DOI:10.1021/jf904150x

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Polyphenolic Apple Extracts: Effects of Raw Material and Production Method on Antioxidant Effectiveness and Reduction of DNA Damage in Caco-2 Cells

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A diet rich in fruits and vegetables is commonly perceived to be associated with reduced cancer risk, attributed to its high content of polyphenols. As apples represent a major polyphenol source in Western countries, we studied differentially produced extracts (1−100 μg/mL): two from different apple juices (AEs), one from pomace (APE), and one peel extract (PE) on their potential to reduce DNA oxidation damage and induce antioxidant defense in Caco-2 cells. Additionally, we measured direct antioxidant capacity (TEAC/ORAC) of the extracts. Quercetin-rich PE and APE most effectively diminished DNA damage and ROS level after 24 h incubation (PE > APE), whereas the AEs were only moderately effective. GPx activity was diminished for all extracts, with AEs > APE > PE. Direct antioxidant activity decreased in the order AEs > PE > APE, displaying no significant correlation with cellular markers. In conclusion, apple phenolics at low, nutritionally relevant concentrations may protect intestinal cells from ROS-induced DNA damage, mediated by cellular defense mechanisms rather than by antioxidant activity.

KEYWORDS: Apple polyphenol extract; DNA damage; antioxidant activity; cellular ROS level; GPx activity; peel; pomace; TEAC; ORAC

INTRODUCTION

Colorectal cancer is the third most common cancer in the world, with prevalence mainly in high-income countries. Especially with respect to this cancer site, food and nutrition are perceived to play a substantial role for prevention and causation. Enhanced consumption of fruits, vegetables and dietary fiber together with physical activity appear related to reduced risk of this disease (*1*).

Since the initiation progress of carcinogenesis involves mutations of the DNA, chemical alteration of DNA bases is supposed to be a crucial factor. As a consequence of increased oxidative stress, DNA oxidation damage can occur with reactive oxygen species (ROS) such as hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), or singlet oxygen ($^1O_2$), leading to mispairing of DNA bases or DNA strand breaks. ROS are generated endogenously from cellular metabolism and inflammatory responses or by exposure to exogenous agents such as ionizing radiation and xenobiotics (*2*, *3*).

Prevention from oxidative stress may be achieved by the uptake of antioxidants. Besides vitamins, polyphenols are powerful dietary antioxidants present in many fruits, vegetables, and their products. Polyphenols consist of different classes with phenolic acids and flavonoids being most frequent in foods, with total intake estimates up to about 1 g/day (*4*). They can act as antioxidants in two ways: by scavenging free radicals and chelation of redox active metal ions (direct antioxidant activity) and by inducing cellular antioxidant defense and repair. A major source of these polyphenols in Western countries are apples and apple juices (whose major polyphenols are depicted in **Figure 1**), with a polyphenol content up to about 5 g/kg fresh weight and 1 g/L, respectively (*5*). Studies in ileostomy patients (*6*) gave insight into the uptake of polyphenols in vivo: Up to 33% of polyphenols originating from 1 L of apple juice containing 250 mg of polyphenols (excluding oligomeric procyanidins) were reported to reach the ileostomy bags/colon after oral uptake. The concentration range examined in our study is representative for this concentration range that is achievable in the colon after apple juice uptake.

Epidemiological studies support a preventive potential of apples and apple juice, especially for lung and colorectal cancer (*7*, *8*), diabetes, and cardiovascular disease (*9*). During pressing, mainly water-soluble polyphenols such as dihydrochalcones or hydroxycinnamic acids are transferred into the juice, whereas the extraction of other (less water-soluble) polyphenols, such as flavonoids, is more difficult. To this end, we used a pomace

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 Published on Web 05/10/2010

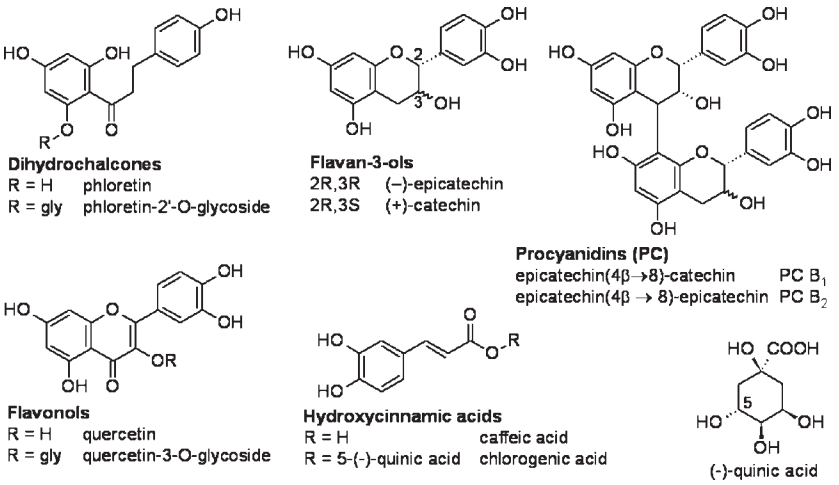

Figure 1. Lead compounds of apple extract polyphenols.

