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Research Section

Occurrence of Emodin, Chrysophanol and Physcion in Vegetables, Herbs and Liquors. Genotoxicity and Anti-genotoxicity of the Anthraquinones and of the Whole Plants

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Abstract—1,8-Dihydroxyanthraquinones, present in laxatives, fungi imperfecti, Chinese herbs and possibly vegetables, are in debate as human carcinogens. We screened a variety of vegetables (cabbage lettuce, beans, peas), some herbs and herbal-flavoured liquors for their content of the 'free' anthraquinones emodin, chrysophanol and physcion. For qualitative and quantitative analysis, reversed-phase HPLC (RP-LC), gas chromatography–mass spectrometry (GC-MS) and RP-LC-MS were used. The vegetables showed a large batch-to-batch variability, from 0.04 to 3.6, 5.9 and 36 mg total anthraquinone per kg fresh weight in peas, cabbage lettuce, and beans, respectively. Physcion predominated in all vegetables. In the herbs grape vine leaves, couch grass root and plantain herb, anthraquinones were above the limit of detection. Contents ranged below 1 mg/kg (dry weight). All three anthraquinones were also found in seven of 11 herbal-flavoured liquors, in a range of 0.05 mg/kg to 7.6 mg/kg. The genotoxicity of the analysed anthraquinones was investigated in the comet assay, the micronucleus test and the mutation assay in mouse lymphoma L5178Y $tk^{+/-}$ cells. Emodin was genotoxic, whereas chrysophanol and physcion showed no effects. Complete vegetable extract on its own did not show any effect in the micronucleus test. A lettuce extract completely abolished the induction of micronuclei by the genotoxic anthraquinone danthron. Taking into consideration the measured concentrations of anthraquinones, estimated daily intakes, the genotoxic potency, as well as protective effects of the food matrix, the analysed constituents do not represent a high priority genotoxic risk in a balanced human diet. © *1999 Elsevier Science Ltd. All rights reserved*

Keywords: anthraquinones; vegetables; diet; mutagens; genotoxicity; cancer risk.

Abbreviations: CBMNT = cytokinesis-block micronucleus test; DMSO = dimethyl sulfoxide; EMS = ethylmethanesulfonate; GC-MS = gas chromatography–mass spectrometry; LC-MS = liquid chromatography–mass spectrometry; MNT = micronucleus test; RP-HPLC = reversed-phase HPLC; TFT = trifluorothymidine.

INTRODUCTION

Hydroxyanthraquinones are the active principles in a large number of plant-derived drugs, such as laxatives from senna (*Cassia senna*), frangula bark (*Rhamnus frangula*) and aloe (Thomson, 1986).

Furthermore, their occurrence in plants has been described in Rheum, Rumex, Polygonum and Leguminosae species (Thomson, 1986). In many fungi imperfecti and some toadstools, anthraquinone derivatives have been discussed as the toxic metabolites and coloring matters (*Dermocybe sanguinea*, *Penicillium* and *Aspergillus* species) (Steglich and Lösel, 1972; Thomson, 1986). Most of the hydroxyanthraquinones are present as pharmacologically inactive glycosides in plant extracts but are thought to be activated by glycosidic cleavage *in vivo* by microorganisms in the intestinal flora (Hattori *et al.*, 1993). The level of occurrence of anthraquinones in food plants can be constitutive

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or induced by infection. In addition, a contamination of foods by enzyme preparations from fungi used in food production has been discussed (Gross *et al.*, 1984). Investigations made by Gross *et al.* (1984) deny a respective contamination, but the necessity for further studies of the presence of naturally occurring anthraquinones in food has been pointed out.

As the anthraquinone content is a quality criterion for plant-derived laxatives, a number of analytical methods have been established for the different anthraquinone derivatives. Mainly extraction with aqueous methanol or acetonitrile and RP-HPLC (reversed-phase HPLC) were used for analysis (Grimminger and Witthohn, 1993; Metzger and Reif, 1996). Finally, capillary zone electrophoresis, micellar electrokinetic capillary chromatography and LC-MS (liquid chromatography–mass spectrometry) analysis have been used for detection and, in some cases, for quantification of the different anthraquinone derivatives (Koyamma *et al.*, 1997; Stuppner and Sturm, 1996).

The genotoxic effects of the 1,8-dihydroxyanthraquinones are still under debate (Brusick and Mengs, 1997). Recently, we have demonstrated unequivocally a genotoxicity of the anthraquinones emodin, aloe-emodin and danthron in mammalian cells (Müller *et al.*, 1996). Here, we present data on the presence of the aglycones emodin, chrysophanol and physcion in vegetables, herbs and liquors, and additional data on the genotoxic effects of the anthraquinones, using the micronucleus test, the single-cell gel electrophoresis assay (comet assay) and the mutation assay in mouse lymphoma L5178Y $tk^{+/-}$ cells. In the micronucleus test, the vegetables were also tested as a whole, in order to investigate putative additional genotoxic or antigenotoxic effects.

MATERIALS AND METHODS

Chemicals

Chrysophanol, danthron, emodin and physcion were purchased from Roth (Karlsruhe, Germany). Cytochalasin B, dimethyl sulfoxide (DMSO), ethylmethanesulfonate (EMS), Hoechst 33258, hypoxanthine, methotrexate, thymidine and trifluorthymidine (TFT) were from Sigma (Deisenhofen, Germany). Test compounds were dissolved in DMSO. The final concentration of DMSO in the cell culture medium did not exceed 1%. The analysed liquors and vegetables were purchased from the local market, worked up immediately, and stored in the refrigerator. Herbs were dried materials from Galke (Gittelde, Germany) and Klenk (Schwebheim, Germany). For RP-HPLC analysis, we used gradient-grade solvents from Fisons.

