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Genotoxic and antigenotoxic effects of catechin and tannins from the bark of *Hamamelis virginiana* L. in metabolically competent, human hepatoma cells (Hep G2) using single cell gel electrophoresis

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Abstract

The genotoxic and antigenotoxic activities of catechin, hamamelitannin and two proanthocyanidin fractions prepared from the bark of *Hamamelis virginiana* L. were investigated in a human derived, metabolically competent hepatoma cell line (Hep G2) using single cell gel electrophoresis (SCGE) for the detection of DNA-damage. DNA-migration was calculated as Olive tail moment (OTM). Catechin and a low-molecular weight proanthocyandin fraction (W_M) caused only slight increases of OTM up to concentrations of 166 μg/ml whereas hamamelitannin and the proanthocyandin fraction with higher molecular weight (W_A) led to a two-fold enhancement of OTM at the same concentrations. These effects were dose-independent. Treatment of the cells with the test compounds in a dose-range of 2–166 μg/ml prior to the exposure to benzo(a)pyrene (B(a)P, 10 μM, 2.5 μg/ml) led to a significant reduction of induced DNA damage which was dose-dependent for all test compounds, except for hamamelitannin. The inhibitory effects of proanthocyanidins were stronger than those of catechin and hamamelitannin; the lowest effective concentrations were about 2 μg/ml. In order to clarify the mechanisms of protection, possible effects of the test compounds on enzymes involved in toxification and detoxification of B(a)P were investigated. While B(a)P toxification by cytochrome P450 was not inhibited by the test compounds, detoxification by glutathion-*S*-transferase (GST) was induced by catechin and W_M. Combination experiments with the ultimate metabolite of B(a)P, (±)-*anti*-benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE; 5 μM, 1.5 μg/ml), revealed strong inhibitory effects, indicating that the observed protective effects were caused by scavenging of the ultimate mutagen by the test compounds. Exposure of Hep G2 cells to the test compounds after B(a)P treatment did not influence B(a)P induced DNA damage, demonstrating that repair mechanisms were not affected.

Keywords: *Hamamelis virginiana* L.; Hamamelidaceae; Catechin; Tannins; Proanthocyanidins; Genotoxicity; Antigenotoxicity; Hep G2 cells; SCGE assay

1. Introduction

Tannins are widely occurring secondary plant products. They are consumed by daily nutrition and occur also in a variety of herbal remedies. The overall daily intake of tannins is estimated to be 400–1000 mg (Desphande et al., 1984). Various reports (Hartmann and Shankel, 1990; Newmark, 1986) about the DNA-protective and anticarcinogenic properties and the lack of adverse effects of these compounds (Desphande et al., 1984) even in high doses designates them as promising chemopreventive agents.

Tannins exhibit antimutagenic activities against various directly and indirectly acting mutagens in vitro in various test systems (Okuda et al., 1984; Schimmer and Lindenbaum, 1995; Chen and Chung, 2000). Previously, we reported on the protective effects of condensed tannins from *Hamamelis virginiana* bark towards nitroaromatic

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mutagens (Dauer et al., 1998). In most of the studies, the *Salmonella* assay or other bacterial test systems were used for the detection of protective effects. However, the predictive value of procaryotic test systems is limited. Furthermore, these systems lack metabolizing enzymes involved in the metabolism of genotoxins and the addition of exogeneous metabolizing systems is necessary when promutagens are used. As tannins may form complexes with proteins (Bate-Smith, 1973) and may precipitate enzymes of the metabolizing system, false positive or artificial results might be obtained in such experimental models. Therefore, in the present investigation, human Hep G2 hepatoma cells were used for the detection of protective effects of tannins. Hep G2 cells have retained the activities of various phase I and phase II enzymes (Natarajan and Darroudi, 1991; Knasmüller et al., 1998) and reflect the metabolism of genotoxins better than bacteria or metabolic incompetent mammalian cells (CHO, V79).

The aim of the present study was the investigation of genotoxic and antigenotoxic effects of different low- and high-molecular tannins in Hep G2 cells. Therefore, as an exemplary promutagen/procarcinogen, benzo(a)pyrene (B(a)P) was chosen. B(a)P is widespread in the environment and occurs in food, ambient and indoor air. B(a)P leads to mutations, chromosome aberrations and sister chromatid exchanges and causes cancer in animals (Lijinsky, 1991). The promutagen B(a)P is activated by cytochrome P4501A1 and P4501A2 to the ultimate mutagen BPDE by twofold epoxidation (Marquardt, 1994), a compound which was also included in subsequent mechanistic investigations. As test compounds, different tannin-derived substances were used in order to deduce structure-activity relationships. To consider the structural diversity of tannins occuring in *Hamamelis virginiana* L. bark, the flavanol catechin, a monomeric unit of condensed tannins, the hydrolysable tannin hamamelitannin and two proanthocyanidin fractions, described by Dauer et al. (2003), were included in the study (Fig. 1).

2. Results and discussion

2.1. Cytotoxic effects

Polyphenols and tannins are known to exhibit cytotoxic effects towards mammalian cells (Kashiwada et al., 1992). In order to specify a non-toxic dose range for genotoxicity and antigenotoxicity studies, cytotoxicity of all test compounds (structures shown in Fig. 1) was evaluated by determination of cell viability and morphological assessment. Cytotoxicity of the polymeric proanthocyanidins W_M and W_A was significantly stronger than that of catechin and hamamelitannin (Fig. 2). Catechin was untoxic under test conditions up to 500 μg/ml and hamamelitannin up to 166 μg/ml, while the non-toxic doses of W_M and W_A were about 10-fold lower (50 μg/ml). No significant difference in cytotoxicity between W_M and W_A was observed (Fig. 2). An abrupt lack of viability could be seen with all test compounds at higher doses. Most likely, this phenomenon was caused by exceeding a toxic and lethal threshold concentration which is substance specific and is lower for the proanthocyanidin fractions compared to catechin and hamamelitannin. Probably, the known high astringency of oligomeric and polymeric proanthocyanidins (Bate-Smith, 1973) was responsible for this phenomenon (astringency describes the strength of the protein precipitating properties of a compound). In comparison, the astringency of catechin and hamamelitannin is very low (Gracza, 1987).

