Anti-leukaemic effects of two extract types of *Lactuca sativa* correlate with the activation of Chk2, induction of p21, downregulation of cyclin D1 and acetylation of α-tubulin

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Abstract. The water extract of the lettuce *Lactuca sativa*, but not the ethyl acetate extract, inhibited the growth of HL-60 leukaemia cells and MCF-7 breast cancer cells. This correlated with the activation of checkpoint kinase 2 (Chk2), the induction of the tumour suppressor p21, and the severe downregulation of the proto-oncogene cyclin D1. The ethyl acetate extract, but not the water extract, induced HL-60 cell death, which correlated with the acetylation of α-tubulin. The acetylation of α-tubulin is indicative for microtubuli stabilisation such as induced by taxol. The calculated amount for human intake would require approximately 3 kg lettuce to reach the required concentration shown to inhibit 50% HL-60 proliferation.

Introduction

Vegetables exert their health effects based on their constituents. The health benefits of lettuce are currently considered to be just due to its low calories rather than the content of compounds with nutritional or even pharmacological value. Studies on the wild lettuce *Lactuca indica* analysed the effects of ethanol extracts in HL-60 cells and demonstrated that this extract type contained polyphenols that were most likely responsible for the inhibition of cell growth and the induction of apoptosis (1,2). In previous studies the extracts of traditional anti-inflammatory healing plants were investigated regarding their *in vitro* anticancer properties (3-5) and extracts of the lettuce *Lactuca sativa* (iceberg) served as controls. *L. sativa* was sequentially extracted with solvents of increasing polarity similar to the medicinal plants investigated. We detected anti-neoplastic activities in two extract types of *L. sativa* and this prompted us to study these extract types in more detail and to correlate their properties with potential molecular mechanisms.

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Materials and methods

Plant material. Lactuca sativa (organic quality) was purchased at a Vienna grocery market, washed thoroughly, freeze dried and immediately extracted.

Extraction. Freeze-dried plant material (18.8 g) (derived from 200 g fresh plant) was ground and sequentially extracted with petroleum ether, dichloromethane, ethyl acetate, methanol, and water (500 ml each). The extracts were evaporated under vacuum resulting in 295.2, 302.7, 238.8, 3237.4 and 2571.8 mg dry extracts, respectively. The residues were dissolved in 1.5, 1.5, 1.0, 2.5 ml ethanol, and 8.0 ml water (respectively) to be tested in HL-60 and MCF-7 cells. For the proliferation- and apoptosis analyses following concentrations were used: 500 μg/ml, 1 mg/ml, and 4 mg/ml dried plant weight (see Table I for respective extract weights), as well as 0.3% EtOH solvent control.

Reagents and antibodies. Hoechst 33258 and propidium iodide were purchased from Sigma. ECL Western blotting substrate Cat no. 32106 was from Pierce. Antibodies: Mouse monoclonal anti-acetylated tubulin clone 6-11B1 Cat no. T6793, and mouse monoclonal anti-ß-actin clone AC-15 Cat no. A5441, were from Sigma. Mouse monoclonal anti α-tubulin (TU-02) Cat no. sc-8035, p21 (C-19) Cat no. sc-397, and anti-cyclin D1 (M-20) Cat no. sc-718 were from Santa Cruz. Polyclonal, anti-phospho-Chk2 (Thr68) Cat no. 2661, anti-Chk2 Cat no. 2662, monoclonal rabbit anti-p44/42 MAP kinase (Erk1/2; 137F5) Cat no. 4695, and mouse monoclonal anti-phospho-p44/42 MAPK (Erk1/2-Thr202/Tyr204) (E10) Cat no. 9106 were from Cell Signaling. Anti-mouse IgG was from Dako and anti-rabbit IgG and high performance chemiluminescence film was from GE Healthcare.

Cell culture. HL-60 promyelocytic leukemia and MCF-7 breast cancer cells were from ATCC. Cells were grown in RPMI-1640 (HL-60) or DMEM (MCF-7) medium supplemented with 10% heat inactivated fetal calf serum, 1%

L-glutamine and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. All media and supplements were obtained from Life Technologies.