General work-up for RP-HPLC analyses

Materials with a high water content (vegetables) were mixed in a Waring blendor and freeze-dried; the powder was stored at 4°C. The powdered materials (10–40 g) were added to a Soxhlet cartridge. After addition of 1,8-dihydroxyanthraquinone (danthron) as internal standard, the material was extracted with acetonitrile for 4–5 hr. Liquid materials (about 200 ml) were acidified with HCl to pH 2.0 and after addition of the standard danthron, extracted three times with equal amounts of ethyl acetate. The extracts were evaporated to dryness and dissolved in 5 ml 80% acetonitrile/20% water (sample solution I) for RP-HPLC analysis.

Further work-up was evaluated for enriching and reducing negative effects of the matrix. The additional work-up was necessary particularly for LC-MS analyses. 0.5 ml of the sample solution I was introduced in a column of 7.0 g silica gel (equilibrated with pentane) and then washed with 10 ml pentane. Elution of the anthraquinones was performed with 20 ml acetone/pentane (50:50). The solvents from the extracts were evaporated and redissolved in 0.5 ml (sample solution II) of 80% acetonitrile/20% water for LC-MS analysis.

General work-up for the analysis of vegetable extracts by GC-MS (gas chromatography–mass spectrometry)

The vegetables were washed, peeled, homogenized in a mixer (Braun Multipractic) and freeze-dried. Extraction of anthraquinones was performed as described (Metzger and Reif, 1996) with the following modifications. The freeze-dried powder (1 g) was mixed with 100 ml 0.2% $NaHCO_3$/acetonitrile (60:40) and danthron was added as internal standard. The suspension was treated for 40 min with ultrasound. Particles were filtered off and the solution was re-extracted with $CHCl_3$. The $CHCl_3$ phase was reduced to dryness, reconstituted with 1 ml $CHCl_3$ and used for further analysis.

General RP-HPLC, LC-MS and GC-MS analysis

RP-HPLC for qualitative and quantitative analysis was performed with a Hewlett-Packard photodiode detector 1040 A, an H-P 9153C workstation, two Knauer HPLC C 64 pumps (analytical) coupled to a Knauer HPLC programmer 50. Separation of the anthraquinones emodin, chrysophanol, physcion and the internal standard danthron was performed on a Knauer Europher Si-100 C-18 RP-column (240×4 mm) with a solvent gradient. Solvent A was acetonitrile/5% methanol and solvent B was water/0.1% acetic acid. Analyses were performed by gradient elution starting with 50% solvent A at 0 min to 100% solvent A at 43 min with a flow rate of 0.8 ml/min. Samples were introduced with a 20 μl sample loop. For quantification, evaluation curves of the four substances were performed in a concen-

tration range of 3.0 mmol/litre to 80 mmol/litre at the anthraquinone-specific wavelength of 435 nm.

To verify the quantitative data of the RP-HPLC analyses we applied a GC-MS analysis using a Fisons GC 8000/MD 800 with mass-selective detector (EI- and CI-mode). Carrier gas was helium with a linear velocity of 50 kPa. Separation was performed on a 15 m fused silica column coated with DB5, (inner diameter 0.25 mm, film thickness 0.25 μm); splitless injection; column temperature 260°C. Quantification was made in the EI+ mode using calibration curves of authentic standards. SIM mode was used with m/z = 240, 254 and 284; mass range of 1.5 amu and a scan time of 2.0 sec was employed. The occurrence of the anthraquinones under study was confirmed by chemical ionization (collision gas methane) and detection of negative ions (NCI mode).

For LC-MS analysis we used a Finnigan TSQ 7000 triple-stage quadrupol mass spectrometer with atmospheric chemical-ionization interface (APCI) in negative mode coupled to an Eurospher Si-100 C-18 RP-column (100 × 2.0 mm) from Knauer and a gradient elution controlled by a Applied Biosystem 140b pump (linear gradient from 60% 10 mM ammonium–acetate in water to 72% acetonitrile in 16 min at a flow rate of 200 ml/min with 5 μl sample loop). Instrument parameters were as follows: ion-source: 760 Torr, rt; corona: 5 μA; E-mult: 1800 V; shit- and auxiliary gas was N_2; capillary: 200°C; collision-gas: argon; pressure 10^{-4} to -10^{-3} bar; CID 10 mV. SIM mode was used with m/z = 240, 254, 270 and 284; mass range of 1.5 amu and a scan time of 2.0 sec was employed. Previous experiments showed that the compounds did not fragment under these conditions. Analysis of the data was performed on a ICIS data system with DEC station 2100.

Cell culture

Mouse lymphoma L5178Y cells were cultured in suspension in RPMI-1640 supplemented with antibiotics, 0.25 mg L-glutamine/ml, 107 μg sodium pyruvate/ml and 10% heat-inactivated horse serum (all from Sigma Chemie GmbH, Deisenhofen, Germany). Cell cultures were grown in an humidified atmosphere with 5% CO_2in air at 37°C.

General work-up for vegetable extracts and lyophilisates used in the genotoxicity assays

Vegetable extracts were obtained by the procedure described in *the general work-up for the analysis of vegetable extracts by GC-MS* (see above), the dry $CHCl_3$ residue was resuspended in DMSO (25 mg/ml). Vegetable lyophilizates were obtained by powdering of the freeze-dried washed vegetable without any extraction step. The dry powder was suspended in DMSO (25 mg/ml). The final concentration of DMSO in the cell culture medium did not exceed 1%.