2.2. Genotoxic effects

Genotoxic effects were investigated in Hep G2 cells using single cell gel electrophoresis (SCGE). The extent of DNA-damage was quantified as Olive tail moment (OTM). B(a)P served as positive control. Whereas catechin and W_M caused only slight increases of OTM compared to the negative control (DMSO), treatment of the cells with hamamelitannin and W_A led to a two-fold increase of OTM at all doses tested. No significant dose-effect correlation was observed (Fig. 3a–d).

The fact that the test compounds caused DNA-migration by themselves was not unexpected because tannins have been described as DNA-damaging compounds in former studies. For example, a significant increase of mosaic spots in *Drosophila melanogaster* (SMART-test) was induced by tannic acid (Szakmary and Knasmüller, 1991). Sanyal et al. (1997) found an increase of micronuclei up to 50% in Hep G2 cells treated with 500 μg/ml tannic acid. Furthermore, induction of micronuclei in cultured V79 Chinese hamster cells by condensed tannins has been described, but due to significant decreases of cell viability at the same doses these results are questionable (Grimmer et al., 1992).

2.3. Antigenotoxic effects against B(a)P and BPDE induced DNA damage

In combination assays, the test compounds were investigated with respect to their antigenotoxic activity against B(a)P and BPDE induced DNA damage. In the DMSO control, the background values of OTM were between 0.62 and 1.85. Exposure of the cells to 10 μM B(a)P resulted in a mean OTM of 8.04–11.23. BPDE (5 μM) led to OTM values of 20.8–34.3.

Catechin, hamamelitannin, W_M and W_A caused significant inhibitory effects against B(a)P induced genotoxicity when the cells were pretreated with these

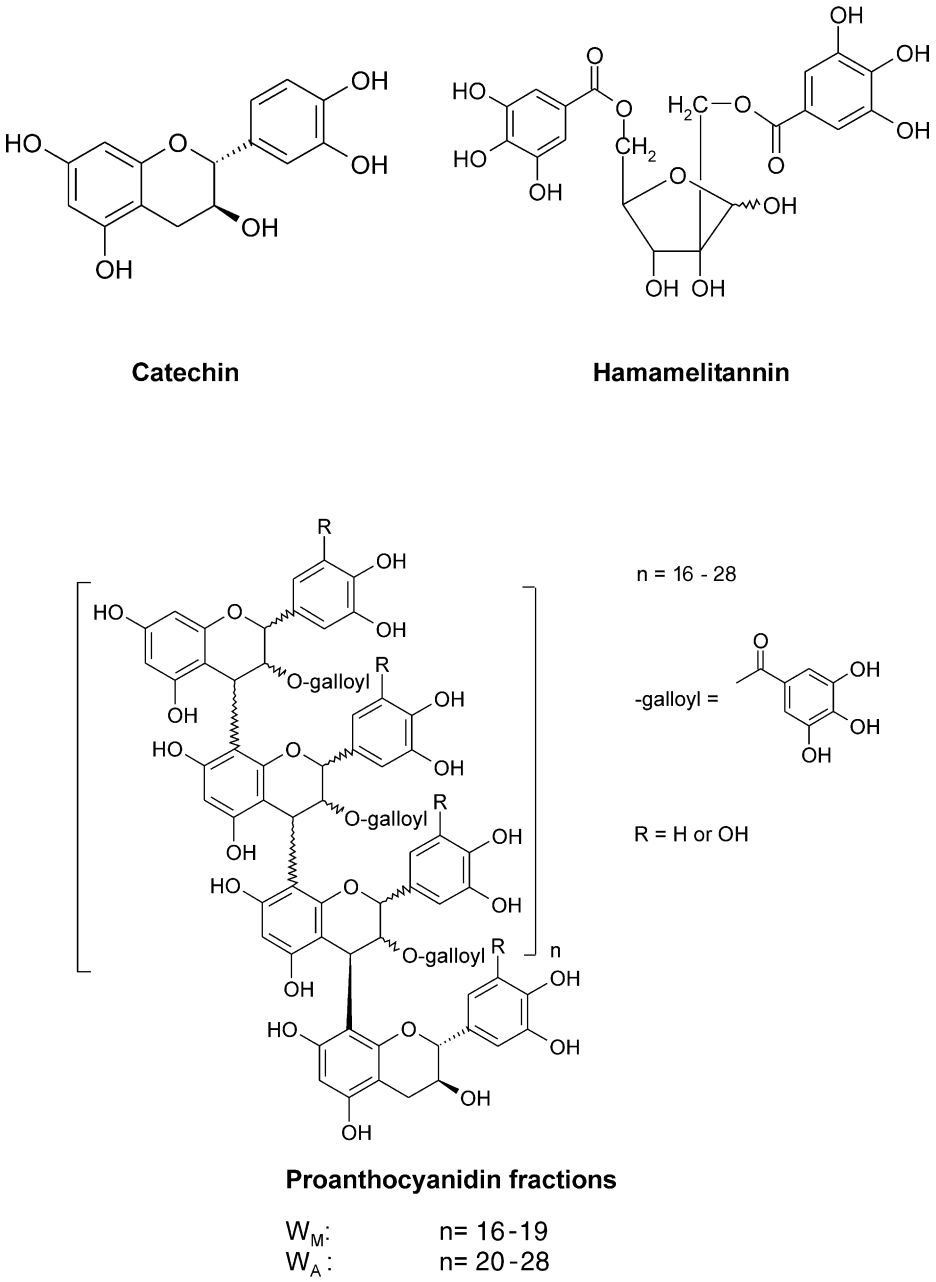

Fig. 1. Chemical structures of the test compounds.

compounds prior to exposure to the mutagen (Figs. 4 and 5). While hamamelitannin caused similar and significant inhibitory activities at all doses tested, catechin, W_M and W_A led to significant and dose-dependent decreases of B(a)P induced DNA damage. Pretreatment with catechin at a concentration of 18 μg/ml caused a 50% reduction of the B(a)P induced genotoxicity. The same concentration of hamamelitannin led to a 33% decrease. Polymeric proanthocyanidins showed even higher inhibition rates. 18 μg/ml of W_M resulted in a 66% reduction, which was the strongest effect observed in our experiments. W_A, the higher molecular weight product, was less active at the same concentration (53% inhibition).