Proliferation inhibition analysis. To determine the anti-proliferative effect of the plant extracts HL-60 cells were seeded in T-25 tissue culture flasks at a concentration of $1x10^5$ cells per ml. MCF-7 cells were seeded at a concentration of $1x10^4$ cells per ml in 24-well plates and grown for 24 h. Then, cells were incubated with increasing extract concentration (corresponding to 500 μg/ml, 1 mg/ml, and 4 mg/ml dried plant; see Table I for respective extract weights) for 72 h. After 24 and 72 h cells were counted with a micro-cell counter and IC_{50} values calculated. Experiments were done in triplicate. The percent of cell divisions compared to the untreated control were calculated as follows:
$[(C_{72\ h\ +\ drug} - C_{24\ h\ +\ drug})/(C_{72\ h\ -\ drug} - C_{24\ h\ -\ drug})] \times 100 = \%$ cell division; C = cell number

Cell death analysis. Hoechst 33258 propidium iodide double staining of the DNA was performed according to the method described (6). HL-60 cells ($1x10^5$ per ml) were seeded in T-25 Nunc tissue culture flask and exposed to increasing concentrations of plant extracts (500 μg/ml, 1 mg/ml, and 4 mg/ml; see Table I for respective extract weights) for 48 h. MCF-7 cells were seeded at a density of $1x10^4$ cells per ml in 24-well plates and grown for 24 h. Then, increasing concentrations of extract were added and the cells were treated for 96 h. Then the cells were washed with PBS, treated with trypsin and resuspended with medium. Hoechst 33258 and propidium iodide were added directly to the cells at final concentrations of 5 and 2 μg/ml, respectively. After 60 min of incubation at 37˚C, the apoptotic nuclear morphology of cells was examined with a Zeiss Axiovert fluorescence microscope and a DAPI filter. This method allows distinguishing apoptosis from necrosis (7).

Western blotting. HL-60 cells were seeded into T-75 Nunc tissue culture flasks and incubated with 50.8 μg/ml ethyl acetate extract, and 547.2 μg/ml water extract (both corresponding to 4 mg/ml dried plant) for 0.5, 2, 4, 8 and 24 h. Then $1x10^6$ cells were harvested (per experimental point), washed twice with ice cold PBS, centrifuged at 1,000 rpm for 5 min, lysed in a buffer containing 150 mM NaCl, 50 mM Tris pH 8.0, %1 Triton-X-100, 1 mM phenylmethylsulfonylfluorid (PMSF) and protease inhibitor cocktail (PIC; from a 100x stock). Then, the lysate was centrifuged at 12,000 rpm for 20 min at 4˚C, and the supernatant was stored at -20˚C until further analysis. Protein samples were mixed with equal volume of 2X SDS electrophoresis loading buffer and were separated by polyacrylamide gel electrophoresis (PAGE) and electro-transferred onto PVDF-membranes (Hybond, Amersham) at 4˚C overnight. Equal sample loading was controlled by staining membranes with Ponceau S. After washing with phosphate buffered saline/Tween-20 (PBS/T) pH 7.2 or Tris buffered saline/Tween-20 (TBS/T) pH 7.6, membranes were blocked for 1 h in blocking solution (5% non-fat dry milk in PBS containing 0.5% Tween-20 or in TBS containing 0.1% Tween-20). Then, membranes were incubated with the first antibody (in blocking solution, dilution 1:500-1:1000) by gently rocking at 4˚C, overnight. Thereafter, the membranes were washed with PBS or TBS and further incubated with the second antibody (peroxidase-conjugated goat anti-rabbit IgG or anti-mouse IgG, dilution 1:2000-1:5000 in PBS/T or TBS/T) for 12 h. Chemiluminescence was developed by the ECL detection kit (Pierce) and then membranes were exposed to high performance chemiluminescence film.

Table I. Drug extract ratio.