In vitro micronucleus test (MNT)/cytokinesis-block micronucleus test (CBMNT)

MNT. Exponentially growing mouse L5178Y cells were treated with the test compounds for 4 hr as indicated in the legends to Figs 5 and 6. The vehicle control was 1% DMSO. After removing the

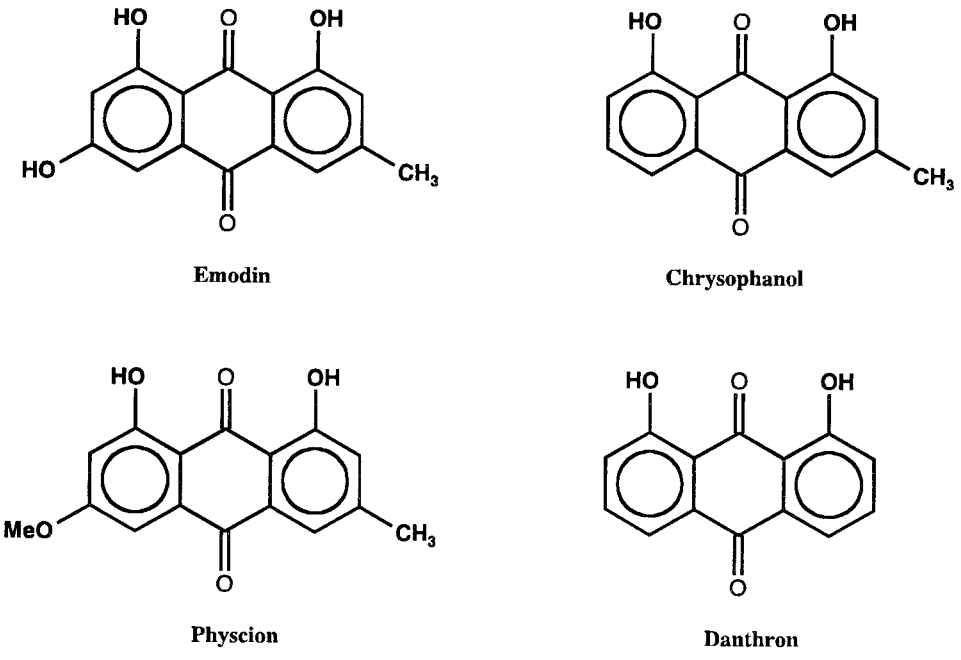

Fig. 1. Chemical formula of the investigated anthraquinones.

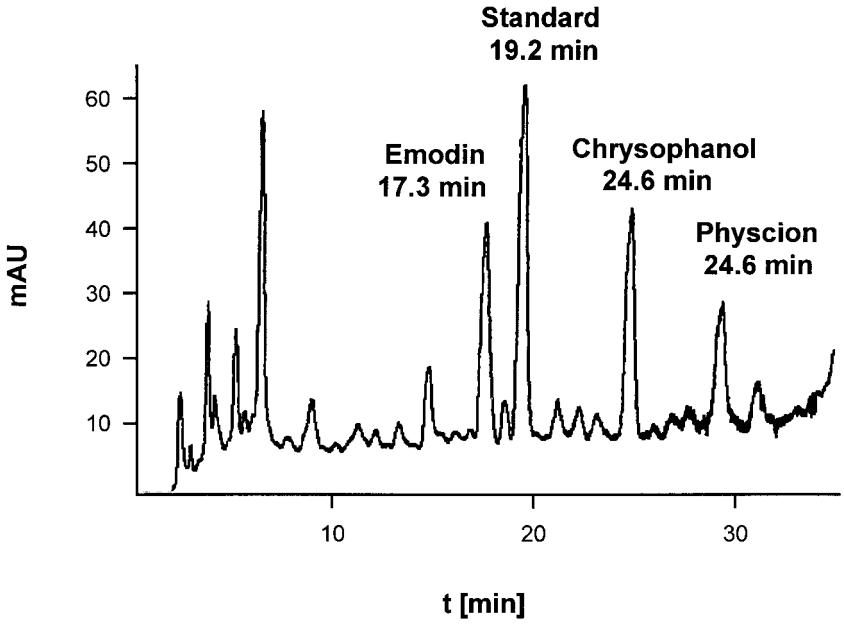

Fig. 2. RP-HPLC separation of anthraquinones of a sample obtained from watercress. Absorbance at 435 nm.

chemicals by centrifugation and medium replacement, the cells were incubated for 15 hr (expression time). The cells were brought onto glass slides by cytospin-centrifugation and fixed with methanol (−20°C, 1 hr). To stain nuclei and micronuclei, the slides were incubated with Hoechst 33258 (5 μg/ml, 3 min), washed twice with Ca- and Mg-free phosphate buffered saline buffer for 2 min and mounted for microscopy. Numbers of nuclei and micronuclei were scored at a magnification of ×1250. Each data point represents the mean of three slides with standard deviation, with 1000 nuclei being evaluated on each slide. All experiments were repeated at least twice with consistent results.

CBMNT. Experiments with cytochalasin B were performed with 6 μg cytochalasin B/ml during substance exposure and recovery time of the cells as described above. To stain binucleated cells, slides were incubated with acridine orange (62.5 μg/ml, 5 min), washed twice with Soerensen buffer for 2 min and mounted for microscopy. Numbers of micronuclei of 1000 binucleated cells were scored and evaluated as described above. All experiments were repeated at least twice with consistent results.

