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Polyphenolic apple juice extracts and their major constituents reduce oxidative damage in human colon cell lines

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Apple juice containing high amounts of antioxidative polyphenols might protect the intestine against oxidative cell damage. We investigated the preventive effectiveness of polyphenolic juice extracts of different origins (cider and table apples) in comparison to their major constituents in human colon cell lines (Caco-2, HT29). Parameters studied were (oxidative) DNA damage (Comet assay), glutathione level (photometric kinetic assay), cellular redox status (dichlorofluorescein assay) and antioxidant capacity. The extracts (50–250 μg/mL) modulated DNA damage and redox status in a concentration-dependent manner at 24-h incubation. The pomace extraction technology, applied for juice preparation, and the preferential selection of cider apple varieties influenced the polyphenolic pattern and increased the biological effectiveness of the extracts. The preventive potential of major juice constituents (1–100 μM, 24 h) strongly differed: rutin, epicatechin and caffeic acid clearly reduced (oxidative) DNA damage (Caco-2), chlorogenic acid efficiently decreased cellular reactive oxygen species level (HT29, Caco-2). The aglyca quercetin and phloretin exhibited the highest preventive/antioxidant capacity in all assays. The stability of the compounds inversely correlated with their preventive effectiveness and might contribute to the observed cell specific sensitivities. In conclusion, apple juice extracts distinctly reduce oxidative cell damage in human colon cell lines, an effect, which in part can be accounted for by their major constituents.

Keywords: Antioxidative effectiveness / Apple juice / Cellular redox status / Oxidative DNA damage / Polyphenols

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1 Introduction

Many degenerative diseases such as cancer, cardiovascular disease, asthma and diabetes have been associated with chronically increased formation of reactive oxygen species (ROS) [1]. For colorectal cancer, a leading cause of cancer death in Western countries, case-control studies have shown an inverse correlation between fruit and vegetable consumption and disease risk [2–4]. It has been suggested that diet related factors might account for up to 80% of the difference in rates between developed and developing countries [5]. Plant foods contain a variety of bioactive compounds including flavonoids/polyphenols. In the Western diet, apples (and apple juice) represent a main source of such compounds, discussed as promising candidates for prevention of cancer and inflammation [6–8]. An important feature of these compounds is their ability to reduce oxidative stress by several mechanisms such as direct scavenging of ROS [9, 10], complexation of metal ions [11] and modulation of cellular response [12]. Combination of such effects contributes to protection by inhibiting cell proliferation and inducing detoxification enzymes as well as apoptosis [13].

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Abbreviations: **ABTS**, 2,2′-azinobis(3-ethylbenzothiazoline-6-sullfonic acid); **AE**, apple juice extract; **APE**, apple pomace extract; CaA, caffeic acid; **ChA**, chlorogenic acid; **DCF**, 2′,7′-dichlorofluorescein; **DTNB**, 5,5′-Dithiobis(2-nitrobenzoic acid); **Epi**, (−)-epicatechin; **FCS**, foetal calf serum; **FI**: fluorescence increase; **FPG**, formamidopyrimidine-DNA-glycosylase; **GSH**, glutathione; **GSSG**, oxidised glutathione; **Md**, menadione; **Plt**, phloretin; **Plz**, phloridzin; **Que**, quercetin; **ROS**, reactive oxygen species; **Rut**, rutin; **TBH**, tert-butylhydroperoxide; **TEAC**, Trolox equivalent antioxidant capacity; **tGSH**, total glutathione

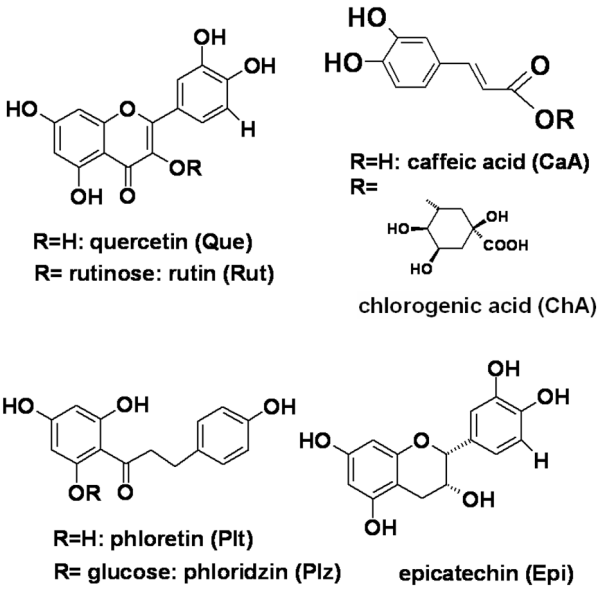

Figure 1. Structures of major apple juice compounds.