extraction technique described in detail elsewhere (*10*): briefly, apple pomace (the residue of the ground and once pressed apples) is treated with cellulase and pectinase enzymes and then pressed again. Furthermore, an extract derived from extraction of flavonol-rich apple peel (*11*) was included in the experiments.

In this study, the potential of polyphenolic extracts from apple juices (AE05, AE06), apple pomace extraction juice (APE), and apple peel (PE) to protect against DNA oxidation damage was assessed in Caco-2 colon carcinoma cells by Comet assay. To discriminate DNA oxidation damage and DNA strand breaks, the assay was performed with/without the repair enzyme formamidopyrimidine-DNA-glycosylase (FPG). To gain information on the potential induction of cellular antioxidant defense, modulation of *tert*-butyl hydroperoxide (TBH)-induced cellular ROS level and cellular glutathione peroxidase (GPx) activity was monitored. GPx is a major ROS decomposing enzyme, regulated by the antioxidant responsive element (ARE), a regulatory sequence involved in the activation of genes associated with protection against oxidative stress, phase II biotransformation, and other cancer-chemoprotective mechanisms (*12*). Additionally, we measured direct antioxidant activity of polyphenolic apple extracts by TEAC and ORAC assay, reflecting major mechanisms of antioxidant action (single electron transfer and hydrogen atom transfer) (*13*), to evaluate their relevance for cell protection. To correlate antioxidant efficacy of the extracts to their polyphenol composition, analytical characterization of the extracts was performed.

MATERIALS AND METHODS

Chemicals, Cells, and Media. All reagents were purchased from Sigma-Aldrich/Fluka (Taufkirchen, Germany). Solvents and chemicals were of analytical grade or complied with the standards needed for cell culture experiments. Caco-2 cells were obtained from Deutsche Sammlung fuer Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany) and medium (1:1 mixture of DMEM with Ham's Nutrient Mix F12: DMEM/F12), fetal calf serum (FCS) and penicillin/streptomycin from Invitrogen (Karlsruhe, Germany). Consumables (flasks, Petri dishes, 96-well plates, etc.) were purchased from Greiner Bio-One (Essen, Germany), except for black 96-well plates, provided from Nunc (Langenselbold, Germany).

Preparation and Analysis of Polyphenol Rich Apple Juice Extracts. Polyphenol rich extracts AE05 and AE06 were obtained from clear apple juices, mainly originating from cider apple varieties harvested in 2005 (AE05) and in 2006 (AE06) (*14*); APE and PE were manufactured from once pressed apple pomace (cv. Bittenfelder, harvested 2006) and apple peels (cv. Granny Smith), respectively. Briefly, after crushing and extraction, the juices were separated and filtered. After adsorption of the polyphenols on adsorber resins and elimination of sugars, organic acids and minerals with water, the polyphenol fraction was eluted with ethanol, concentrated, freeze-dried and stored cool, excluding light and moisture (*14*, *15*). For APE, after the first crushing, pomace was treated with pectinases and cellulase before extraction as described (*10*, *15*). For the production of PE, table apple peels were extracted with hot water; subsequently this aqueous extract was loaded on the adsorber column and treated as described above.

Polyphenols, oligosaccharides and the amount of oligomeric procyanidins in the extracts were determined using HPLC-DAD (diode array detector), HPAEC (high performance anion exchange chromatography), and photometric detection of total anthocyanidins (after procyanidin hydrolysis), respectively (*14*).

Antioxidant Capacity of the Extracts (TEAC and ORAC Assay). Antioxidant capacity of the extracts was assessed by TEAC and ORAC assay with Trolox as antioxidant standard.