Comet assay

Comet assays were performed according to Singh *et al.* (1988) with slight modifications. Mouse L5178Y cells were incubated with the test compounds for 4 hr as indicated in the legend to Fig. 4. Cells were washed and embedded in low melting agarose (0.5%) which was layered onto fully frosted microscope slides that had been coated with a layer of 0.75% normal agarose (diluted in Ca- and Mg-free PBS buffer). A final layer of 0.5% low melting agarose was added on top. Slides were immersed in a jar containing cold lysing solution (1% Triton X-100, 10% DMSO and 89% 10 mM Tris/1% Na–lauryl-sarcosine/2.5 M NaCl/ 100 mM Na_2 EDTA (pH 10) for lysis at 4°C (1 hr). Slides were treated for 15 min in electrophoresis buffer (300 mM NaOH/1 mM Na_2 EDTA (pH 13) and exposed to 25 V/300 mA for 20 min. Preincubation and electrophoresis were performed in an ice-bath. Slides were neutralized for 3 × 5 min in 0.4 M Tris, pH 7.5, and DNA was stained by adding 50 μl ethidium bromide (20 μg/ml) onto each slide. Cells were analysed with a ×1250 magnification and using computer-aided image analysis. Images of at least 50 cells (25 from each of two slides) were evaluated by the use of the software program NIH Image 1.54 (NIH, USA). Two areas were selected in each picture: the whole cellular DNA including the tail region of the comet and a region containing only the tail region of the comet. The integrated densities (sum of the grey values of each pixel in the selection) were measured in each selection and the percentage in the tail region of the comet was calculated. This number represents the amount of DNA in the tail and is referred to as "tail (%)" in Fig. 5. Medians and standard errors for each treatment are given. All experiments were repeated at least twice.

Mutation assay

Cultures of mouse L5178Y cells were treated with methotrexate before each experiment to kill pre-existing TFT-resistant cells. To accomplish this, cells were incubated for 12 hr in culture medium plus methotrexate (0.3 μg/ml), thymidine (9 μg/ml), hypoxanthine (15 μg/ml) and glycine (22.5 μg/ml).

Table 1. Emodin, chrysophanol and physcion in vegetables, analysed by RP-HPLC and GC-MS. Data are given as range, in mg/kg ($n \geqslant 3$) and were not corrected for recovery

	Sum of anthraquinones[a]		Emodin		Chrysophanol		Physcion		Recovery of standard (%)
	fresh[b]	dry[c]	fresh[b]	dry[c]	fresh[b]	dry[c]	fresh[b]	dry[c]	
Latuca sativa var. capitata cv. 'Cabbage lettuce'	0.06–5.9	1.0–174	0.02–0.03	⩽0.5	0.01–0.03	⩽0.5	0.03–5.8	0.5–173	95
Phaseolus vulgaris Beans	0.05–36	0.4–342	0.02–0.06	0.2–0.7	0.02–3.5	0.12–33	0.01–32	0.1–308	129
Pisum sativum Gardenpeas	0.04–3.6	0.2–21	0.02–0.03	0.1–0.2	–	–	0.02–3.6	0.1–21	59
Brassica chinensis Chinese cabbage	–	–	–	–	–	–	–	–	84
Latuca sativa var. capitata cv. 'Iceberg salad'	< 0.04	< 0.74	< 0.01	< 0.24	< 0.01	< 0.17	< 0.02	< 0.34	99
Cichorium intybus var. folio. cv. 'Chicory'	–	–	–	–	–	–	–	–	69
Cichorium intybus var. folio. cv. 'Radicchio'	–	–	–	–	–	–	–	–	97

– not detectable; < above limit of detection, but not quantifiable; [a]maximum sum of all anthraquinones detected; [b]mg/kg fresh weight; [c]mg/kg dry weight.

The cells were incubated for at least 48 hr in the same medium without methotrexate. To measure chemically-induced mutants using the *in situ* procedure (Spencer *et al*., 1994), cultures containing 1×10^6 cells in 5 ml medium were treated with DMSO (final concentration 1%; vehicle control) or 1,8-dihydroxyanthraquinones with increasing doses until insolubility. EMS was used as a positive control. Incubation was performed for 4 hr, then the cells were washed twice with fresh medium. After that, from each tested culture, 0.5×10^6 cells were added to 50 ml semi-solid culture medium (containing 0.25% granulated agar, Baltimore Biological Laboratories, USA), plated into two plastic 100-mm culture dishes and allowed to solidify at room temperature. TFT-resistant cells were selected by adding an overlay of TFT (final concentration 8 μg/ml) in 15 ml semi-solid medium after an expression time of 42 hr. Cloning efficiency was determined by adding 600 cells to 100 ml semi-solid medium in three 100-mm culture dishes. All plates were incubated for a total of 9 to 12 days at 37°C in 5% CO_2 for colony growth. An automatic colony counter was used to count the number of TFT-resistant colonies. Experiments were repeated at least twice.

Mutagenicity of emodin, chrysophanol and physcion in the Ames test

These experiments were performed as described (Maron and Ames, 1983), using *Salmonella typhimurium* TA100 and TA2638 in the presence or absence of liver microsomes from Aroclor 1254-pretreated male rats.

RESULTS

Occurrence of anthraquinone–aglycones in vegetables, herbs and herbal-flavoured liquids

We used RP-HPLC analysis for the quantitative determination of the content of the aglycosidic anthraquinones emodin, chrysophanol and physcion

Table 2. Emodin, chrysophanol and physcion in herbs, analysed by RP-HPLC. Data are given as range, in mg/kg ($n \geqslant 3$) and were not corrected for recovery

	Sum of anthraquinones[a]	Emodin	Chrysophanol	Physcion	Recovery of standard (%)
Vitis vinifera Grape vine leaves	0.4–0.8	0.1–0.2	0.1–0.2	0.2–0.4	91
Rhizoma graminis Couch grass root	0.2–0.7	0.06–0.2	0.05–0.2	0.08–0.3	110
Plantagines lanceolatae Plantain herb	0.4–0.7	0.1–0.2	0.1–0.2	0.2–0.3	105
Fagopyrum esculentum Buckwheat	–	–	–	–	74
Artemisia annua Wormwood	< 0.1	< 0.1	–	–	130

– not detectable; < above limit of detection, but not quantifiable; [a]maximum sum of all anthraquinones detected.