The intestine is exposed to substantial levels of ingested oxidants such as lipid peroxides or iron that might adversely affect intestinal integrity by increasing the free radical production in the colon [14, 15]. Intestinal ROS formation might also be induced by certain food constituents that might be subjected to redox cycling [16]. Chronic gut inflammation observed in inflammatory bowel diseases (*e.g.*, ulcerative colitis and Crohn's disease) is also supposed to be associated with enhanced production of oxidants, derived from infiltrating macrophages and neutrophils [17, 18]. Moreover, the intestinal synthesis of glutathione (GSH), the most important intracellular antioxidant, is impaired in patients with inflammatory bowel disease [18]. Whether fruit-derived polyphenols protect against ROS-mediated damage in colon cells is not clear yet.

In the present *in-vitro*-study, a potential preventive effect of flavonoid/polyphenolic apple juice extracts (A(P)Es), prepared from cider apples with high amounts of these antioxidants and to lesser extent from table apples, was investigated in the human colon cell lines Caco-2 and HT29. Additionally, major extract constituents (see Fig. 1) such as the flavonol rutin (Rut), the dihydrochalcone phloridzin (Plz), the flavanol (–)-epicatechin (Epi) and the hydroxycinnamic acid derivatives caffeic acid (CaA) and chlorogenic acid (ChA) were identified and quantified by HPLC and included in the biological tests. The aglyca quercetin (Que) and phloretin (Plt), released from juice constituents into the intestinal tract by bacterial and intracellular glycosidases and brush border enzymes (*e.g.*, lactate Plz hydrolase) [19], were also tested. To simulate pathological situations with increased oxidative stress, cells were additionally treated with the oxidants menadione (Md) or tert-butylhydroperoxide (TBH). The vitamin K analogue, Md, directly acts as oxidant by redox cycling, generating superoxide ($O_2^{-\bullet}$), but also indirectly by depletion of GSH via aryl adduct formation [20]. Oxidative cell damage (DNA damage, GSH level/status), cellular redox status, total antioxidant capacity of extracts/compounds and stability of major compounds under cell incubation conditions were monitored.

2 Materials and methods

2.1 Chemicals, cells and media

Quercetin (dihydrate, Que), rutin (quercetin-3-rutinoside, Rut), chlorogenic acid (ChA), phloridzin (Plt-2′-glucoside, hydrate, Plz), caffeic acid (CaA) and epicatechin (Epi) were purchased from Sigma-Aldrich (Taufkirchen, Germany) and phloretin (Plt) from Roth (Karlsruhe, Germany). Md, TBH and reduced/oxidised glutathione (GSH/GSSG), glutathione reductase, NADPH, Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were provided by Sigma-Aldrich. The 2′,7′-dichlorofluorescin-diacetate and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Fluka GmbH (Deisenhofen, Germany). Agarose, low and normal melting, was obtained from Bio-Rad GmbH (Munich, Germany). The formamidopyrimidine-DNA-glycosylase (FPG) enzyme was provided by A. R. Collins (Oslo, Norway).

All organic solvents and other chemicals were of analytical grade or complied with the standards needed for cell culture experiments. BCA protein detection kit was obtained from Uptima (Montluçon, France).

Caco-2 and HT29 cells were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) (Braunschweig, Germany), and DMEM, DMEM/Nutrient Mix F12 (1 : 1) medium, foetal calf serum (FCS), and penicillin/streptomycin from Invitrogen GmbH (Karlsruhe, Germany).

2.2 Apple juice extracts (AE)

2.2.1 General description

Phenolic AE were produced from juices of different, mainly cider apple varieties, harvested in the Research Institute Geisenheim and from local orchards as described [21, 22]. Briefly, after crushing and extraction on a horizontal press (Bucher HP L-200), the juices were treated with pectinase, separated and cross-flow filtered (0.2 µm). After adsorption of the polyphenolic fraction on adsorber resins, sugars,

organic acids and minerals were eliminated with water; subsequently the polyphenolic fraction was eluted with ethanol 96%, concentrated, and freeze-dried. The resulting extracts were stored cool, under exclusion of light and moisture. Extracts (AE01, AE02, and AE04) were obtained from juices and APE03 from the pomace extraction juice, respectively.

2.2.2 AE01

This extract was obtained from Boskoop apples harvested 2001 using clarified juice, adsorber resin XAD16 HP (Rohm & Haas, Frankfurt, Germany).

2.2.3 AE02

A mixture of different apple varieties, harvested 2002 was used, namely table apples (20%, mainly Jonagold), Topaz (25%), Bohnapfel (17.5%), Winterrambour (22.5%) and Bittenfelder (15%); clarified juice, adsorber resin XAD16 HP, as described [22].

2.2.4 APE03

Mixture of different table apple varieties (Melrose, Granny Smith, Golden Delicious, Jonagold) harvested 2003 was used for obtaining extraction juice from once pressed apple pomace. Enzyme preparation for pomace extraction (2 h, 50°C) was under following conditions: Rohapect MA-1, Rohapect AF2 (pectinases), Rohament CL (cellulase), 300 ppm each; adsorber resin P-495 (Bucher-Alimentech, Switzerland).

2.2.5 AE04

A mixture of different juices and fruit varieties: 50% clear apple juice, see AE02, 10% extraction juice and 40% clarified juice of different varieties (Schafsnase, Bohnapfel, Winterrambur), harvested 2002, adsorber resin P-495 was used.