In order to evaluate the mechanisms of protective effects, further experiments were performed. In post incubation experiments, Hep G2 cells were incubated with B(a)P prior to the addition of the test compounds. No inhibitory effects were observed with all test compounds (data not shown), indicating that DNA repair mechanisms were not affected. These findings were in contradiction to results reported previously for tannic acid. Sanyal et al. (1997) demonstrated a reduction of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) induced

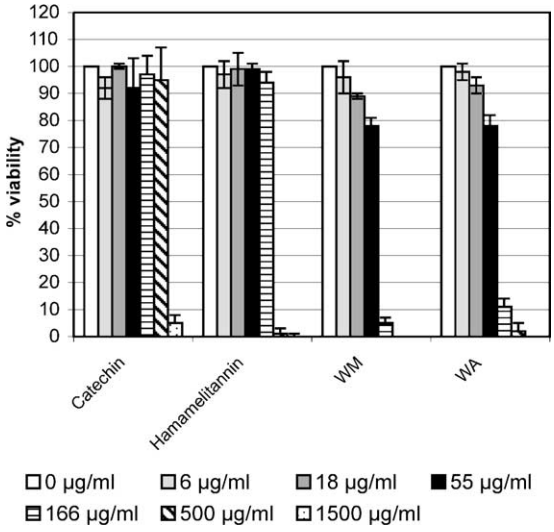

Fig. 2. Effects of catechin and tannins from *Hamamelis virginiana* on Hep G2 cell viability. Results are the mean ± S.D. ($n = 3$).

micronucleus frequencies in Hep G2 cells by 5-50 µg/ml tannic acid in posttreatment experiments. Posttreatment of Chinese hamster ovary cells with 3.3 µg/ml tannic acid for 24 h exhibited a 50–70% reduction of chromosome aberrations induced by UV rays, mitomycine C and methyl methane sulfonate (Sasaki et al., 1988). Similar effects were demonstrated with human embryonic cells and repair deficient human cells (Sasaki et al., 1989).

Protective effects could only be observed when cells were pretreated with the test compounds. Alteration of enzyme activities involved in toxification or detoxification of the promutagen B(a)P or direct interactions of the test compounds with B(a)P or its reactive metabolites thus could be responsible for the effects. To clarify the underlying mechanism, cells were analyzed for CYP4501A (ethoxyresorufin dealkylation) and GST activities after incubation with the test compounds.

None of the test compounds caused an inhibition of CYP4501A (Fig. 6a). Therefore, inhibition of B(a)P

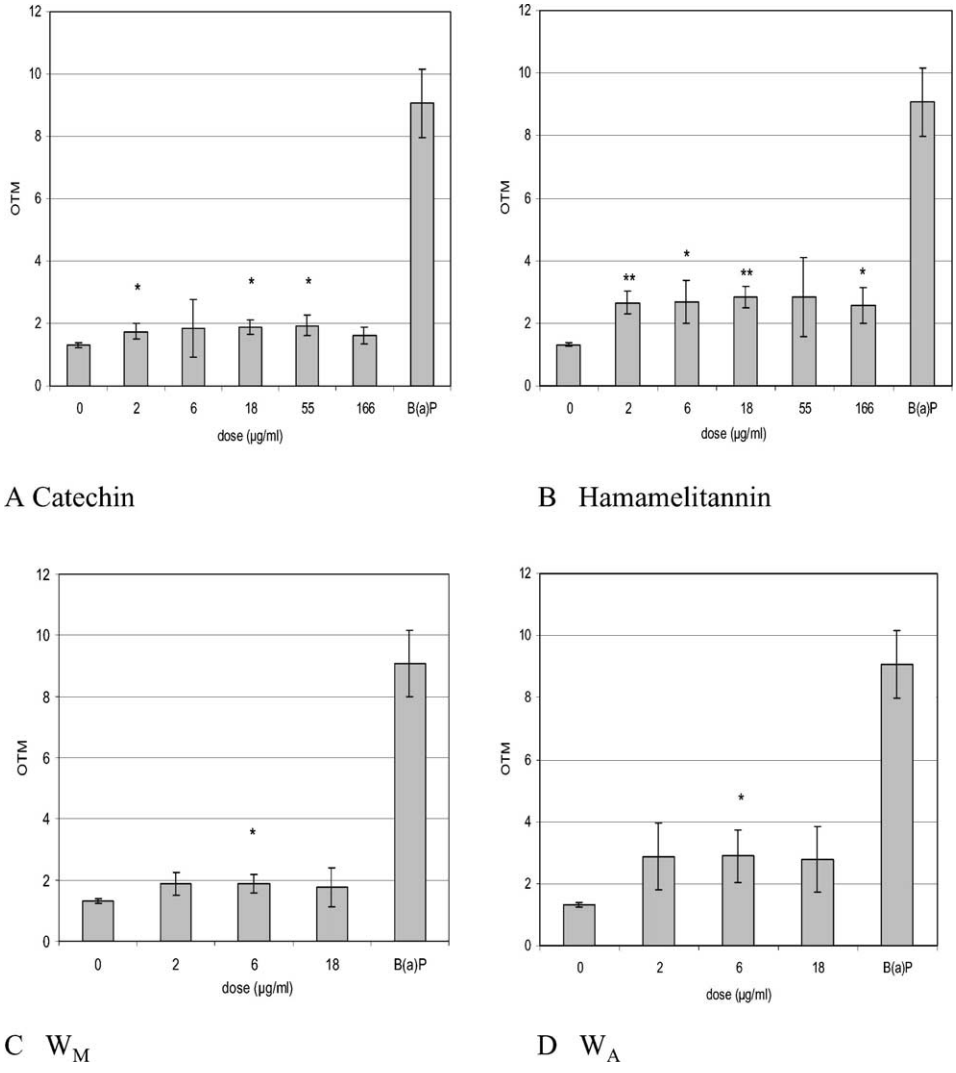

Fig. 3. Effects of catechin and tannins from *Hamamelis virginiana* on OTM using SCGE with Hep G2 cells. Results are the mean ± S.D. ($n = 3$). Stars indicate statistical significance versus control (Students test; *: $P < 0.05$,**: $P < 0.01$).