Table 3. Emodin, chrysophanol and physcion in herbal flavoured liquors, analysed by RP-HPLC. Data are given as range, in mg/kg ($n \geqslant 3$) and were not corrected for recovery

	Sum of anthraquinones[a]	Emodin	Chrysophanol	Physcion	Recovery of standard (%)
K1	–	–	–	–	140
K2	0.54–0.86	0.08–0.14	0.36–0.58	0.09–0.14	104
K3	0.07–0.08	0.02–0.03	0.03–0.04	0.016–0.020	67
K4	1.03–1.28	0.24–0.30	0.64–0.79	0.15–0.18	92
K5	–	–	–	–	109
K6	0.05–0.07	0.01–0.02	0.02–0.03	0.01–0.02	88
K7	6.08–7.60	2.03–2.55	2.91–3.65	1.14–1.40	95
K8	–	–	–	–	98
K9	0.05–0.08	0.02–0.03	0.02–0.03	0.010–0.017	114
K10	–	–	–	–	95
K11	0.05–0.06	0.010–0.016	0.02–0.03	0.010–0.017	108

– not detectable; [a]maximum sum of all anthraquinones detected.

(Fig. 1). The limit for quantification based on signal to noise (1:8) was 1.77 mmol/litre for emodin, 1.38 mmol/litre for chrysophanol and 2.34 mmol/litre for physcion in the sample solution. Because LC-MS-analysis was not more sensitive than RP-HPLC, LC-MS analysis was only performed to verify anthraquinone occurrence in the selected materials. Additionally, we applied a GC-MS method to quantify the contents of chrysophanol and physcion in selected vegetables. The respective detection limits in the sample solutions were 30 μmol/litre for chrysophanol and 4 μmol/litre for physcion. External standard method was used for quantification.

In Table 1, anthraquinone concentrations measured in a number of vegetables are shown. Data are given as a range, demonstrating large batch-to-batch variability. Physcion predominated, with maximum contents in beans (32 mg/kg fresh weight), followed by lettuce ($\leqslant$5.8) and peas ($\leqslant$3.6). The RP-HPLC and GC-MS methods used for determination of anthraquinone contents, produced similar concentrations and ranges.

Anthraquinone concentrations in the herbs are given in Table 2. No preponderance of any specific compound is seen here. Compared with the vegetables, concentrations range at the lower end of the respective batch-to-batch variability.

As liquors contain herbal ingredients, we investigated several common and often consumed herbal-flavoured liquors (assigned K1 to K11; Table 3). In seven of 11 analysed herbal-flavoured liquors all three anthraquinones were detected in a ranking chrysophanol > emodin > physcion. The concentrations of the individual compounds varied between 0.02 mg/kg and 3.7 mg/kg. With the liquors, matrix effects were less marked so that detection limits down to 0.010 mg/kg were possible, based on the possibility to work up large amounts of materials.

Genotoxicity of snthraquinones in Salmonella typhimurium and mouse lymphoma cells

Emodin, chrysophanol and physcion showed no mutagenicity in the Ames test with TA100 and TA2638 with or without metabolic activation using a S9-mix from Aroclor 1254-pretreated male rats (data not shown).

Mutagenic activity in eukaryotic cells was measured by the induction of TFT-resistance in mouse L5178Y cells (Fig. 2). Emodin induced a moderate increase in relative mutation frequency (> twofold over control); (see also Müller *et al.*, 1996). In a similar concentration range, chrysophanol and physcion did not meet the criterion of a twofold mutation frequency when compared with the solvent control. Toxicity as shown by a reduction in the cloning efficiency was low in the applied dose range for emodin and chrysophanol. For physcion, a reduction of the cloning efficiency to less than 50% of the control value was observed at the higher concentrations tested.

With respect to the induction of micronuclei in the same cell line, we have previously shown a dose-dependent increase for emodin (Müller *et al.*, 1996). Chrysophanol and physcion were much less potent and the effect was not clearly concentration dependent (Fig. 3). In the comet assay performed in a similar dose range, none of the three anthraquinones showed an effect, in the dose range tested (Fig. 4).

Genotoxicity and anti-genotoxicity of the vegetables

To assess whether the vegetables as a whole contain additional genotoxic constituents, the lyophilisates of lettuce, beans and peas were tested in the micronucleus test in mouse lymphoma cells, alone and in combination with the known genotoxic (and carcinogenic) anthraquinone danthron (IARC, 1990; Müller *et al.*, 1996). None of the vegetable