Dried plant material	0.5 mg	1.0 mg	4.0 mg
Solvent	Corresponding to extract		
Petroleum ether	7.9 μg	15.7 μg	62.8 μg
Dichloromethane	8.5 μg	16.1 μg	64.4 μg
Ethyl acetate	6.4 μg	12.7 μg	50.8 μg
Methanol	86.1 μg	172.2 μg	688.8 μg
Water	68.4 μg	136.8 μg	547.2 μg

Quantification of total phenolics. The content of total phenolics was determined with Folin-Ciocalteu reagent according to Chen *et al*. Briefly, 200 μl of extract solution (1 mg/ml) were mixed with 200 μl Folin-Ciocalteau reagent (50% in water). After 3 min, 4 ml of 2% Na_2CO_3 solution was added to the mixture. After 30 min the absorbance was read at 750 nm. The quantification was performed by external standardisation with gallic acid calibration curves ($r^2 = 0.9998$). All analyses were performed in duplicate.

Statistical analysis. All experiments were done in triplicate and analysed by t-test (GraphPad Prism 4.0 program).

Results

Anti-carcinogenic activity of L. sativa extracts. Increasing concentrations of *L. sativa* extracts (corresponding to 0.5, 1 and 4 mg/ml dried plant material; see Table I) were tested in HL-60 leukemia, and MCF-7 breast cancer cells. Whereas extracts of petroleum ether, dichloromethane, ethyl acetate, and methanol had almost no effect on the proliferation, the water extract significantly inhibited the proliferation in both cell lines (Fig. 1). The calculated concentrations of the water extract that inhibited 50% proliferation (IC_{50}) in HL-60 cells was 451.4 μg/ml (corresponding to ~3.3 mg/ml dried plant weight), and in MCF-7 the IC_{50} was 1094.4 μg/ml (corresponding to ~8.0 mg/ml dried plant weight). Further, the ethyl acetate extract, but not the water or the other extracts induced apoptosis in HL-60 cells. In MCF-7 cells neither of the extracts induced apoptosis. In general, the steady state level of necrotic cell death was higher in MCF-7 cells than in HL-60 cells (Fig. 2).

The water extract down-regulates cell cycle protagonists. HL-60 cells were exposed to 50.8 μg/ml ethyl acetate extract, and 547.2 μg/ml water extract (both corresponding to 4 mg/ml dried plant) for increasing times to analyse protein activation and expression by Western blot analysis. The water extract