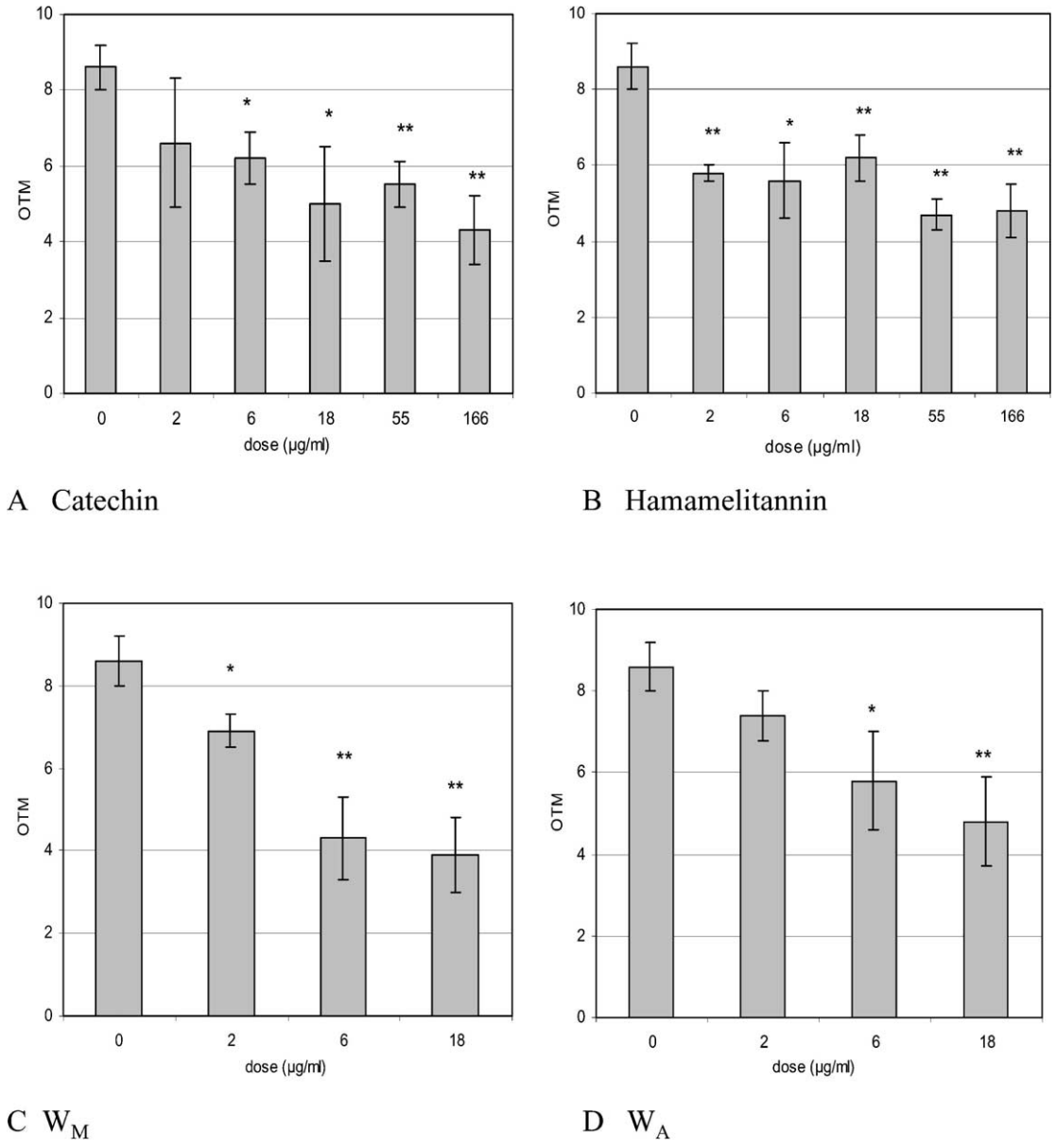

Fig. 4. Effects of catechin and tannins from *Hamamelis virginiana* on B(a)P (10 µM) induced DNA damage using SCGE with Hep G2 cells, method: pretreatment. Results are the mean ± S.D. ($n = 3$). Stars indicate statistical significance versus control (Students test; *: $P < 0.05$, **: $P < 0.01$).

toxification could not be responsible for the antigenotoxic effects. In contrary, induction of CYP4501A was found with W_M, W_A and hamamelitannin. These findings were in contradiction to observations made with *Salmonella typhimurium* where inhibitory effects on CYP450 activities by catechin and polymeric proanthocyanidins have been reported (Desphande et al., 1984; Nagabhushan et al.; 1988; Catterall et al., 2000). Tannins are known to possess protein precipitating properties. Decrease of enzyme activities might have been caused by denaturation of proteins of the exogeneous metabolizing system in these studies, giving false positive and artificial results.

Analysis of glutathione-*S*-transferase (GST) activity after treatment of the cells with the test compounds revealed an induction of GST by catechin and W_M. W_A and hamamelitannin had no significant effects (Fig. 6b). Therefore, only for catechin and W_M an enhancement of B(a)P detoxification by GST induction could be a possible mechanism of protection. In previous studies, an induction of GST by tannic acid could be shown in vivo when tannic acid was fed to mice (Athar et al., 1989).