TEAC assay was performed using the ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) radical decolorization assay as described (*16*, *17*). Briefly, phenolic extracts, dissolved in DMSO, were mixed with ABTS radical solution (absorbance adjusted to 0.700 ± 0.02) and allowed to stand for 6 min at 30 °C prior to absorbance reading at 734 nm using a multiplate reader (Synergy 2; BioTek, Bad Friedrichshall, Germany). ABTS radicals were generated by treatment of an aqueous ABTS solution with potassium persulfate. Solvent controls and Trolox standard curve (0−15 μmol/L) were run in each assay. Percentage decolorization and TEAC value were calculated as described (*16*).

ORAC was measured using the method of Ou et al. (*18*). Briefly, apple juice extracts and Trolox standards (0−500 μmol/L) were mixed with fluorescein solution in black 96-well plates and equilibrated at 37 °C. After 10 min, AAPH (2,2′-azobis(2-methylpropionamidine)dihydrochloride) was added and the decrease of fluorescence was monitored for 90 min (ex/em 485/528 nm) using a multiplate fluorescence reader (Synergy 2; BioTek, Bad Friedrichshall, Germany). The area under curve and ORAC values were calculated as described (*18*).

ORAC and TEAC values are expressing the concentration of a Trolox solution (in mmol/L) having an antioxidant capacity equivalent to an extract solution of 1 mg/mL.

Cell Culture and Incubation. Caco-2 cells were maintained in 175 cm^2 flasks in DMEM/F12 (1:1) supplemented with 20% FCS, 100 U/mL penicillin and 100 μg/mL streptomycin, at 37 °C, 5% CO_2 and 95% relative humidity. For the experiments, cells were seeded in Petri dishes or cell culture plates in DMEM/F12 (1:1), supplemented with 20% FCS and penicillin/streptomycin. After a 24 h growing period, cells were incubated with polyphenolic extracts (dissolved in DMSO) in serum reduced medium (10% FCS; final DMSO concentration 0.1%) for another 24 h. Under these conditions, cell viability and artifactual H_2O_2 generation in the medium were ascertained to be > 80% (trypan blue exclusion assay) and < 2 μM (FOX1 assay), respectively (*14*).

DNA Damage (Comet Assay). To detect DNA damage, the alkaline single-cell gel electrophoresis (Comet assay) was performed as described (*2*, *19*). Briefly, 2.5×10^5 cells per 6 cm Petri dish were incubated as described above. For induction of DNA (oxidation) damage, cells were treated with the redox-cycler menadione (Md, 6 μmol/L, 1 h in serum-free medium) and isolated by trypsin treatment (*20*). Thereafter, $4 \times 50{,}000$ cells were centrifuged, and the pellet was mixed with low melting agarose, applied onto a precoated microscope slide, coverslipped and kept on ice until solidification. After removal of the cover glass, slides were immersed in lysis buffer overnight at 4 °C. Thereafter, slides were washed and covered either with enzyme buffer or with FPG enzyme solution, sealed with a cover glass and incubated at 30 min at 37 °C. The FPG protein recognizes oxidized purine bases, cuts them out and nicks the DNA at the respective sites, resulting in additional DNA damage (*21*, *22*). After DNA unwinding (pH 13.5, 20 min, 4 °C) and horizontal gel electrophoresis (20 min, 25 V, 300 mA), slides were washed, stained with ethidium bromide and analyzed using a fluorescence microscope (Axioskop 20, filter set 15, Zeiss, Germany) with computerized image analysis (Comet Assay IV, Perceptive Instruments, Suffolk, GB), scoring 2×50 cells per slide. DNA migration was expressed as mean tail intensity (TI: DNA in the comet tail in percent of total DNA). Results are given as relative TI in % of Md-treated control.

Intracellular ROS Level (DCF Assay). Modulation of cellular ROS level after incubation with polyphenols was quantified by dichlorofluorescin (DCF) assay according to Wang and Joseph (*23*), with slight modifications (*14*). Briefly, 2.4×10^4 cells/well were seeded in 96-well plates and incubated as described above. Subsequently, cells were washed with PBS, treated for 30 min with 2′,7′-dichlorofluorescein diacetate (DCFH-DA; final concentration 50 μM in PBS pH 7.0; 1% DMSO v/v), washed and incubated with the oxidant *tert*-butyl hydroperoxide (TBH, 250 μM in PBS) for 30 min at 37 °C. The increase of fluorescence (FI), resulting from oxidation of the probe 2′,7′-dichlorofluorescein to the fluorescent DCF, was measured at 0 and 30 min after TBH addition in a microplate reader (ex 485 nm; em 525 nm; Synergy 2; BioTek, Bad Friedrichshall, Germany). All treatments and fluorimetric determination were performed in the dark. FI was calculated as described (*23*) and expressed as relative FI in % of TBH-treated control.