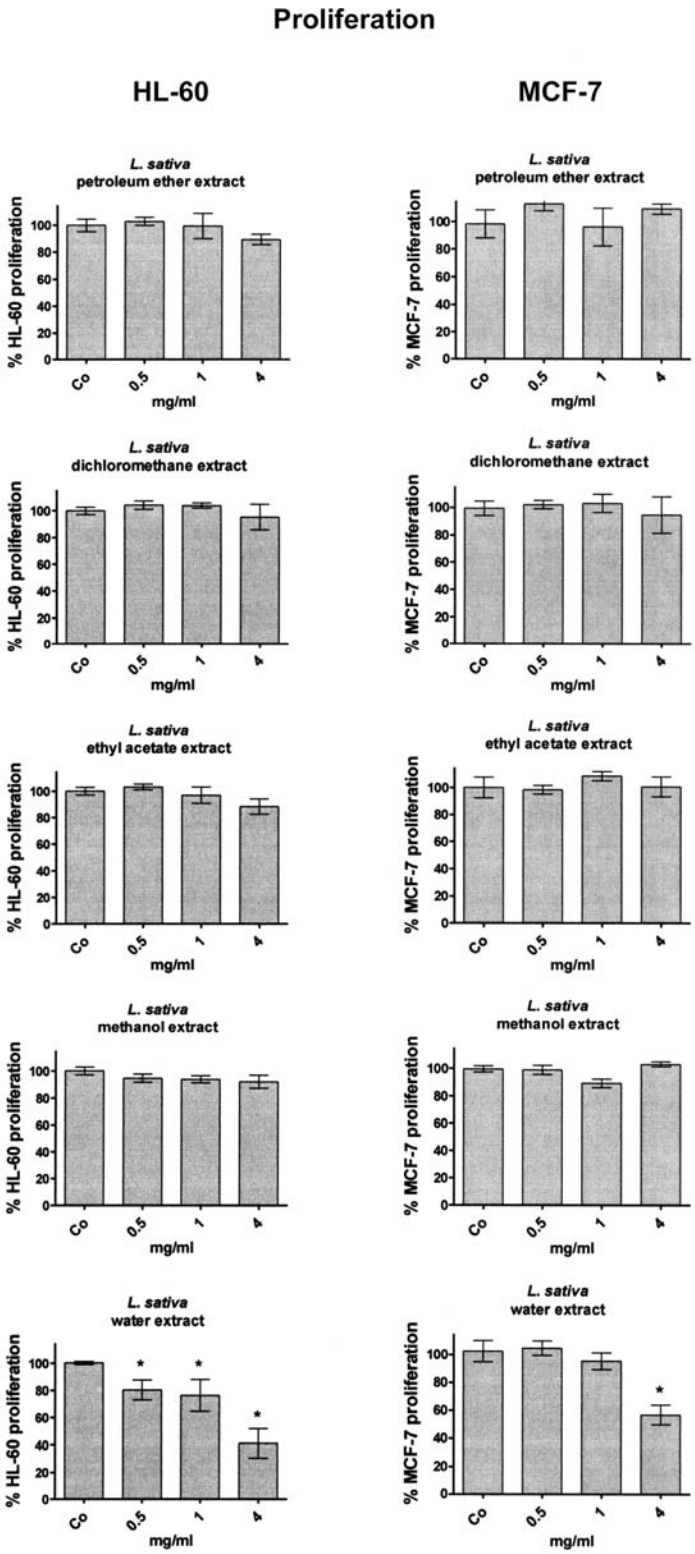

Figure 1. Anti-proliferative effects of *L. sativa* extracts. HL-60 cells were seeded into T-25 tissue culture flasks ($1x10^5$ cells/ml), grown for 24 h to enter logarithmic growth phase, whereas MCF-7 were seeded in 24 well plates, and allowed to attach for 24 h. Then cells were incubated with: petroleum ether extract (7.9, 15.7 and 62.8 μg/ml); dichloromethane extract (8.5, 16.1 and 64.4 μg/ml); ethyl acetate extract (6.4, 12.7 and 50.8 μg/ml); methanol extract (86.1, 172.2 and 688.8 μg/ml); and water extract (68.1, 136.8 and 547.2 μg/ml), each extract type corresponding to 0.5, 1, and 4 mg/ml dried plant material, and counted after 24, 48 and 72 h. The percentage of proliferation between this time span was calculated in comparison to controls. Controls received 0.3% EtOH which was the ethanol concentration cells had to experience together with the highest extract concentration. The other samples were also adjusted to equal ethanol concentrations to achieve similar solvent conditions. Controls were considered as cells with a maximal proliferation rate (100%). Experiments were done in triplicate. Error bars indicate SEM, and the asterisks significance compared to control ($p<0.05$).

caused phosphorylation of checkpoint kinase 2 at Thr68 (phospho-Thr68-Chk2) within 30 min and therefore, its activation. This was accompanied by a decrease of the proto-oncogene cyclin D1 after 8 h (Fig. 3a) and provided an explanation for cell cycle inhibition. On the other hand, the ethyl acetate extract had no effect on Chk2, but after 24 h