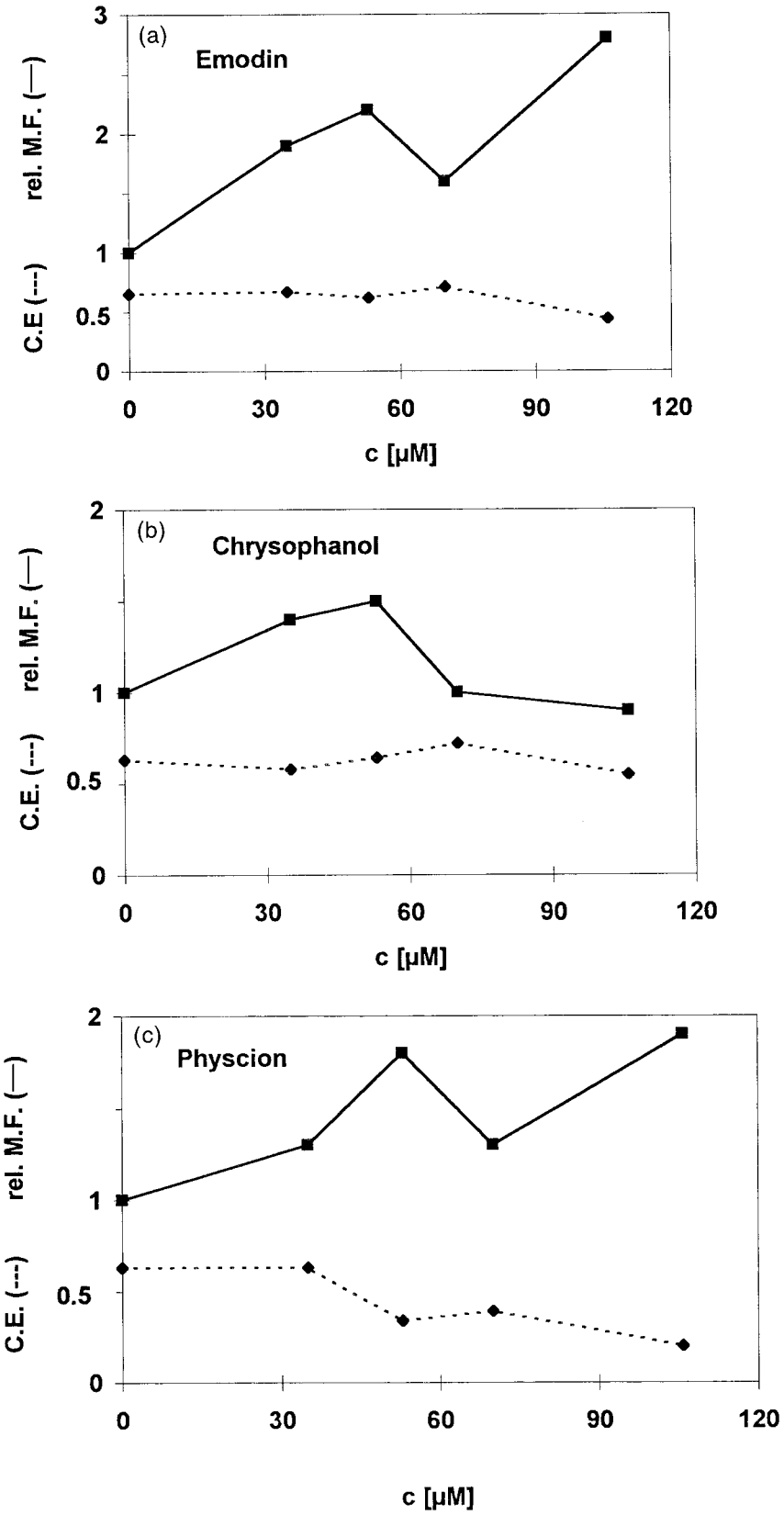

Fig. 3. Mutation assay with emodin, chrysophanol and physcion in mouse lymphoma $tk^{+/-}$ cells. Relative mutation frequencies (rel. M.F.) and cloning efficencies (C.E.) are given. The results of one of at least three, reproducible, experiments are shown. Control (1% DMSO): C.E.: 0.63; positive control (ethyl methanesulfonate 2 mM): rel M.F. 8.0; C.E. 0.52.

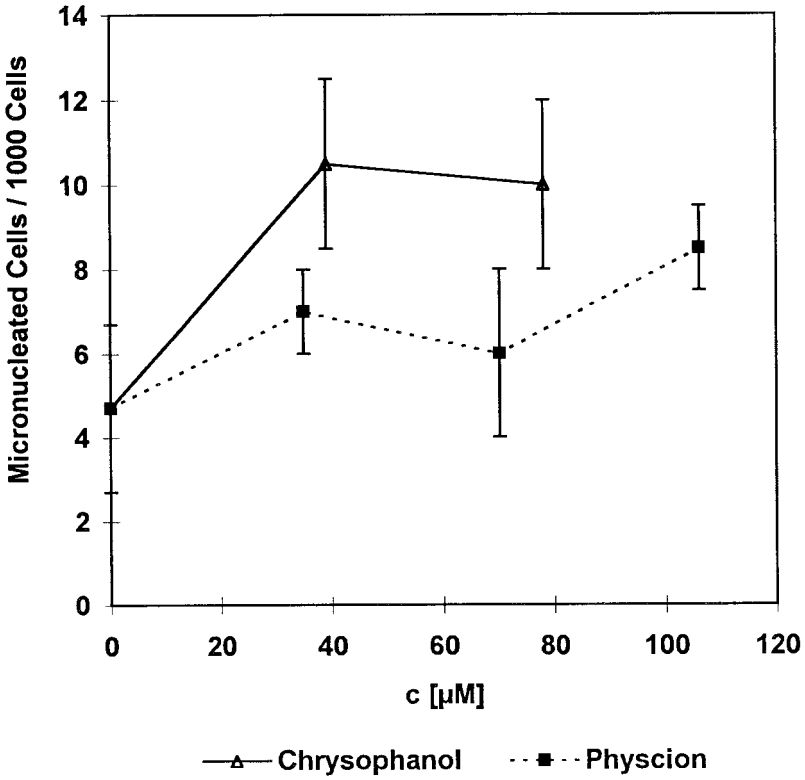

Fig. 4. Micronucleus test in mouse lymphoma cells with chrysophanol and physcion. Mean and standard deviation is given (n = 3). Positive control: ethyl methanesulfonate (2 mM): 20 micronucleated cells/1000 cells. The results of one of at least three, reproducible, experiments are shown.

lyophilisates increased the frequency of micronuclei (Fig. 5). Surprisingly, the genotoxicity of danthron was reduced significantly by the addition of the vegetable lyophilisates.