GPx Activity. Cells (1.5×10^6 seeded in 9 cm Petri dishes) were incubated as described above, harvested using trypsin/EDTA (0.5% v/v), submitted to cell lysis with PBS + 0.1% Triton X-100 and centrifuged (10000*g*, 15 min, 4 °C). In the supernatant, protein was determined (BCA protein quantification kit, Uptima, Monluçon, France) as well as GPx activity, according to Paglia and Valentine (*24*). This photometric activity assay is based on reduction of TBH by GPx under GSH oxidation and subsequent reduction of GSSG by glutathione reductase/NADPH. Briefly, an aliquot of the supernatant was added to PBS (62.5 mM NaH_2PO_4, 6.25 mM EDTA), containing 0.25 mM NADPH, 2.5 mM GSH, 1.25 U/mL glutathione reductase, and 1.25 mM sodium azide. The GPx-independent decay of NADPH was then monitored for 5 min at 340 nm at 37 °C. After addition of TBH, NADPH consumption was measured again (5 min, 340 nm). Calculation of GPx activity was performed as described (*24*), and GPx activity was expressed as U/mg protein, with 1 unit defined as reduction of 1 μmol of NADPH per minute. Results are given as relative activity in % of solvent control.

Statistics. Data on biological markers and antioxidant capacity were obtained from $n \geq 3$ independent experiments, each performed at least in duplicate. Results were analyzed for significant difference ($p < 0.05$) to the respective oxidant-treated (Comet assay, DCF assay) or solvent control (GPx assay) by Student's *t* test (unpaired, two-sided).

Linear regression analysis was used to assess correlations between antioxidant capacity values and extract composition. Results are expressed as coefficient of correlation *R* (Pearson coefficient), with $R = 0$ displaying no linear correlation at all and $R = 1$ (or -1) exhibiting a perfect positive (or inverse) linear correlation.

RESULTS AND DISCUSSION

Composition and Antioxidant Capacity of the Extracts. Analytical data of the extracts are summarized in **Table 1**. Polyphenols (including oligomeric procyanidins) represent the major proportion of extract constituents (58−88%), consisting of oligomeric procyanidins and low molecular phenolics (flavan-3-ols, dihydrochalcones, hydroxycinnamic acids, and quercetin glycosides). The pattern of polyphenols/procyanidins varied substantially between the extracts: Whereas AE05 is rich in hydroxycinnamic acids (especially chlorogenic acid), oligomeric procyanidins are the predominant constituents of AE06. As quercetin glycosides are predominantly located in the apple peel (*11*), PE is especially rich in these compounds. The low amount of hydroxycinnamic acids in the PE produced from the apple variety Granny Smith is consistent with the distinctly low concentration of these compounds in table apples, compared to cider apples (*5*). Oligosaccharides, found as minor constituents (9−38%), are supposed to be transferred into the extracts attached to the polyphenol fraction (*25*), whereas unbound sugars are known to be efficiently eliminated during extract production by rinsing the adsorber resin with water (*26*). The pomace extract APE contains especially high amounts of oligosaccharides, presumably as a result of cellulase/pectinase treatment before extraction.

Table 1. Antioxidant Capacity and Composition (% of Total Extract) of the Apple Extracts AE05, AE06, APE, and PE[a]

	AE05	AE06	APE	PE
	Antioxidant Capacity (mM Trolox)[b]			
ORAC	6.1	6.1	4.5	5.8
TEAC	4.1	6.5	3.4	4.7
	Composition (%)			
monomeric polyphenols	37.7	31.0	22.7	26.0
flavan-3-ols	1.7	8.1	5.2	4.8
phloretin glycosides	5.7	3.8	3.4	3.6
hydroxycinnamic acids	30.0	18.7	13.6	1.7
quercetin glycosides	0.4	0.3	0.6	16.3
oligomeric procyanidins[c]	24.0	57.0	35.0	44.0
polyphenols + procyanidins	61.7	88.0	57.7	70.0
oligosaccharides	13.0	8.7	38.2	24.0
total	74.7	96.7	95.9	94.0

[a] Analytical data of AE05, AE06, and APE taken from Bellion et al. (*14*) for comparison. [b] TEAC and ORAC values are given in mmol/L Trolox (concentration of a Trolox solution having the same antioxidant capacity as a 1 mg/mL solution of the extract). [c] Data represent the sum of oligomeric procyanidins (photometric analysis after acid hydrolysis).