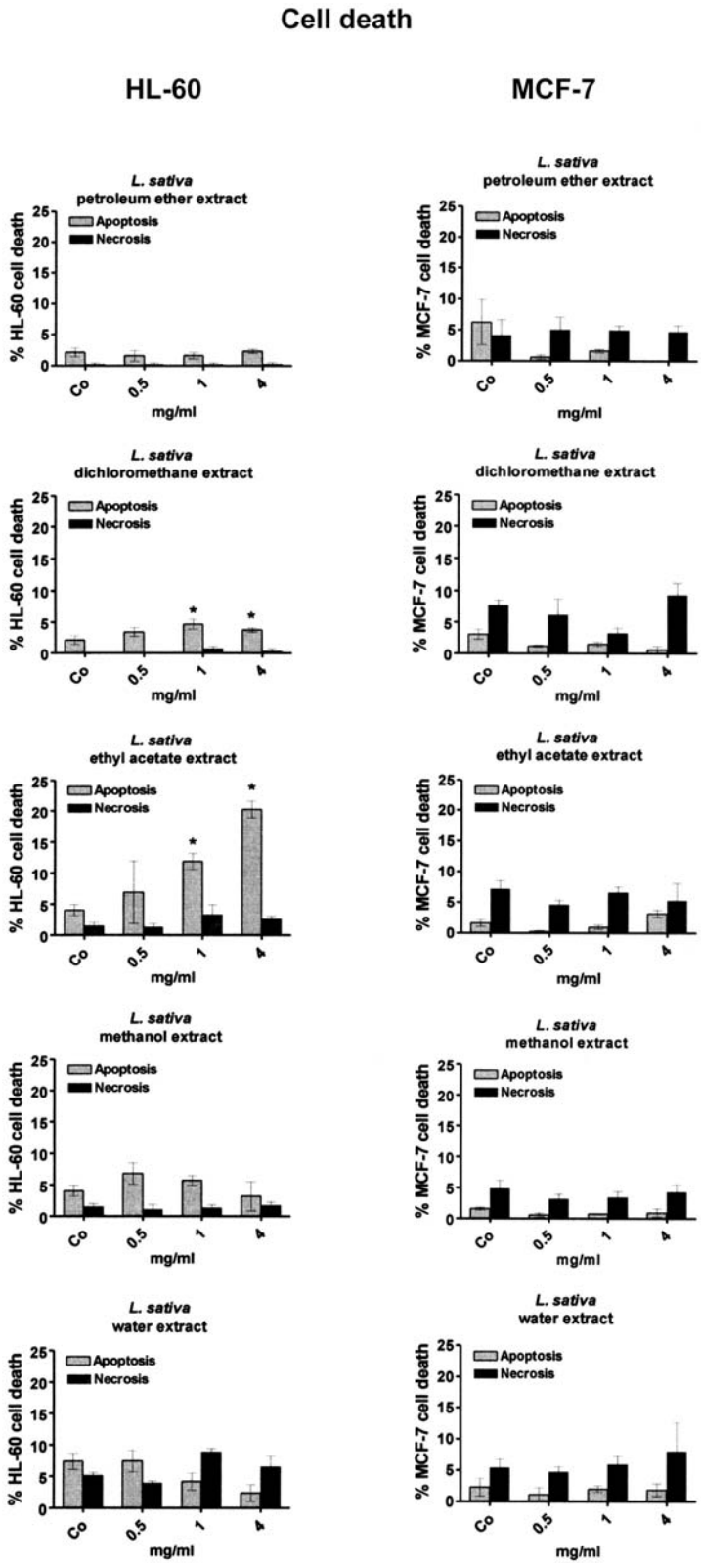

Figure 2. Induction of apoptosis and necrosis by *L. sativa* extracts. HL-60 cells were incubated for 48 h and MCF-7 cells for 96 h with: petroleum ether extract (7.9, 15.7 and 62.8 μg/ml); dichloromethane extract (8.5, 16.1 and 64.4 μg/ml); ethyl acetate extract (6.4, 12.7 and 50.8 μg/ml); methanol extract (86.1, 172.2 and 688.8 μg/ml); and water extract (68.1, 136.8 and 547.2 μg/ml), each extract type corresponding to 0.5, 1 and 4 mg/ml dried plant material. 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 apoptosis or necrosis (see Materials and methods) were counted and percentages of vital, apoptotic and necrotic cells were calculated. Experiments were done in triplicate. Error bars indicate SEM, and the asterisks significance compared to control ($p<0.05$).

also this extract down-regulated cyclin D1. The water extract induced rapid, strong, and transient p21 up-regulation, whereas the ethyl acetate extract induced p21 only weakly (Fig. 3b). HL-60 cells are p53-deficient (8), and therefore, the up-regulation of p21 was most likely mediated through the activation of Erk1/2 (9,10).