In subsequent tests, BPDE was used instead of B(a)P in order to clarify whether the phenolic compounds interacted directly with the ultimate mutagen. In these experiments, cells were preincubated with the test compounds. BPDE was then added to the medium without removing the tannins, allowing extra- and intracellular reactions of the test compounds with BPDE. Strong

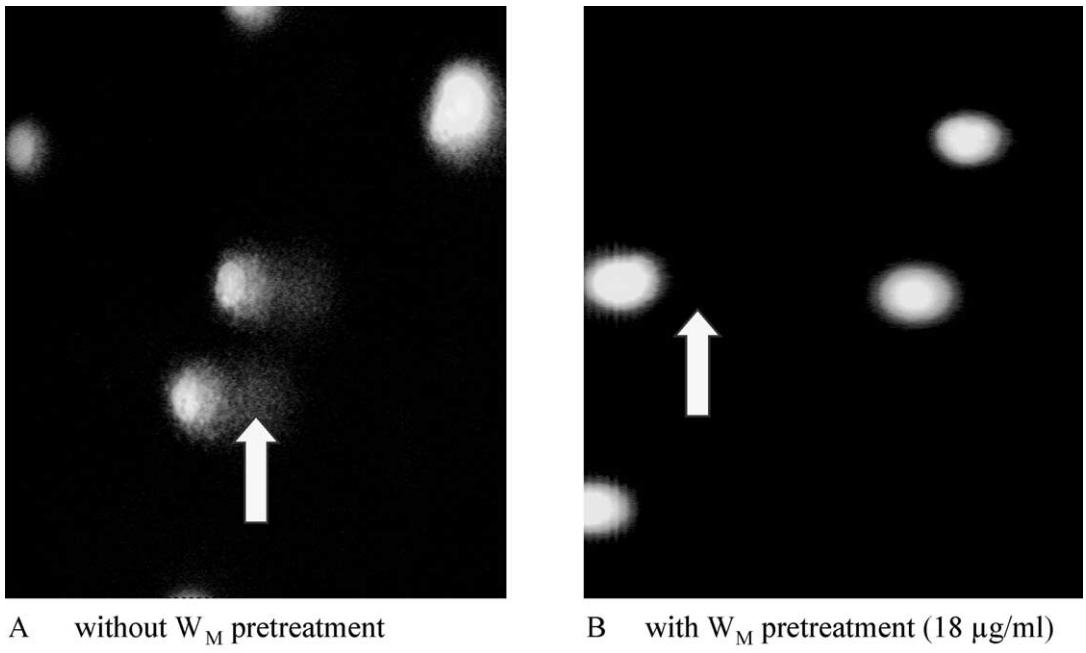

Fig. 5. Effect of W_M pretreatment (18 µg/ml) on B(a)P induced comet formation in Hep G2 cells. The arrow indicates the site of comet formation respectively the lack of comet formation.

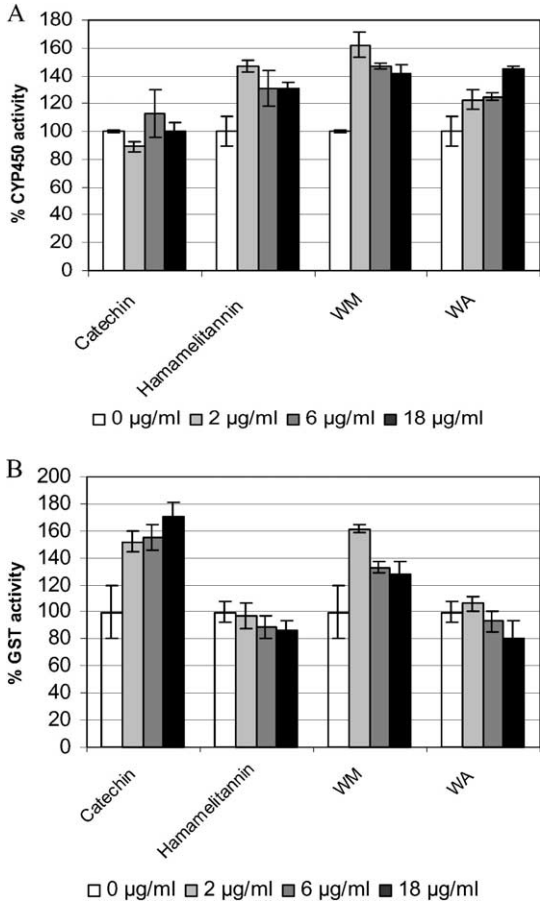

Fig. 6. Effects of catechin and tannins from *Hamamelis virginiana* on cytochrome P4501A (6a) and GST (6b) enzyme activities in Hep G2 cells. Results are the mean ± S.D. ($n = 2$).

inhibitory effects with inhibition rates up to 90% were found with catechin and the tannins (Fig. 7). These results led to the conclusion that scavenging of BPDE was the main mechanism of the observed protective effects. As the flavanol catechin, which is not a tannin, was effective, too, an intact proanthocyanidin (polyflavanol) structure seemed not to be necessary for the effect. As hamamelitannin, not possessing a flavanol structure, also was effective, we concluded that a flavanol structure was not a structural requirement for protective effects. Probably, the observed effects were based on a di- or trihydroxyphenol structure. Likely, the test compounds inactivated BPDE by adduct formation. In previous studies, catechin has been reported to interact directly with B(a)P metabolites and to inhibit the binding of [^{3}H]-B(a)P metabolites to calf thymus DNA (Nagabhushan et al., 1988). The reaction of polyphenolic compounds with BPDE was also shown with ellagic acid (Wood et al., 1982) and other hydrolysable tannins. It was demonstrated that polyphenols reacted with ultimate mutagens of polycyclic aromatic hydrocarbons by nucleophilic attack of the epoxide structure to yield phenol-mutagen-adducts. Because polyphenols are proton donors, they also may catalyze the hydrolytic degradation of BPDE (Okuda et al., 1984).