2.2.6 Analysis of A(P)E

Dry extracts were dissolved in 10% methanol (1 g/L). Polyphenols were separated on a 1090 HPLC/photo diode array system (Hewlett-Packard; Böblingen, Germany), equipped with a 250 × 4.6 mm Aqua 5-µm C18 column and protected with a 4 × 3-mm C18 ODS security guard (Phenomenex; Aschaffenburg, Germany). Compounds were separated using an ACN/acetic acid gradient according to Schieber *et al.* [23] and detected at 280 (flavanols), 320 (phenol carbonic acids) and 360 nm (flavonols). Individual compounds were quantified by comparison with external standards.

2.3 Antioxidant capacity of extracts and major compounds

The Trolox equivalent antioxidant capacity (TEAC) of apple juice extracts and of major constituents/aglyca was determined with the ABTS radical cation decolourisation assay, according to Re *et al.* [24], with slight modifications. The water-soluble vitamin E analogue Trolox was used as antioxidant standard.

Briefly, compounds and extracts, dissolved in DMSO, were added to ABTS solution, which had been pre-activated by 2.45 mM potassium persulfate for 12 – 16 h in the dark and made up with PBS so as to reach an extinction of 0.7 ± 0.02. In the reaction mixture (pH 7.4), final concentrations of compounds and extracts were 0 – 10 µM and 0 – 10 µg/mL, respectively (1% DMSO v/v). After 6 min at 30°C, absorbance at 734 nm was measured. Solvent controls and Trolox standard curve (0 – 15 µM) were run in each assay. The percentage inhibition of absorbance and the TEAC value were calculated as described [24]. TEAC values are expressed in mM Trolox, equivalent to the antioxidant capacity of 1 mM solution of the major compounds and of 1 mg/mL of the A(P)E, respectively. Additionally, the TEAC was monitored during 24-h incubation of compound/extract in PBS (37°C, 5% CO_2) and expressed as relative TEAC in percent of initial value (% of t_{0h}).

2.4 Cell culture

HT29 cells were maintained in 175-cm^2 flasks in DMEM, supplemented with 10% FCS and 100 U/mL penicillin and 100 µg/mL streptomycin, at 37°C, 5% CO_2 and 95% saturated atmospheric humidity. Caco-2 cells were cultured under identical conditions but using the medium DMEM/Nutrient Mix F12 (1:1), supplemented with 20% FCS.

2.5 Cellular redox status (DCF assay)

Oxidative stress was quantified in cells by the DCF assay according to Wang and Joseph [25], with slight modifications. Briefly, cells were seeded (3×10^4 HT29/well into black/clear-bottom 96-well plate, 2×10^4 Caco-2/well into 48-well plate), cultivated for 24 h, and incubated with antioxidants for 2 or 24 h. After washing with PBS (pH 7.3), cells were incubated for 30 min with dichlorfluoresceindiacetate, dissolved in DMSO (final concentration 50 µM, DMSO: 0.5% v/v in PBS, pH 7.0), washed (PBS) and incubated with TBH (250 µM) in PBS (pH 7.0) for 40 min. The increase of fluorescence (FI), resulting from oxidation of the non-fluorescent product dichlorofluorescein to DCF by intracellular ROS, was measured at 0 and 40 min in a microplate reader. All dye and oxidant incubations, washing steps and fluorimetric determination were performed in

the dark. FI was calculated as $[(F_{40\,min} - F_{0\,min})/F_{0\,min}] \times 100$ as described [25]. Results are expressed as rel. FI (in % of TBH control).

2.6 Incubation with polyphenols (± treatment with Md)

Step 1: Cells were seeded in petri dishes (Caco-2: Comet assay, 2.5×10^5, ∅ 6 cm; GSH, 7.5×10^5, ∅ 10 cm; HT29: Comet assay, 1×10^6, GSH, 1.5×10^6, both ∅ 6 cm), cultivated in growth medium for 24 h, washed twice with PBS (pH 7.3) and incubated 24 h with polyphenols (1 – 100 μM) or with A(P)E (10 – 250 μg/mL), both dissolved in DMSO (final concentration 0.1% v/v) in medium with reduced FCS (HT29: 5% and Caco-2: 10%, respectively).

Step 2: In most experiments, cells were additionally washed twice (PBS) and treated with Md (3 – 5 μM for Caco-2 and 20 μM for HT29 cells, 1 h in serum-free medium).

After incubation, cells were washed twice (PBS) and isolated by trypsin (0.5% w/v) treatment. Cell suspensions were used for determination of cytotoxicity, DNA damage or GSH level/status.

2.7 Viability

Cytotoxicity was routinely monitored in cell suspensions by trypan blue exclusion. Viability was expressed as viable cells in percent of total cells (absolute viability in %). Each experiment was performed in duplicate. All results obtained with cellular assays (see Section 3.) were based on experiments with viabilities > 85%.