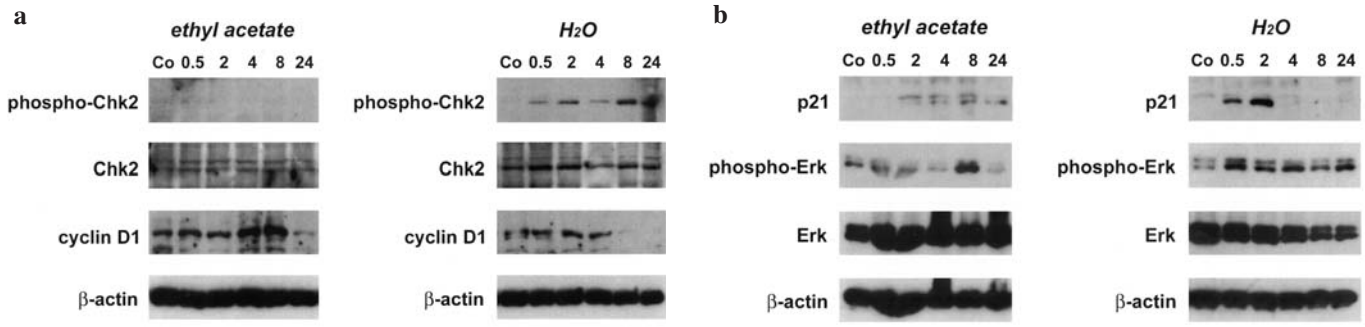

Figure 3. Analysis of cell cycle-related protein and phospho-protein expression. HL-60 cells ($1x10^6$ cells) were seeded into T-75 tissue culture flasks and allowed to grow for 24 h when cells were incubated with 50.8 μg/ml ethyl acetate and 547.2 μg/ml water extracts (each extract type corresponding to 4 mg/ml dried *L. sativa*) for 0.5, 2, 4, 8 and 24 h. Then, isolated protein samples were subjected to electrophoretic separation and subsequent Western blot analysis using antibodies against: (a) phospho-Thr68-Chk2, Chk2, cyclin D1; (b) p21, phospho-Thr202/Tyr204-Erk, and Erk. Equal sample loading was controlled by Poinceau S staining and ß-actin analysis.

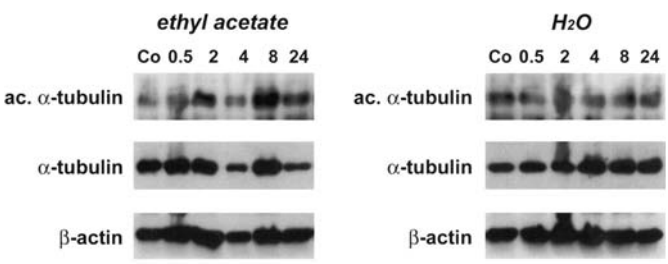

Figure 4. Western blot analysis of tubulin acetylation. HL-60 cells ($1x10^6$ cells) were seeded into T-75 tissue culture flasks and allowed to grow for 24 h when cells were incubated with 50.8 μg/ml ethyl acetate and 547.2 μg/ml water extracts (each extract type corresponding to 4 mg/ml dried *L. sativa*) for 0.5, 2, 4, 8 and 24 h. Then, isolated protein samples were subjected to electrophoretic separation and subsequent Western blot analysis using antibodies against: acetylated-α-tubulin and α-tubulin. Equal sample loading was controlled by Poinceau S staining and ß-actin analysis.

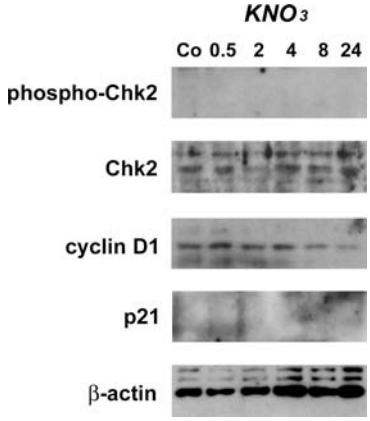

Figure 5. Analysis of cell cycle-related protein and phospho-protein expression. HL-60 cells ($1x10^6$ cells) were seeded into T-75 tissue culture flasks and allowed to grow for 24 h when cells were incubated with 4 μg/ml KNO_3 for 0.5, 2, 4, 8 and 24 h. Then, isolated protein samples were subjected to electrophoretic separation and subsequent Western blot analysis using antibodies against: phospho-Thr68-Chk2, Chk2, cyclin D1; p21. Equal sample loading was controlled by Poinceau S staining and ß-actin analysis.