Besides enzymatic toxification pathways, free oxygen radicals also may contribute to the bioactivation of B(a)P (Marquardt, 1994). Catechin and tannins are known to possess antioxidative properties. Hence, although it was demonstrated that an inactivation of the ultimate mutagen BPDE was the main mechanism of protection, it cannot be excluded that antioxidative

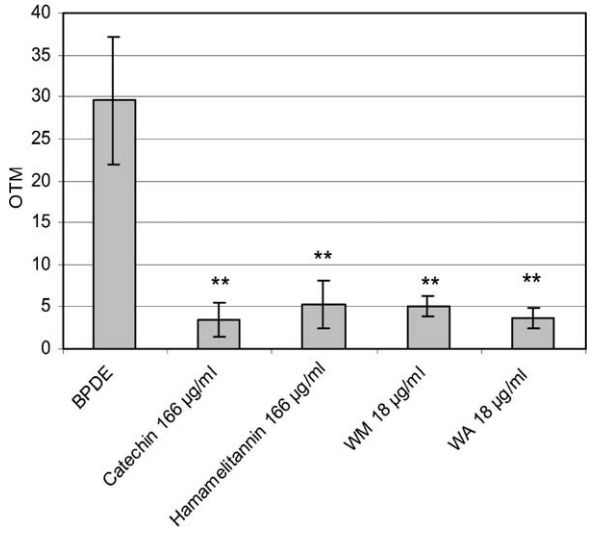

Fig. 7. Effects of catechin and tannins from *Hamamelis virginiana* on BPDE induced DNA damage (5 µM) using SCGE with Hep G2 cells, method: combined pre- and simultaneous treatment. Results are the mean ± S.D. ($n = 3$). Stars indicate statistical significance versus control (Students test; **: $P < 0.01$).

properties of the test compounds may contribute to the observed antigenotoxic effects.

In conclusion, it could be demonstrated that catechin and tannins from the bark of *Hamamelis virginiana* L. exhibit protective effects against B(a)P and BPDE induced DNA damage in Hep G2 cells by inactivation of the ultimate mutagen. Various contradictions to earlier findings (mainly in bacterial test systems) stress the importance to use adequate test systems. In the case of tannins, systems which require the addition of exogeneous metabolic activation systems seem not to be suitable. Additionally, it was obvious that the Hep G2 cell line is a suitable laboratory model for the detection of genotoxicity and antigenotoxicity of tannins. Especially high molecular proanthocyanidins, which are very widespread in herbal remedies, fruits and vegetables and which have not yet been investigated extensively due to their difficult analytical approach, may contribute to a reduction of genetic damage caused by genotoxins present in air and daily nutrition.

3. Experimental

3.1. Test compounds

(+)-Catechin and hamamelitannin were purchased from C. Roth (Karlsruhe, Germany). Extraction and isolation of polymeric proanthocyanidins from *Hamamelis* bark was reported previously (Dauer et al., 2003). In brief, powdered bark (400 g) was percolated with acetone-water (7:3; 2.5 l). After filtration, acetone was removed in vacuo (40 °C) and the aqueous layer was extracted with petroleum benzene (4 × 300 ml, discarded) and subsequently with ethyl acetate (5 × 300 ml, discarded). The aqueous residue was freeze-dried to yield 47 g of solid, brownish product (W). 18.0 g W were chromatographed on Sephadex LH-20 (column 500 × 45 mm) with ethanol (8.5 l) (W_E, yield: 7.61 g), methanol (6.0 l) (W_M, yield: 5.30 g) and acetone-water (7:3; 2 l) (W_A, yield: 2.39 g). W_E contained about 60% carbohydrates and dimeric to oligomeric proanthocyanidins. W_M and W_A contained polymeric proanthocyanidins.

3.2. Proanthocyanidin analysis

Phytochemical characterization of polymeric proanthocyanidins (W_M and W_A) was reported previously (Dauer et al., 2003). Qualitative composition of polymeric proanthocyanidins was determined by complete thiolytic degradation, isolation of degradation products and structure elucidation by ^{1}H-NMR spectroscopy. W_M and W_A are procyanidin-prodelphinidin-copolymers with a di- and tri-hydroxylation of the B-ring at a ratio of about 1.3:1. The polymers are completely galloylated at position 3, except for the chain terminating units which are catechin (95%) and gallocatechin (5%). 2,3-*cis* Stereochemistry within the chain predominates. Determination of molecular weight was performed by two independent methods: complete thiolytic degradation, separation of the degradation products by HPLC and calculation of the degree of polymerisation by the ratio of integrals of thioethers to flavanols. In comparison, non-aqueous gel permeation chromatography of the peracetates in $CHCl_3$ (polystyrol calibration) was performed. Molecular weights found by thiolysis were 7400–8900 for W_M (DP of 17–20) and 9500–13,000 for W_A (DP 21–29).

3.3. Hep G2 cell culture and single cell gel electrophoresis (SCGE)

3.3.1. Chemicals and solutions for SCGE

B(a)P, ethidium bromide and DMSO were from Sigma (Deisenhofen, Germany), Agarose was from Merck (Darmstadt, Germany). (±)-*anti*-Benzo(a)pyrene-7,8-diol-9,10-epoxide (BPDE) was kindly provided from the "Biochemisches Institut für Umweltkarzinogene" (Großhansdorf, Germany). Dulbeccos minimal essential medium (DMEM) and phosphate buffered saline (pH 7.4) were from Gibco (Paisley, Scotland). All other chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany). Lysis solution contained 2.5 M NaCl, 0.2 M NaOH, 100 mM EDTA-Titriplex and was supplemented with 10% DMSO and 1% Triton X-100. The composition of the electrophoresis buffer was 0.3 M NaCl, 0.04 M NaOH and 1 mM EDTA-Titriplex. For additional information see Singh et al. (1988).