All extracts exhibited distinct antioxidant capacities, with TEAC and ORAC values of 3.4−6.5 and 4.5−6.1 mM Trolox, respectively, at 1 mg/mL extract concentrations (**Table 1**). Both assays displayed an almost similar ranking for the extracts, AE06 being most effective, followed by AE05 and PE; the lowest antioxidant capacity was obtained with APE. This suggests that both modes of antioxidant action studied (hydrogen atom transfer and single electron transfer (*13*)) contribute almost equally to the antioxidant activity of the complex polyphenol mixture in the extracts.

Furthermore, antioxidant capacity (ORAC) of selected extract polyphenols, representing different phenolic subgroups, was assessed and compared to the respective published TEAC values (*15*): (−)-epicatechin (ORAC/TEAC: 3.9/3.2 mM Trolox), phloridzin (5.4/3.6), phloretin (4.1/3.8), chlorogenic acid (4.2/1.3), caffeic acid (4.3/1.3), rutin (5.5/3.7), and quercetin (6.9/4.8). Highest effectivity was observed for the flavonols quercetin and rutin, which, however, might not contribute to the extract antioxidant capacity, due to their low amounts, except for PE. Other constituents present in higher concentrations, such as chlorogenic acid or phloridzin, despite lower TEAC/ORAC values might well contribute.

Linear regression analysis (**Table 2**, left column) showed a distinct positive correlation between ORAC values of the extracts

Table 2. Correlation between Extract Antioxidant Capacity (ORAC/TEAC) and Composition, Obtained by Linear Regression Analysis. The Value given is the Coefficient of Correlation *R*, the One in Parentheses is the Respective *p* Value.[a]

	AE05, AE06, APE, PE ($n = 4$)		extended no. of extracts ($n = 9$)[b]	
	ORAC	TEAC	ORAC	TEAC
monomeric polyphenols	0.779 (0.212)	0.248 (0.743)	0.895 (0.001)	−0.201 (0.612)
flavan-3-ols	−0.047 (0.949)	0.720 (0.294)	−0.330 (0.385)	0.679 (0.049)
phloretin glycosides	0.533 (0.456)	−0.129 (0.879)	0.651 (0.059)	−0.315 (0.411)
hydroxycinnamic acids	0.287 (0.698)	0.001 (0.992)	0.600 (0.083)	−0.178 (0.641)
quercetin glycosides	0.154 (0.860)	−0.021 (0.997)	−0.175 (0.640)	0.031 (0.907)
oligosaccharides	−0.941 (0.056)	−0.749 (0.243)	−0.837 (0.004)	−0.493 (0.173)
oligomeric procyanidins	0.220 (0.788)	0.844 (0.161)	0.098 (0.785)	0.715 (0.031)
procyanidins + polyphenols	0.603 (0.399)	0.996 (0.005)	0.614 (0.072)	0.479 (0.192)

[a] Left column: regression analysis based on data from **Table 1**. Right column: extended analysis with values from 9 different polyphenol-rich apple extracts. [b] Data from studied apple extracts (AE05, AE06, APE, PE) and from five previously described extracts (AE01−04; AE07) (*14*, *15*).

(including data from five previously described extracts (AE01−04 and AE07) (*14*, *15*)) and their amount of hydroxycinnamic acids and dihydrochalcones, representing the major proportions of monomeric polyphenols. Correspondingly, a linear dependence of ORAC results on the concentration of total low molecular weight polyphenols was obtained. TEAC values were mainly correlated with the concentration of flavan-3-ols and their oligomers, the procyanidins. For the oligosaccharide fraction, a strong inverse correlation to extract antioxidant capacity was found.

These findings were strengthened by an extended regression analysis (**Table 2**, right column), which included other apple juice extracts (AE01−04; AE07) with known composition and TEAC values (*14*, *15*). ORAC of AE01, AE02, AE03, AE04, AE07 (6.6, 6.7, 5.9, 6.9, and 6.3 mM Trolox) and TEAC of AE07 (5.3 mM Trolox) were also determined. The results support the above-described relevance of total monomeric polyphenols, especially hydroxycinnamic acids and dihydrochalcones on ORAC values of the extracts, whereas TEAC was probably stronger influenced by oligomeric procyanidins and flavan-3-ols. This agrees with the known values for the monomers catechin/epicatechin (3.2/3.6 mM Trolox (*27*)) and their dimers procyanidin B_1/B_2 (6.5/7.6 mM Trolox (*27*)). Since the latter showed nearly twice the antioxidant capacity of the monomers, apple procyanidins with a higher degree of polymerization may exhibit still stronger antioxidant capacities.