2.8 Oxidative DNA damage (Comet assay)

Alkaline single-cell gel electrophoresis was performed according to Singh *et al.* [26] and Collins *et al.* [27], with slight modifications [28, 29]. Briefly, $4 \times 60\,000$ cells were centrifuged (10 min, 4°C, 400 × g) and the pellet was mixed with low melting agarose, distributed onto a microscope slide (pre-coated with agarose), submitted to lysis and treated with FPG enzyme to recognise/cut off oxidised purine bases, resulting in additional DNA damage [27]. 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, viewed microscopically (Zeiss Axioskop 20, filter set 15, exc.: BP 546/12; em.: LP 590) and analysed by computerised image analysis (Perceptive Instruments, Suffolk, GB), scoring 2×50 images (two gels) per slide. DNA migration is expressed as mean tail intensity (TI%) from one slide. Total DNA damage (with FPG treatment) was presented as rel. TI% (relative to the Md control, Md = 100%). Coefficient of variation (intra assay) of the method was 7.3%.

2.9 GSH level/status (kinetic photometric assay)

Total glutathione (tGSH: GSH + GSSG) and GSSG were measured by determination of 5-thio-2-nitrobenzoate (TNB) [30, 31], with slight modifications. Cell suspensions (see Section 2.5) were washed with PBS, centrifuged (10 min, 4°C, 400 × g) twice and resuspended in 370 μL phosphate buffer (106 mM K_2HPO_4, 18.3 mM KH_2PO_4 + 6.29 mM Na_2EDTA). Aliquots (2×10 μL) were submitted to photometric protein determination, 350 μL of cell suspension was used for protein precipitation (10% SSA), centrifugation ($12\,000 \times g$) and determination of tGSH and GSSG.

For tGSH determination, in a 96-well plate, supernatants (10 μL) were added to 190 μL of a freshly prepared reaction mixture, containing 4 μL NADPH (20 mM), 20 μL DTNB (6 mM), 2 μL GSH reductase (50 U/mL) and 164 μL phosphate buffer. TNB formation rate of samples and of GSH standards (1 – 80 μmol/L) was monitored in a microplate reader (MWGt, Sirius HT injector, 412 nm) after 10 min.

For GSSG determination [32], supernatants and standards (1 – 32 μM) were reacted with 50% triethanolamine (50 μL) and vinylpyridine (10 μL) for 1 h in a thermomixer at 26°C. The 20-μL aliquots were assayed as described for tGSH determination.

Each experiment was performed in duplicate. Results on tGSH and GSSG were expressed as nmol GSH/mg protein and used to calculate GSH and GSH status (= reduced GSH in % of tGSH). Coefficient of variation (intra assay) of the method was 2.5%.

2.10 Stability of major compounds (HPLC analysis)

The stability of polyphenols was investigated within 24 h under the cell incubation conditions (see Section 2.6, step 1) with or without cells. At different time points, cell supernatant (200 μL) was mixed with acetone (400 μL) to precipitate proteins. The mixture was centrifuged at $12\,000 \times g$ (5 min, 4°C) and the supernatant was analysed immediately by HPLC-UV at 280 nm for Plt, Plz, Epi, 320 nm for ChA and CaA, and 370 nm for Que and Rut. Jasco HPLC system was used PU-980, LG 980-02, DG-980-05 with UV-detector GAT LCD 500; column: Phenomenex aqua (see Section 2.2). Eluents were A: water/acetic acid 98:2; B: water/ACN/acetic acid 500:500:5 with the gradient (for A): 0 min 90%, 30 min 45%, 50 min 0%, 55 min 90%; for Q and Pt: 0 min 90%, 10 min 45%, 20 min

0%, 22 min 90%, 23 min 0%, 24 min 90%, 26 min 0%, 28 min 90% flow: 1 mL/min). Rel. peak areas (% of initial value) were determined and the half-life ($t_{1/2}$) was calculated from the curves fitted to exponential decay of first order (origin 7.5).

2.11 Statistics

Results of cell assays are reported as mean ±SD of 3–6 independent experiments. Data of samples treated with flavonoid + oxidant and oxidant controls were analysed for significant difference ($p < 0.05$) by one sample *t*-test (one sided). Samples, treated only with polyphenols were compared to respective solvent controls by independent *t*-test (one sided).

3 Results and discussion

3.1 Composition of apple juice/pomace extracts (A(P)E)

By adsorber resin technology, the polyphenolic fraction of apple juices was separated from other constituents such as carbohydrates and the majority of organic acids and minerals.

Constituents of the A(P)E, identified and quantified by HPLC-PDA, are listed in Table 1. The pattern of characterised polyphenols (up to 50% of total) shows substantial variations in the proportion of individual constituents. For APE03 (originating from apple pomace), especially high concentrations of Que and Plt derivatives were found whereas ChA was observed only at 10% the respective amount in the juice extracts. Enzymatic treatment of pomace is known to release phenols from the apple peal, resulting in an increase of the dihydrochalcone Plz, of Que glycosides, and of Epi and CaA, as compared to the corresponding premium juice [33]. AE01 (produced from Boskoop apples) exhibited 15–20-fold higher amounts of CaA than the other extracts. These differences in the amounts of constituents might be relevant for the preventive effectiveness of the A(P)E.

