Nat Prod Rep* 23, 699 - 734 (2006)

55. Javier Arriaga-Giner, Juan Borges-del-Castillo, Teresa Manresa-Ferrero, Purificacion Vazquez-Bueno, Francisco Rodriguez-Luis, S. Valdes-Iraheta: Eudesmane derivatives from *Pluchea odorata*. *Phytochemistry* 22, 1767-1769 (1983)

Abbreviations: ASE accelerated solvent extractor, ASR anisaldehyde sulphuric acid reagent, CC-I column chromatography I, CC-II column chromatography II, CHCl3 chloroform, CH2Cl2 dichloromethane, SDS-PAGE sodiumdodecylsulfonate polyacrylamide gel electrophoresis, PE petroleum ether, PIC Protease Inhibitor Cocktail, PMSF phenylmethylsulfonylfluorid, TLC thin layer chromatography, VLC vacuum liquid chromatography

Key Words: *Pluchea odorata*, anti-neoplastic, apoptosis, HL-60, genotoxic, H2AX, cyclin D1, Cdc25A, Cdc2, acetylated tubulin

Send correspondence to: Ruxandra Popescu, Department of Pharmacognosy, University of Vienna, Althanstrasse 14, A-1090, Vienna, Austria, Tel: 43-1-4277-55261, Fax: 43-1-4277-9552, E-mail: ruxandra.popescu@univie.ac.at

http://www.bioscience.org/current/volE3.htm