The inhibitory effect of the vegetables on the genotoxicity of danthron was further investigated by (i) using extracts of the lyophilisates and (ii) using the cytokinesis-block micronucleus test (CBMNT). With this modification of the assay, potential interference of the vegetable constituents with the cell cycle can be controlled for. Inhibition or delay of cell division could prevent formation of micronu-

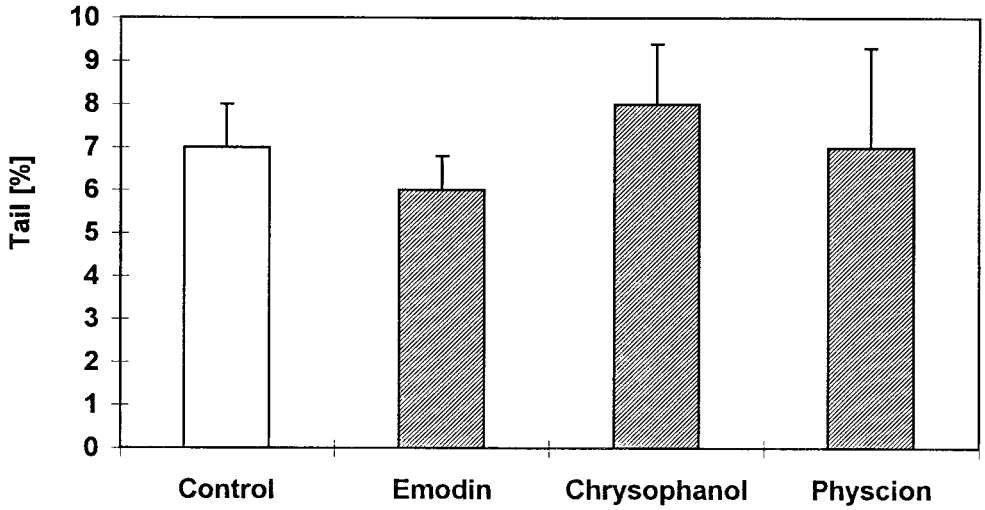

Fig. 5. Comet assay with mouse lymphoma cells. Control 1% DMSO (solvent control), emodin: 56 µM, chrysophanol 70 µM, physcion 70 µM. Median and standard error is given. The results of one of at least three, reproducible, experiments are shown.

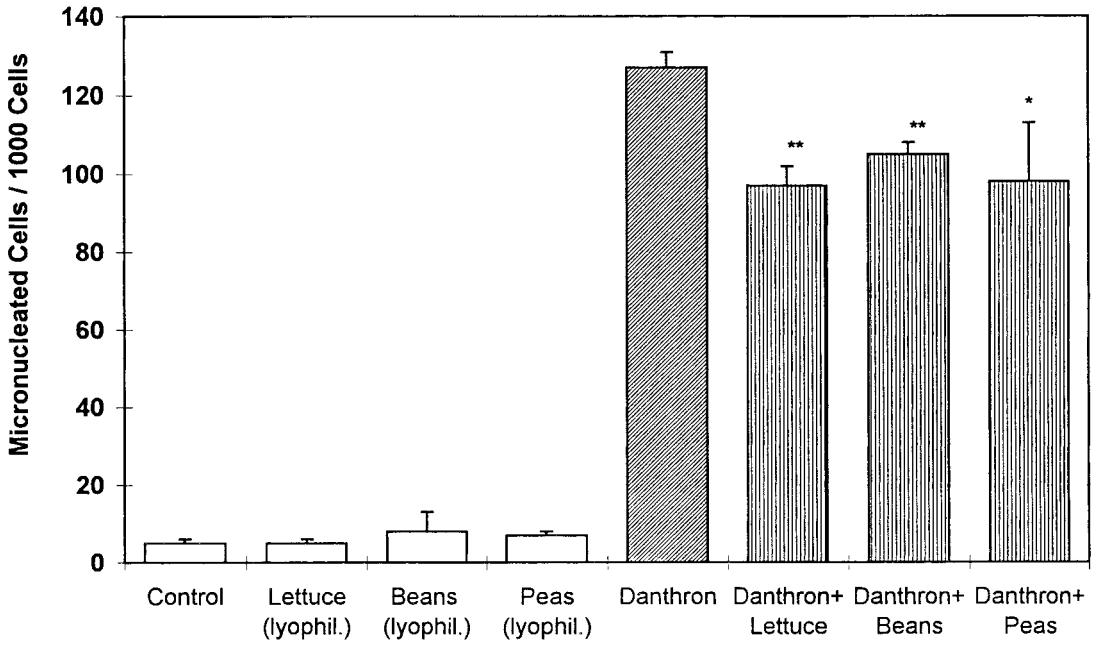

Fig. 6. Micronucleus test in mouse lymphoma cells with a lyophilisate of lettuce, beans and peas (250 μg/ml suspended in DMSO) and danthron (7.5 μg/ml) used as a positive control. Control: 1% DMSO. Mean and standard deviation is given (n = 3). Significant decreases of micronuclei frequencies of the coincubation (danthron + vegetable lyophilisate) compared to danthron alone are indicated by asterisks (Student's *t*-test: $^{**}P < 0.01$, $^{*}P < 0.05$). The results of one of at least three, reproducible, experiments are shown.

clei. Cytochalasin B is used to inhibit cytokinesis of the cells without affecting mitosis (Fenech, 1993). This results in binucleated cells. As micronuclei are only expressed after mitosis is completed, the CBMNT method offers the advantage to assess only the cells that have undergone mitosis. As a measure of a potential interference with the cell cycle, the ratio binucleated to mononucleated cells can also be determined.

The lettuce extracts (residue of the $CHCl_3$-extract suspended in DMSO, unfiltered and 0.2 μm-filtered) did not induce higher micronucleus frequencies as compared with the control (Fig. 6, open bars). The positive control danthron induced a high frequency of micronuclei as shown above. When co-incubated with danthron, both types of extracts produced a marked reduction of the micronucleus frequency. The unfiltered lettuce extract abolished the micronucleus induction completely, whereas the filtered extract inhibited the genotoxicity of danthron to a somewhat lower degree (Fig. 6). The ratio of binucleated cells to mononucleated cells (5.3 ± 0.3 in the controls) was not affected by any treatment, indicating the absence of an effect on the cell cycle.