Table 1. Amounts of identified compounds in apple juice extract (A(P)E) in mg/g

Compound	AE01 [mg/g]	AE02[a]) [mg/g]	APE03 [mg/g]	AE04 [mg/g]
Procyanidin B1	2.9	7.0	6.2	n.d.
Procyanidin B2	16.0	15.1	18.4	12.1
Epicatechin[b])	11.8	19.2	17.7	12.5
Phloretin-2-xyloglucoside	42.7	66.15	31.7	68.9
Phloridzin	34.7	27.9	78.9	48.0
Chlorogenic acid	171.8	181.5	19.2	183.2
3-coumaroylquinic acid	16.0	9.5	3.0	9.4
4-coumaroylquinic acid	72.4	77.3	5.0	66.0
5-coumaroylquinic acid	7.0	10.4	3.8	39.8
Caffeic acid	102.7	4.8	4.0	7.5
Quercetin-3-rhamnoside	3.2	4.1	25.1	4.3
Quercetin-3-galactoside	0.9	0.8	8.1	1.8
Quercetin-3-glucoside	1.4	1.4	12.3	1.5
Quercetin-3-rutinoside[b])	1.8	2.6	49.1	4.5
Total	485.3	427.75	282.5	460.5

a) According to [22].
b) Bold: major compounds used for biological assays. n.d.: Not detected.

3.2 Trolox equivalent antioxidant capacity (TEAC)

The antioxidative capacity of juice extracts and major compounds is summarised in Table 2. The A(P)E showed rather similar Trolox equivalent antioxidant capacity (TEAC) values, ranging from 3.4–4.2 mM Trolox. The TEAC values of the major compounds, determined under identical conditions, agreed with previous findings [34], except for Rut, Plz and Epi, for which somewhat lower values have been published (Rut 2.4; Epi 2.5 [34] Plz 2.38 mM [35]). The TEAC values of the A(P)E (in mg/mL) are in the lower range of the respective compound capacities. The difference in TEAC values of AE02 and AE04, having almost identical contents of the characterised constituents (see Table 1), suggests that other, not yet identified compounds play an additional role.

During 24-h incubation in PBS at physiological pH and 37°C, the antioxidative capacity of all A(P)E was found to decrease moderately down to 75% of t_{Oh} (Table 2b) independently of their polyphenolic composition. Under similar conditions, we found indications for the antioxidative capacity of Que, Epi and Rut also to decrease down to 80–60%. The TEAC value of Plz remained nearly unchanged whereas Plt, CaA and ChA showed even an increase of antioxidative capacity. Taken together, the results suggest decomposition products with antioxidant capacities differing from the respective parent compound to be formed under physiological conditions. Specific products might even give rise to an increase of antioxidative potential.

3.3 Modulation of cellular redox status

The A(P)E (50–250 µg/mL) reduced TBH-induced ROS production at 24-h incubation of HT29 cells in a composition- and concentration-dependent manner: AE02 > APE03 ≈ AE04 > AE01 (Fig. 2a). The most effective AE02 decreased the ROS level down to 70% of TBH control. Considering ROS level of the DMSO control (dotted line, Fig. 2a) the TBH-induced ROS production is diminished

Table 2. Trolox equivalent antioxidant capacity (TEAC) of apple juice extracts (A(P)E) and major compounds (a) TEAC, according to [24] (b) rel. TEAC after 24 h incubation in PBS (37°C), in % of the initial value (t = 0 h)[a)]

Compound	(a) TEAC [mM][b)]		(b) rel. TEAC [% of t_{0h}]
	1 mM[b1)]	1 mg/mL[b2)]	
Extracts			
AE01		3.4 ± 0.1	74 ± 4
AE02		3.7 ± 0.0	76 ± 4
APE03		4.2 ± 0.0	73 ± 9
AE04		4.0 ± 0.1	76 ± 6
Major compounds			
Quercetin	4.8 ± 0.1	14.2 ± 0.3	60 ± 9
Phloretin	3.8 ± 0.2	13. ± 0.6	116 ± 2
Rutin	3.7 ± 0.1	5.6 ± 0.2	85 ± 4
Phloridzin	3.6 ± 0.2	7.5 ± 0.4	103 ± 11
Epicatechin	3.1 ± 0.2	10.6 ± 0.7	79 ± 5
Caffeic acid	1.3 ± 0.0	7.2 ± 0.2	150 ± 15
Chlorogenic acid	1.3 ± 0.1	3.6 ± 0.1	155 ± 11

a) TEAC and rel. TEAC values are means ± SD obtained from six to eight and from three to four independent experiments, respectively.

b) The mmolar concentration of a Trolox solution having an antioxidant capacity equivalent (b1) to 1.0 mM solution or (b2) to 1.0 mg/mL solution of the test sample [36].

by more than 50%. In Caco-2 cells, a decrease of ROS level was also observed, (AE02 ≈ AE01 > APE03 > AE04, Fig. 2b). In line with this, antioxidant activity after 24-h incubation with an apple extract (Golden Delicious) had been described as well in differentiated TBH-treated Caco-2 cells [37].