3.3.2. Hep G2 cell culture

The Hep G2 cells were kindly provided by the German Cancer Research Center, Heidelberg. Stock cultures of the cells (in DMEM with 10% DMSO) were stored at −140 °C.

Hep G2 cell culture was carried out according to the methods described previously (Uhl et al., 1999). For experiments, culture medium was removed from the culture flasks when cells formed a confluent cell layer at the bottom of the flasks. Cells were detached with trypsin (0.1%, 1 ml per flask). The number of viable cells was determined with a counting chamber (Neubauer) after staining with Erythrosine B. Subsequently, the cells were subcultivated in multiwells (Falcon, 12 wells, 3 ml culture medium/well, 5×10^5 cells/well). After 24 h incubation at 37 °C and 5% CO_2, test compounds or mutagen (dissolved in DMSO) were added (the final DMSO concentration was 1% at all dose levels).

3.3.3. Determination of cytotoxicity and genotoxicity

For the determination of acute toxicity, the cells were exposed to the test compounds in concentrations between 2 and 1500 μg/ml for 24 h. Cell morphology was then evaluated microscopically and viability was determined with a counting chamber after staining with Erythrosine B. Only doses that did not cause changes in cell morphology and led to viability rates >80% were tested in the SCGE experiments.

For evaluation of genotoxicity the cells were exposed to the test compounds in a concentration range between 2 and 166 μg/ml for 24 h, followed by SCGE.

3.3.4. Determination of antigenotoxic effects towards B(a)P and BPDE

3.3.4.1. Combination experiments with B(a)P. B(a)P was used at a dose of 2.5 μg/ml (10 μM) in all experiments. For the pretreatment experiments, cells were exposed to the test compounds for 24 h. After removal of the medium, cells were washed twice with PBS and then exposed to B(a)P in fresh culture medium another 24 h. For posttreatment, cells were exposed to B(a)P for 24 h. Subsequently, after removal of the medium and washing, cells were exposed to the test compounds in fresh medium for another 24 h.

3.3.4.2. Combination experiments with BPDE. In order to investigate direct interactions of catechin and tannins with BPDE a combined pretreatment and simultaneous treatment was performed. BPDE was used at a dose of 1.5 μg/ml (5 μM). The cells were incubated with the test compounds for 24 h. Afterwards, without removing the medium, BPDE was added for another 24 h.

3.3.5. Single cell gel electrophoresis (SCGE)

SCGE was performed on the basis of the guidelines developed by Tice et al. (2000) and according to the protocol of Uhl et al. (1999). After exposure of the cells to the putative protective compounds and/or B(a)P or BPDE, the culture medium was removed from the wells; the cells were washed twice with PBS, removed with trypsin (0.1%, 200 μl/well) and resuspended in fresh culture medium. The cell suspension was passed through a syringe (0.8 × 40 mm) to isolate the cells. Then the number of viable cells was determined. Aliquots of cell suspensions containing 1×10^5 viable cells were centrifuged (10 min, 350 *g*). The pellets were subsequently resuspended in 100 μl agarose (0.7%, 35 °C) and spread on glass slides precoated with agarose 0.5%. The slides were covered with a coverslip and allowed to solidify on ice. After a few minutes, the coverslips were removed and the slides were placed in lysis solution for 1 h. The slides were then placed in 0.3 M NaOH for 20 min and were subjected to alkaline electrophoresis (25 V, 300 mA, 25 min, 4 °C). After neutralisation with TRIS buffer, the slides were exposed to ethidium bromide (25 μM in water, 80 μl/slide) for DNA-staining and covered with a coverslip.

3.3.5.1. Evaluation of results. The slides were evaluated under a fluorescence microscope (Leica DMLS, objective: PL Fluotar 40x / 0.70 PH2) with a commercially available image analysis system (KOMET® Version 3.1, Kinetic Imaging Ltd., Liverpool, UK). The dimension of comet formation was scanned by a CCD-camera and calculated as Olive tail moment (Olive et al., 1990). Each experiment was performed in triplicate (three independent cell cultures on different days). For each dose, 102 cells were evaluated for comet formation, and mean and standard deviation of OTM was calculated. Statistical significance was evaluated using the Student's test (unpaired test).

3.3.6. Enzyme measurements

GST activity was measured according to Habig et al. (1974) using 1 mM 1-chloro-2,4-dinitrobenzene as substrate. The formation of the glutathion conjugate at 25 °C was followed for 10 min at 340 nm. The CYP4501A activity was assayed using 10 nM ethoxyresorufin as a substrate. The formation of resorufin at 37 °C was followed for 40 min (excitation wavelength 522 nm, emission was measured at 586 nm) (Prough et al., 1978). The protein content was assayed according to Lowry et al. (1951). Due to low enzyme activities in Hep G2 cells (Grant et al., 1988), measurements require high cell numbers. Therefore, experiments were performed in duplicate with two independent cell cultures on different days. For each dose, 2 photometric measurements were performed.

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References

Athar, M., Khan, W.A., Mukthar, H., 1989. Effect of dietary tannic acid on epidermal, lung and forestomach polycyclic aromatic hydrocarbon metabolism and tumorigenicity in SENCAR mice. Cancer Research 49, 5784–5788.

Bate-Smith, E.C., 1973. Haemanalysis of tannins: the concept of relative adstringency. Phytochemistry 12, 907–912.

Catterall, F., Souquet, J.-M., Cheynier, V., Clifford, M.N., Ioannides, C., 2000. Modulation of the mutagenicity of food carcinogens by oligomeric and polymeric procyanidins isolated from grape seeds: synergistic genotoxicity with *N*-nitrosopyrrolidine. Journal of the Science of Food and Agriculture 80, 91–101.

Chen, S.-C., Chung, K.-T., 2000. Mutagenicity and antimutagenicity studies of tannic acid and its related compounds. Food and Chemical Toxicology 38, 1–5.