DISCUSSION

We have shown for the first time the presence of 'free' (non-glycosidic) anthraquinones in a number of vegetables. Large batch-to-batch variability and the small number of analysed samples prevented a reliable estimation of an average exposure level. Based on the highest concentration detected, an intake of 4 mg anthraquinones in a serving of 100 g beans would be deduced. Furthermore, anthraquinone glycosides could be present at up to 10-fold higher concentrations, as determined for aloe-emodin and emodin in senna (Grimminger and Witthohn, 1993). For laxative drugs, the maximum therapeutic daily dose is 30 mg anthraquinone glycoside. For senna, a maximum of up to 4 mg anthraquinones per day is used (Kabelitz and Reif, 1994). Therefore, the dose of total anthraquinones that could be associated with the consumption of 100 g beans might occasionally be of the same order as the maximum daily dose of anthraquinones taken up with the use of a senna laxative.

For the herbs, human exposure to anthraquinones is expected to be negligible, in view of both concentrations determined and estimated daily amount of herbs used. The anthraquinones in liquors might be of interest only in the case of an abuse. Because of the observed ranking chrysophanol > emodin > physcion, also seen in rhubarb root extracts, the use of this root extract as a part of the herbal ingredients of the spirits can be assumed. However, for a toxicological assessment of liquors, the anthraquinone content is considered irrelevant.

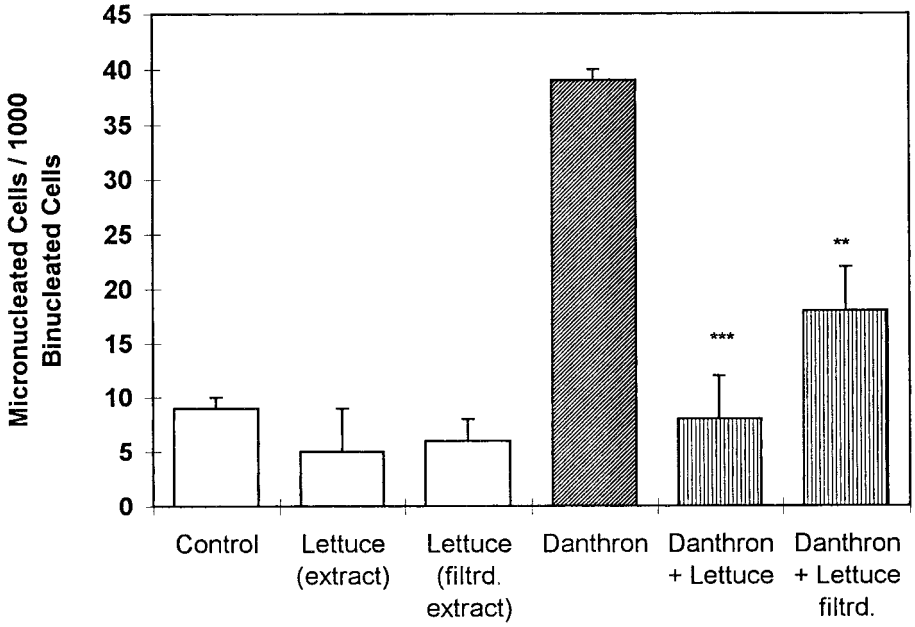

Fig. 7. Cytokinesis-block micronucleus test (CBMNT) in mouse lymphoma cells with lettuce extracts (250 μg/ml, unfiltered and 0.2 μm filtered), and with danthron (7.5 μg/ml), in the presence and absence of the extracts. All incubations were performed with 6 μg/ml cytochalasin B; control: 1% DMSO. Mean and standard deviation is given (n = 3). Significant decreases of micronuclei frequencies of the coincubation (danthron + lettuce) compared to danthron alone are indicated by asterisks (Student's *t*-test: $^{***}P < 0.001$; $^{**}P < 0.01$). The results of one of at least three, reproducible, experiments are shown.

For emodin, we have shown an unequivocal genotoxic potency in mammalian cells (Müller *et al.*, 1996). On the contrary, chrysophanol and physcion showed borderline effects in the tests used, and physcion was cytotoxic at higher concentrations. It should be mentioned here, however, that chrysophanol can be metabolized by a cytochrome P450-dependent reaction (Mueller *et al.*, 1998) to the known genotoxin aloe-emodin (Müller *et al.*, 1996). Therefore, a genotoxification pathway relevant for the situation *in vivo* may be deduced for chrysophanol.

Previously published data from mammalian mutation assays for anthraquinones by other investigators were not consistently positive (see for example Bruggemann and Van der Hoeven, 1984; Westendorf *et al.*, 1990). However, the earlier data related to the hemizygous hprt locus, a locus known to be less sensitive (especially for the detection of compounds that induce large deletions or multi-locus lesions; see McGregor *et al.*, 1996). In the present study, we used the highly sensitive heterozygous thymidine kinase (tk) locus in the L5187Y cell line (Combes *et al.*, 1995; Stopper and Müller, 1997).

Although three common vegetables have been shown to contain anthraquinones, data on their genotoxicity alone should not be used to derive a human health risk. The vegetables as a whole contain numerous other constituents some of which might also have protective effects (Ames, 1983; Mitscher *et al.*, 1996). We have shown that lyophilisates of lettuce, beans, and peas suspended in DMSO slightly, but significantly, reduced the induction of micronuclei by danthron. Chloroform extracts of unfiltered lyophilisates had an even stronger protective effect. The additional anti-genotoxic factors were possibly released only under heat and ultrasound as used in the extraction procedure.

In conclusion, together with the strong epidemiological evidence for a cancer-protective effect of vegetable consumption, it could be postulated that the mutagenicity from substances taken up with vegetables is more than compensated by protective effects. For the evaluation of a putative human health risk from dietary mutagens, the assessment should not be based exclusively on measured concentrations of mutagens.

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