When the incubation time was limited to 2 h, no such protective effects were observed with the A(P)E (10 – 250 μg/mL). This suggests that direct scavenging of ROS by parent compounds might be less relevant and that other mechanisms, detectable after longer-term incubation, are involved in preventive effectiveness such as redox-regulated cell response [38].

The major compounds ChA, Plt and Que were effective at 10 – 30 μM in HT29 (Fig. 2c) and at 3 – 30 μM, including CaA, in Caco-2 cells (data not shown). At 100 μM, a reincrease of ROS level was observed which is probably due to prooxidative actions under *in vitro* conditions [39]. In cell culture, especially the presence of transition metals (*e.g.*, Cu(II) and Fe(III) as constituents of incubation media) and a higher pH level can stimulate artifactual ROS production and autoxidation of polyphenols [40, 41]. In contrast, Rut, Plz and Epi (1 – 100 μM) did not modulate cellular ROS levels to any detectable extent. A decrease of TBH-induced ROS in HepG2 cells level had been already described for Que [42].

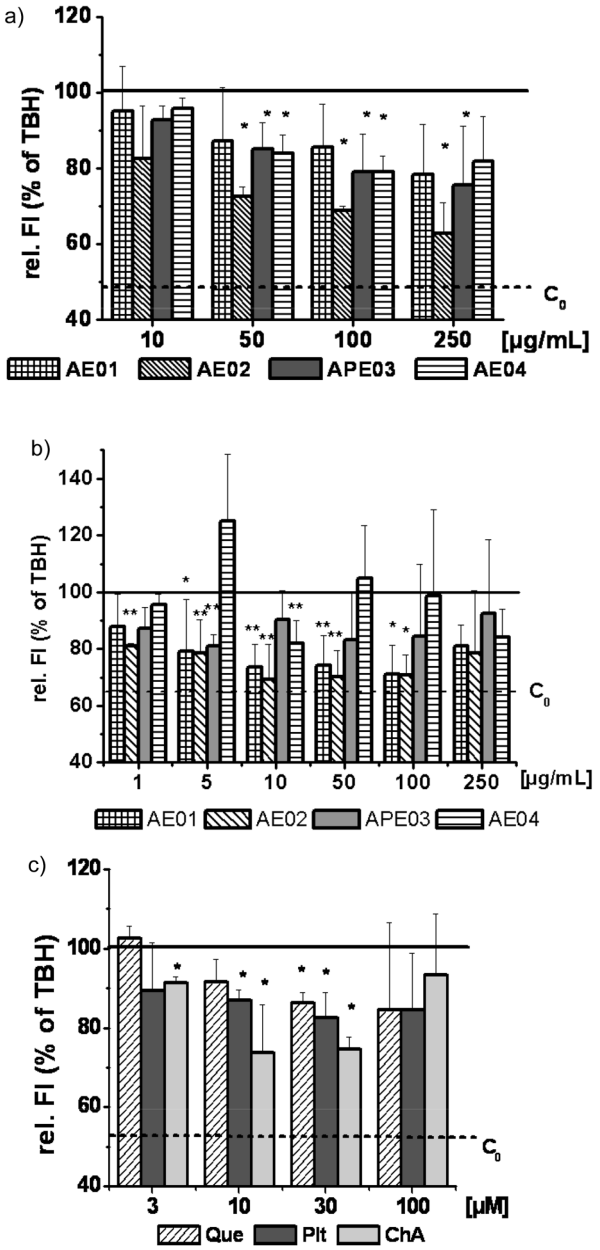

Figure 2. Effects of A(P)E (a) (10 – 250 μg/mL) in HT29 cells and (b) (5 – 250 μg/mL) in Caco-2 cells and (c) the aglyca Que and Plt and ChA (1 – 100 μM) on TBH-induced ROS in HT29 cells, measured by DCF oxidation. Values are expressed as increase of fluorescence (rel. FI, in % of TBH control), as mean ± SD of three independent experiments, each performed in quadruplicate. DMSO control: dotted line; */** significantly lower than TBH control, *$p < 0.05$; **$p < 0.01$.

3.4 Modulation of Md-induced (oxidative) DNA damage

Distinct decrease of DNA damage was observed at incubation of Caco-2 cells with AE01 and APE03 (Fig. 3a). Concentration- response curves, resembling a "U-shape", were