The ethyl acetate extract induces tubulin acetylation. The ethyl acetate extract, but not the water extract, induced the acetylation of α-tubulin in HL-60 cells (Fig. 4). This implicated that the ethyl acetate extract stabilized microtubules (11-14) and therefore, might have triggered apoptosis similar to taxol (15), which was in fact observed between 12.7 and 50.8 μg/ml (corresponding to 1 and 4 mg/ml dried plant, respectively), whereas the water extract neither induced α-tubulin acetylation nor apoptosis.

Analysis of a potential KNO_3 effect. We investigated whether the observed cytostatic and cytotoxic effects of the extracts were due to remnants of KNO_3, a fertiliser used in lettuce farming. Residual amounts are frequently found in *L. sativa* and the legislation in the European Union allows a maximal residual fertiliser concentration of 4 μg KNO_3/g plant weight, which is however never reached in the 'iceberg' variant. Nevertheless, HL-60 cells were exposed to 4 μg/ml KNO_3 for 0.5, 2, 4, 8 and 24 h, but this had no effect on Chk2 activation or on p21 expression, whereas Cyclin D1 expression was slightly reduced after 8 h (Fig. 5). Also α-tubulin did not become acetylated (data not shown). Therefore, the majority of the observed effects were not induced by fertilizer remnants.

Total phenolic content of L. sativa ethylacetate- and water extract. Numerous polyphenolic compounds possess anti-neoplastic properties and are found in many plants. To estimate whether polyphenolics could have contributed to the anti-neoplastic effects of the ethyl acetate- and water extracts of *L. sativa* the amount of total phenolics was determined by the Folin-Ciocalteu method. The total phenolic content (expressed as gallic acid equivalents; g.a.e.) was 2.85 mg g.a.e./ 119.38 mg ethyl acetate extract and 37.57 mg g.a.e/ 1285.92 mg water extract (both corresponding to 100 g fresh plant). These low concentrations argue against an anti-proliferative and antiapoptotic effect derived from phenolic constituents.

Discussion

The present investigation studied five increasingly polar extracts of lettuce *L. sativa*, a commonly consumed side dish, regarding their anticancer properties, in particular the inhibition of proliferation and the induction of leukemia cell death because these are hallmarks for anti-neoplastic activities.

In HL-60 cells *L. sativa* ethyl acetate extract triggered apoptosis at concentrations of 12.7 and 50.8 μg/ml (corresponding to 1 and 4 mg dried plant weight/ml cell culture medium, respectively). At a concentration of 50.8 μg/ml the ethyl acetate extract induced the acetylation of α-tubulin within 2 h, which indicated a fundamental disturbance of the fine tuned dynamic equilibrium between polymerised/depolymerised microtubuli that is mandatory for proper chromosome segregation during mitosis. Taxol induces apoptosis through the stabilisation of microtubules, which is reflected by increased tubulin acetylation (12,13). By contrast, the ethyl acetate extract of *L. sativa* did not affect the viability of MCF-7 breast cancer cells.