Modulation of DNA Damage. After 24 h incubation, all extracts significantly reduced Md-induced DNA strand breaks, predominantly at low concentrations (1−10 μg/mL; **Figure 2**). Among the extracts, PE was the most effective, exhibiting a preventive potential over the full concentration range tested (**Figure 2d**) with a maximum reduction down to 46% followed by the pomace extract APE (maximum reduction down to 53%) and the apple juice extracts AE05 and AE06 (down to 50 and 72%, respectively).

Similar concentration−effect curves were also observed for total DNA damage (strand breaks plus FPG-sensitive sites) (**Figure 2**). Relative tail intensities, however, were approximately doubled by FPG treatment, suggesting that FPG-sensitive (oxidative) DNA modifications count for about 50% of total damage. DNA protecting effects, evoked by 1−10 μg extract/mL, were nullified or even reversed at increasing concentrations (10−100 μg/mL).

We do not attribute this increased DNA damage to extracellular peroxide generation, since previous experiments (24 h incubation of apple juice extracts in DMEM/F12 medium) showed at best marginal formation (< 2 μM) as well as efficient decomposition of H_2O_2 (*14*). At higher extract concentration, however, intracellular ROS production (*28*) might well contribute to the observed augmentation of DNA damage.

To some extent, the observed efficacy of the extracts against DNA damage can be attributed to specific phenolic constituents (*15*). In PE, for example, the outstanding amount of quercetin glycosides is supposed to account for its high DNA protective potential since quercetin has been identified as efficient reducer of DNA damage in Caco-2 cells (*15*, *29*). Correspondingly, the other (less effective) extracts contain practically no quercetin glycosides but high amounts of chlorogenic acid (13−30%), found to be ineffective against DNA damage (*15*).

Cellular ROS Level. Modulation of cellular ROS level in Caco-2 cells after 24 h incubation with apple extracts is shown in **Figure 3**. PE was the most effective, reducing cellular ROS level by 30% at all concentrations tested (1−100 μg/mL); its strong activity is supposed to be due to the high amount of quercetin glycosides (16.3%), as quercetin was found to exhibit distinct ROS-reducing potential under the same conditions (*20*). AE06 and APE (1−3 and 1−30 μg/mL, respectively) were also potent reducers of TBH induced ROS level; at higher concentrations, however, an increase of ROS level was observed, which could result from intracellular prooxidative effects, similar to the elevation of DNA damage shown above and in previous publications (*15*). AE05 was practically ineffective, except for a slight reduction by 12% at 100 μg/mL. For AE05, which exhibited high TEAC and ORAC values similar to PE, direct antioxidant capacity did not cause a reduction of ROS level at the concentration range tested.

GPx Activity. Modulation of GPx activity after incubation with apple extracts is shown in **Figure 4**. All extracts concentration dependently downregulated GPx activity to a various extent: lowest reduction of GPx activity was caused by PE (significant at ≥30 μg/mL), followed by APE (significant at ≥10 μg/mL). AE05 and AE06 were the most effective inhibitors of GPx activity, showing significant reduction already at concentrations ≥3 μg/mL. It is not clear at present whether the observed GPx inhibition by apple polyphenols is based on direct compound−gene/protein interaction or on polyphenol-mediated effects (such as ROS generation).

Similar to our findings, a reduction of GPx as well as catalase (CAT) and superoxide dismutase (SOD) activity was described at incubation of HL60 cells with the flavonoid fisetin (*30*). Furthermore, the authors assumed fisetin-mediated generation of ROS; beneficial and/or toxic actions of fisetin, however, were ascribed to modulation of signaling cascades rather than to its antioxidant potential (*30*). After H_2O_2 treatment of HeLa cells, GPx, CAT, and SOD activities were downregulated to a differential extent, dependent on the nature of ROS (*31*).

On the other hand, a rise of GPx activity was reported after short-time incubation of Caco-2 cells with moderate H_2O_2 concentrations (*32*). Correspondingly, GPx activity is known to be