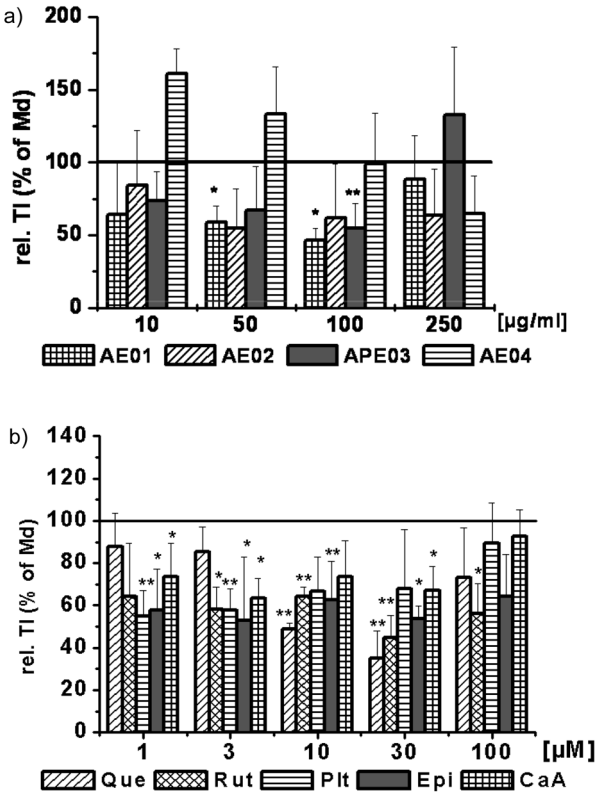

Figure 3. Effect of (a) A(P)E and (b) of Que, Rut, Plt, Epi and CaA on Md-induced DNA damage (+ FPG) in Caco-2 cells. Values are expressed as rel. TI% (in % of Md control) as mean ± SD of three to four independent experiments. */**significantly lower than Md control, *$p < 0.05$; **$p < 0.01$.

obtained reaching a minimum at 100 µg/mL. AE02 and AE04 were only weakly effective at 50 and 250 µg/mL, respectively. Whilst with APE03 a strong increase of DNA damage was observed at 250 µg/mL, the other extracts did not show such effects. In HT29 cells, AE01 produced a similar dose-response curve (10 – 250 µg/mL) whereas AE04 was only protective at high concentration (250 µg/mL). AE02 and APE03 did not decrease DNA damage in all concentrations tested. Considering the results of both cell lines, the ranking of preventive effectiveness was AE01 > APE03 > AE04 ≈ AE02.

Effects of the major compounds on DNA damage in Caco-2 cells are shown in Fig. 3 b: pre-incubation (24 h) with Que, Plt and CaA achieved a concentration dependent modulation (U-shape curve). Rut and Epi decreased DNA damage at all concentrations tested (1 – 100 µM), Plz and ChA (1 – 100 µM) were not effective. In HT29 cells (data not shown), Que and Plt were preventive already at threefold lower concentrations as compared with Caco-2 cells. Aherne and O'Brien [43] have observed a preventive action of Que (24 h, 50 µM) against Md-mediated (10 µM, 30 min) DNA damage in Caco-2 cells (measured without FPG), whereas Rut (24 h, 50 µM) was not significantly effective (Aherne, [43]). When TBH was used instead of Md, both flavonols were able to diminish DNA damage [43].

The observed re-increase of DNA damage at high concentrations of APE03, Plt and Que agrees well with the observed increase of ROS level (see Section 3.3).

3.5 GSH

Since GSH synthesis is known to be induced in response to phenolic antioxidants [44], effects of A(P)E and of major compounds on modulation of tGSH level were also studied. The basic tGSH levels of Caco-2 and HT29 cell (DMSO controls) were 59 ± 20 nmol/mg protein ($n = 15$) and 80 ± 20 nmol/mg protein ($n = 20$), respectively. Incubation with A(P)E (10 – 250 µg/mL) did not modulate tGSH level in HT29 cells (data not shown). In Caco-2 cells, a slight but significant increase of tGSH up to 110% of control was observed for AE02 (50 µg/mL, $p < 0.05$, $n = 3$), inversely correlating with the decrease of Md-induced (oxidative) DNA damage.

The apple juice constituents (1 – 100 µM, $n = 3$) did not affect the tGSH level in both cell lines (data not shown). In HT29 cells, CaA and Epi even caused a GSH depletion down to 80 – 90% at concentrations > 10 µM ($p < 0.05$, $n = 3$). This depletion might result from GSH-conjugate formation following oxidation of polyphenolic structures to quinoid and hydroxylated products as has been described for CaA, ChA [45] and catechins [46].

The aglyca Que or Plt (1 – 100 µM, 24 h) increased tGSH level (Fig. 4). Que, the most active compound, induced tGSH up to 150 – 160% of control at 30 and 10 µM in Caco-2 and in HT29 cells, respectively. Similar elevation of GSH levels by the key enzyme γ-glutamylcysteine synthetase had been found for Que in HepG2 cells [47].

With Plt, a slight induction (up to 120% of control, Fig. 4) was achieved at low micromolar concentrations. High concentrations of Plt and of Que, however, resulted in a strong (re-)increase of tGSH level, which might be due to prooxidative stimulation and ROS formation, followed by induction of the ARE [47]. Quinone-induced oxidative stress has been found to elevate GSH in rat lung epithelial L2 cells [48].

Modulation of GSH status (reduced GSH in % of tGSH) by major polyphenols and A(P)E was not detectable in our experiments (status > 93% in both, controls and treated cells). Thus, no indication for increased GSSG formation was obtained, as has been described for other, flavone and flavanone structures resulting from prooxidant or autoxidation reactions [49].