Investigations have shown that *L. indica* induced also apoptosis in HL-60 cells (2). The induction of HL-60 cell death by *L. indica* ethanol extract was due to phenolic compounds, in particular quercetin. Polyphenolic compounds are plant constituents with well studied anti-neoplastic properties i.e. pro-apoptotic, and cell cycle inhibitory activities (16-19). A variety of polyphenolic compounds were investigated in different lettuce genotypes, for example quercetin, kaempferol, luteolin, apigenin, and chrysoeriol derivates (20,21). The total phenolic content in *L. sativa* (expressed as gallic acid equivalents) was 2.85 mg g.a.e./100 g fresh weight in the ethyl acetate extract, and 37.57 mg g.a.e/100 g fresh weight in the water extract. This was 10-fold less than the total content of phenolic compounds found earlier in lettuce varieties (20). This discrepancy seemed to be linked to the different extraction conditions. The low concentration of phenolics in the *L. sativa* ethyl acetate extract might be due to the better solubility of many phenolics and tannins, in methanol. Thus, the amount of phenolic compounds in the water extract of *L. sativa* was low too, because this extraction was subsequent to that with methanol. The active principle in the aqueous extract seems to be unstable, because the pro-apoptotic effect gradually faded over extended storage time. Unlike the ethyl acetate extract, the water extract of *L. sativa* showed an anti-proliferative effect in HL-60 and also in MCF-7 cells. Rapid phosphorylation and activation of Chk2, the downregulation of Cyclin D1, and upregulation of p21 was most likely causal for the retardation of cell proliferation upon treatment with 547.2 μg water extract/ml cell culture medium (corresponding to 4 mg/ml dried plant weight). Through the decrease of Cyclin D1 Cdk 4 and Cdk 6 become inactivated (22,23) resulting in an inhibition of the cell cycle progression. Furthermore, the tumour suppressor p21 became upregulated. p21 has been identified in cyclin A, cyclin E and Cdk2 immunoprecipitates and specifically inhibits i.e. Cdk2 (24). The upregulation of p21 was independent of p53, which is a major regulator of p21, because HL-60 cells are p53 negative (8). Additionally, the water extract induced long-lasting phosphorylation of MAP-kinase and therefore, conflicting mitotic signalling. The anti-proliferative activity in the water extract was more stable throughout storage and this evidenced that two distinct compounds, a stable one inhibiting proliferation, and a labile one inducing apoptosis, are contained in *L. sativa* 'iceberg'. The ethyl acetate extract produced only a slight decrease of cyclin D1 and had no effect on Chk2, whereas the water extract did not influence the acetylation of tubulin and this is consistent with the observation that the ethyl acetate extract induced cell death but not cell cycle inhibition, whereas the water extract induced growth arrest but not apoptosis.

We took into account that contamination with fertiliser residues may have caused the cellular effects seen in the experiments. The amount of contaminating nitrate markedly varies depending on the kind of vegetable, genetic factors, agricultural practices, climatic conditions, degree of maturity, and light (25). Whereas *L. sativa* 'rucola' frequently contains higher amounts of contaminating fertiliser than allowed by the EU legislation, *L. sativa* 'iceberg' contains on average 5-times lower KNO_3 concentrations. To rule out that the observed effects were due to KNO_3, a fertilizer of which residual amounts are frequently found in *L. sativa*, HL-60 cells were treated with 4 μg/ml KNO_3 (4.5 μg KNO_3/g fresh plant weight is the EU upper limit for groceries such as lettuce (26). We tested an approximately 50-fold higher KNO_3 concentration in HL-60 than allowed by the EU for human consumption (because we calculated the KNO_3 concentration for the weight of dried plant material and not, as the EU, for the weight of fresh plants). The experiments evidenced that KNO_3 had no effect on Chk2 or on p21. Also the other tested parameters were unchanged (data not shown). Only Cyclin D1 expression was slightly reduced after 8 h, but this was due to a 50-fold KNO_3 excess that was unlikely to be present in the water extract. Therefore the anti-proliferative and pro-apoptotic effects of *L. sativa* were not induced by fertilizer residues but by plant-derived compounds, although it cannot be entirely ruled out that KNO_3 accumulated in the water extract throughout the sequential extraction steps (though not 50-fold).

The calculated intake for a person (~75 kg) would require the consumption of ~300 g dried, or 3 kg fresh *L. sativa*, which is admittedly high. But this amount could be taken as squash-juice or any other kind of liquid form, since the anti-proliferative activity is water soluble. The findings warrant further research to elucidate the active principles and the molecular mechanisms in more detail, which were responsible for the distinct anti-cancerogenic effect of *L. sativa*.

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