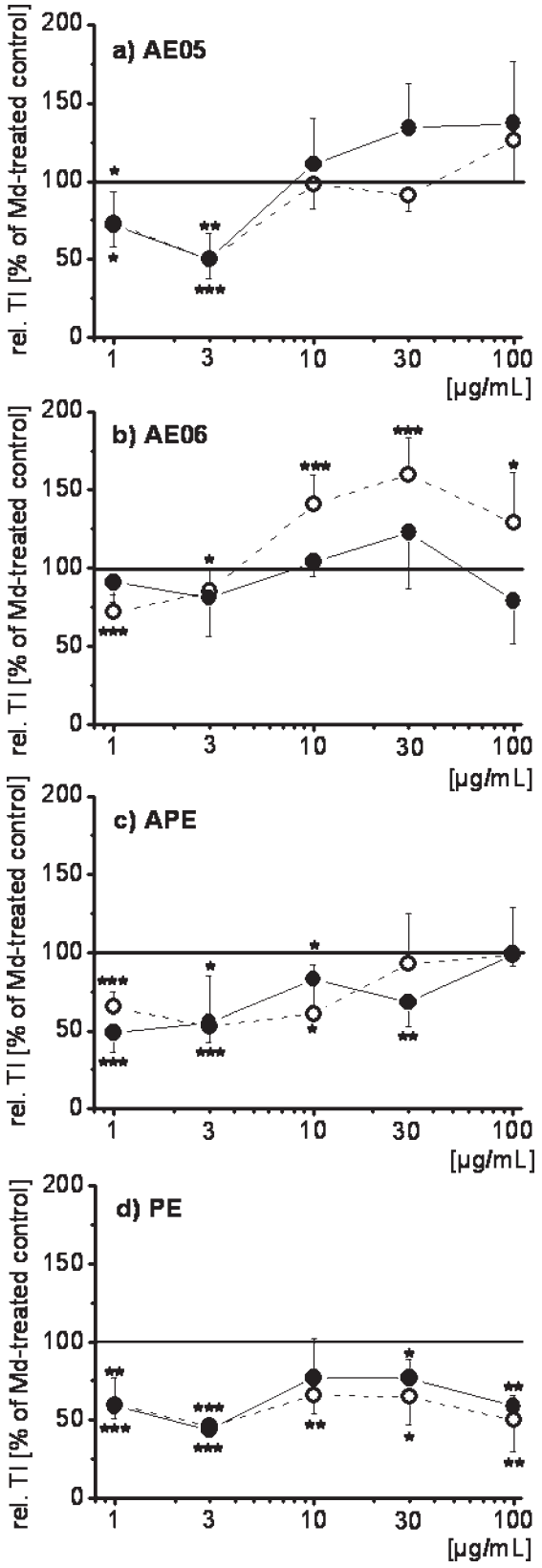

Figure 2. Menadione (Md)-induced DNA damage in Caco-2 cells after 24 h incubation with the apple extracts (**a**) AE05 (**b**) AE06, (**c**) APE, and (**d**) PE, in % of Md-treated control (relative TI). Open circles, DNA strand breaks (− FPG); full circles, total DNA damage (+ FPG); = strand breaks + DNA oxidation damage. Mean and SD of $n = 3-5$ independent experiments. Mean TI values were 0.96%/1.8% (−/+ FPG) for the untreated controls and 5.2%/10.3% (−/+ FPG) for the Md-treated controls. Significant change to Md-treated control: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

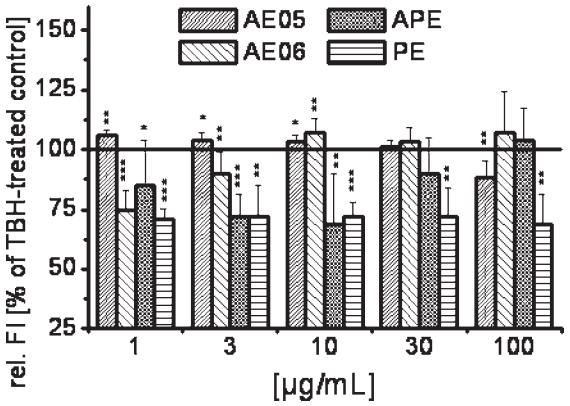

Figure 3. Modulation of TBH-induced cellular ROS-level in Caco-2 cells after 24 h incubation with apple extracts; $n = 3-5$ (mean ± SD); significantly lower than TBH-treated solvent control: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