Dauer, A., Metzner, P., Schimmer, O., 1998. Proanthocyanidins from the bark of *Hamamelis virginiana* exhibit antimutagenic properties against nitroaromatic compounds. Planta Medica 64, 324–327.

Dauer, A., Rimpler, H., Hensel, A., 2003. Polymeric proanthocyanidins from the bark of *Hamamelis virginiana* L. Planta Med. 69, 89–91.

Desphande, S.S., Sathe, S.K., Salunkhe, D.K., 1984. Chemistry and safety of plant polyphenols. Advances in Experimental Medicine and Biology 177, 457–495.

Gracza, L., 1987. Adstringierende Wirkung von Phytopharmaka. Deutsche Apotheker Zeitung 127, 2256–2258.

Grant, M.H., Duthie, S.J., Gray, A.G., Burke, M.D., 1988. Mixed function oxidase and UDP-glucuronosyltransferase activities in the human Hep G2 hepatoma cell line. Biochemical Pharmacology 37, 4111–4116.

Grimmer, H.R., Parbhoo, V., McGrath, R.M., 1992. Antimutagenicity of polyphenol-rich fractions from *Sorghum bicolor* grain. Journal of the Science of Food and Agriculture 59, 251–256.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione-*S*-transferases. The first enzymatic step in mercapturic acid formation. Journal of Biological Chemistry 249, 7130–7139.

Hartmann, P.E., Shankel, D.M., 1990. Antimutagens and anticarcinogens: a survey of putative interceptor molecules. Environmental Molecular Mutagenesis 15, 145–182.

Kashiwada, Y., Nonaka, G., Nishioka, I., Chang, J., Lee, K., 1992. Antitumor agents, 129. Tannins and related compounds as selective cytotoxic agents. Journal of Natural Products 55, 1033–1043.

Knasmüller, S., Parzefall, W., Sanyal, R., Ecker, S., Schwab, C., Uhl, M., Mersch-Sundermann, V., Williamson, G., Hietsch, G., Langer, T., Darroudi, F., Natarajan, A.T., 1998. Use of metabolically competent human hepatoma cells for the detection of mutagens and antimutagens. Mutation Research 402, 185–202.

Lijinsky, W., 1991. The formation and occurrence of polynuclear aromatic hydrocarbons associated with food. Mutation Research 259, 251–261.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Ranfall, R.J., 1951. Protein measurements with the Folin-Phenol reagents. Journal of Biological Chemistry 193, 265–275.

Marquardt, H., 1994. Chemische Kanzerogenese. In: Marquardt, H., Schäfer, S.G. (Eds.), Lehrbuch der Toxikologie. BI-Wissenschaftverlag, Mannheim, pp. 124–147.

Nagabhushan, M., Amonkar, A.J., Nair, U.J., Santhanam, U., Ammigan, N., D'Souza, A.V., Bhide, S.V., 1988. Catechin as an antimutagen: its mode of action. Journal of Cancer Research and Clinical Oncology 114, 177–182.

Natarajan, A.T., Darroudi, F., 1991. Use of human hepatoma cells for in vitro metabolic activation of chemical mutagens/carcinogens. Mutagenesis 6, 399–403.

Newmark, H.L., 1986. Plant phenolics as inhibitors of mutational and precarcinogenic events. Canadian Journal of Physiology and Pharmacology 65, 461–466.

Okuda, T., Mori, K., Hayatsu, H., 1984. Inhibitory effects of tannins on direct-acting mutagens. Chemical and Pharmaceutical Bulletin 32, 3755–3758.

Olive, P.L., Banath, J.P., Durand, R.E., 1990. Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells using the "comet" assay. Radiation Research 122, 86–94.

Prough, R.A., Burke, M.D., Mayer, R.T., 1978. Direct fluorometric methods for measuring mixed function oxidase activity. Methods in Enzymology 52, 372–377.

Sanyal, R., Darroudi, F., Parzefall, W., Nagao, M., Knasmüller, S., 1997. Inhibition of the genotoxic effects of heterocyclic amines in human derived hepatoma cells by dietary bioantimutagens. Mutagenesis 12, 297–303.

Sasaki, Y.F., Imanishi, H., Ohta, T., Watanabe, M., Matsumoto, K., Shirasu, Y., 1988. Suppressing effect of tannic acid on UV and chemically induced chromosome aberrations in cultured mammalian cells. Agricultural and Biological Chemistry 52, 2423–2428.

Sasaki, Y.F., Imanishi, H., Ohta, T., Watanabe, M., Matsumoto, K., Shirasu, Y., 1989. Suppressing effect of tannic acid on the frequencies of mutagen-induced sister-chromatid exchanges in mammalian cells. Mutation Research 213, 195–203.

Schimmer, O., Lindenbaum, M., 1995. Tannins with antimutagenic properties in the herb of *Alchemilla* species and *Potentilla anserina*. Planta Medica 61, 141–145.

Singh, M.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Experimental Cell Research 175, 184–191.

Szakmary, A., Knasmüller, S., 1991. Effects of tannic acid on spontaneous and induced somatic mutations in *Drosophila melanogaster*. Mutagenesis 6, 225–228.

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.-C., Sasaki, Y.F., 2000. Single cell gel / comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environmental Molecular Mutagenesis 35, 206–221.

Uhl, M., Helma, C., Knasmüller, S., 1999. Single-cell gel elctrophoresis assays with human-derived hepatoma (Hep G2) cells. Mutation Research 441, 215–224.

Wood, A.W., Huang, M.-T., Chang, R.L., Newmark, H.L., Lehr, R.E., Yagi, H., Sayer, J.M., Jerina, D.M., Conney, A.H., 1982. Inhibition of the mutagenicity of bay-region diol epoxides of polycyclic aromatic hydrocarbons by naturally occurring plant phenols: exceptional activity of ellagic acid. Proceedings of the National Academy of Sciences USA 79, 5513–5517.