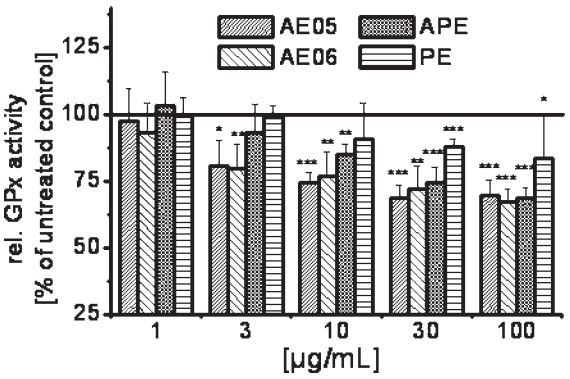

Figure 4. Modulation of GPx activity in Caco-2 cells after 24 h incubation with apple extracts; $n = 3-5$ (mean ± SD); significantly different from solvent control: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

increased when culturing cells with elevated oxygen tension (*33*) as is the case already under standard cell culture conditions, compared to the *in vivo* situation (approximately 150 mmHg vs 1−10 mmHg) (*34*). Thus, the observed downregulation of antioxidant defense by polyphenols might also be a consequence of intracellular ROS scavenging, leading to a decreased oxidant status. Decreased GPx activity may partially impede the cellular protection against oxidative stress in the challenge assays (Comet and DCF assay), as extracts with higher potential to reduce GPx activity diminished oxidative DNA damage and cellular ROS level less effectively.

Taken together, the polyphenol-rich apple extracts from juice (AE05, AE06), pomace (APE), and peel (PE) exhibited a distinct cell free antioxidant capacity (ORAC and TEAC) in the order AE06 > PE ≈ AE05 > APE, according to their total amount of monomeric polyphenols and oligomeric procyanidins. Monomeric polyphenols with distinct antioxidant capacity strongly account for the antioxidant capacity of the extracts; especially the amounts of hydroxycinnamic acids/dihydrochalcones and of flavanols were found directly correlated to the observed ORAC and TEAC values, respectively. Since procyanidins are also strong antioxidants (*27*), they are similarly supposed to contribute to the observed TEAC/ORAC values. For example, AE06, containing 57% oligomeric procyanidins, exhibited the highest ORAC/TEAC values within the tested extracts.

In the cellular assays, the extracts protected against DNA oxidation damage and diminished the TBH-induced ROS level,

predominantly in the lower concentration range tested (1−10 μg/mL). Since both markers were modulated quite similarly, protection against DNA oxidation damage might be influenced by the observed reduction of cellular ROS level. Highest protective activity was observed for PE, followed by APE, whereas the juice extracts AE05 and AE06 were distinctly less effective, corresponding to their low flavonol concentration. Quercetin glycosides are supposed to contribute substantially to the protective effects of PE, since both, quercetin and its glycoside rutin, reduced cellular ROS level and Md-mediated DNA damage efficiently (*15*, *20*, *29*). The contribution of procyanidins is difficult to assess, as the degree of polymerization is not known and oligomers with >3 flavanol units are poorly absorbed into cells (*35*). Besides the original apple juice polyphenols, also their intestinal degradation products/metabolites have been found to contribute to the observed antioxidant activity of apple juice extracts (*20*).

GPx activity was reduced by all extracts in the order AE06 ≈ AE05 > APE > PE, which largely matches TEAC results and polyphenol/procyanidin concentrations. Downregulation of GPx activity, which has also been reported for other polyphenols, may be caused by a decrease of intracellular ROS level by radical scavenging polyphenols.

In conclusion, polyphenol-rich extracts from apple effectively diminish DNA oxidation damage by reduction of cellular ROS level. This preventive effectiveness is attributable to induction of cellular defense rather than to the radical scavenging activity of polyphenols/procyanidins and might well contribute to the reported health benefits of apples and apple juices.

ABBREVIATIONS

AAPH, 2,2′-azobis(2-methylpropionamidine)dihydrochloride; ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); AE05, polyphenol rich extract from apple juice, produced 2005; AE06, polyphenol rich extract from apple juice, produced 2006; APE, polyphenol rich extract from pomace extraction juice, produced 2006; DAD, diode array detector; DCF, 2′,7′-dichlorofluorescin; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; FPG, formamidopyrimidine-DNA-glycosylase; GPx, glutathione peroxidase; HPAEC, high performance anion exchange chromatography; Md, menadione; ORAC, oxygen radical absorbance capacity; PBS, phosphate buffered saline; PE, polyphenol rich extract from apple peels; ROS, reactive oxygen species; TBH, *tert*-butyl hydroperoxide; TEAC, Trolox equivalent antioxidant capacity; TI, tail intensity.

ACKNOWLEDGMENT

The authors gratefully acknowledge the gift of FPG enzyme by Prof. A. R. Collins (University of Oslo, Norway). They also thank S. Schmidt for competent assistance

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Received for review November 25, 2009. Revised manuscript received March 10, 2010. Accepted April 22, 2010. The authors thank the Federal Ministry of Education & Research (Grant No. 01EA0501) for financial support.