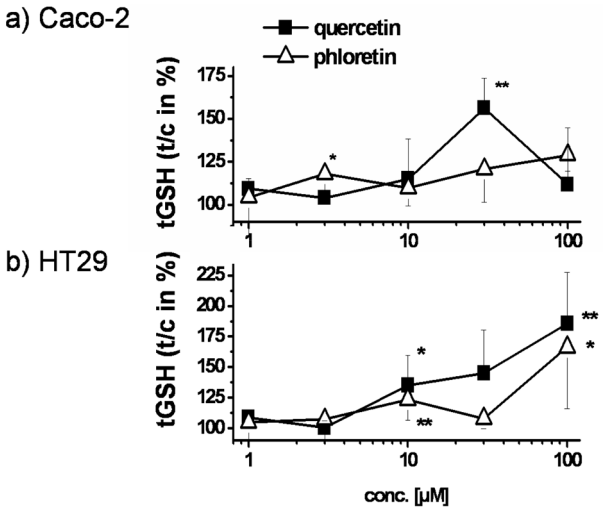

Figure 4. Effects of the aglyca Que and Plt (1 – 100 µM) on tGSH level (a) in Caco-2 and (b) HT29 cells after 24-h incubation. Values are expressed as rel. tGSH (in % of DMSO control) as mean ± SD of three independent experiments. */**significantly higher than DMSO control:* $p < 0.05$; ** $p < 0.01$.

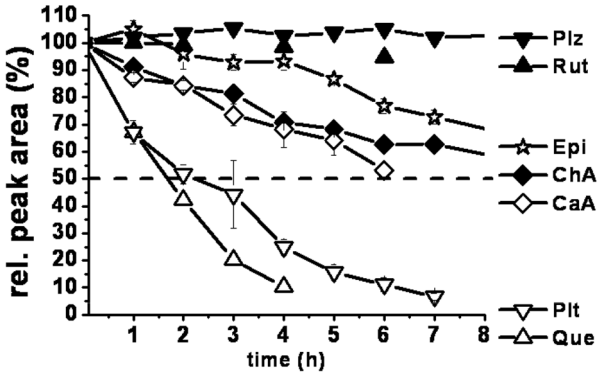

Figure 5. Stability of major compounds (30 µM) in HT29 cells (DMEM + 5% FCS) within 24 h. Values are expressed as rel. peak area (in % of t = 0) as mean ± SE of two independent experiments (except for Plz and Rut: $n = 1$).

3.6 Stability

The decay of parent compounds during incubation of HT29 cells in DMEM is shown in Fig. 5. Substantial decrease was observed for the aglyca Que and Plt ($t_{1/2}$: 2 h, both). The hydroxycinnamic acid derivatives, CaA and ChA, as well as Epi, appeared to be more stable under these conditions ($t_{1/2}$ CaA: 7 h; ChA: 11 h; Epi: 13 h) and the glycosides Rut and Plz remained almost unchanged (rel. peak $area_{24\,h}$: Plz 95%, Rut 80%). No generation of the respective aglyca Que and Plt was indicated. When the incubation was performed without cells, most compounds were less stable ($t_{1/2}$: Que, 0.5 h; Plt, 1.2 h; ChA, 6 h; rel. peak $area_{24\,h}$: Rut, 65%, Plz, 90%), except for Epi and CaA ($t_{1/2}$ 14 and 13 h, respectively), pointing to the relevance of chemical degradation processes, *e.g.*, generation of carboxylic acids and formation of polymeric products, as described for Que [50]. Additional peaks (HPLC), only detected in presence of HT29 cells, indicated formation of metabolites from Que, Plt and ChA similar to findings of Boulton *et al.* [50] and van der Woude *et al.* [51] for Que. Phase II metabolites, resulting from glucuronidation and methylation of Que were identified in HT29 cells, whereas incubation of human intestinal S9 revealed characteristic sulfation and lack of methylation [51].

In DMEM/F-12 mix with/without Caco-2 cells, Que and Rut were more stable than in DMEM/ HT29 cells ($t_{1/2}$ for Que in medium: 1.3 h; with cells: 3.5 h; Rut: rel. peak $area_{24\,h} > 90\%$), probably due to differences in buffer/pH, as reported by Agullo *et al.* [52]. The observed lack of metabolic transformation agrees with findings of Boulton *et al.* [50] on fate of Que in Caco-2 cells.

These factors in addition to cell-specific uptake might explain the differing sensitivity of HT29 and Caco-2 cells towards modulation of (oxidative) cell damage and GSH synthesis.

4 Concluding remarks

Taken together, oxidative cell damage was reduced at 24-h incubation with apple juice/pomace extracts, A(P)E, at concentrations 50 – 100 µg/mL. These are in the lower range of polyphenols in apple juice (about 10 – 400 µg/mL) [53, 54] and correspond to an effective concentration range of 40 – 75 µM for the sum of tested major constituents, considering an average molecular mass of 400 and a portion of approximately 30% of total A(P)E.

Some major juice constituents were already preventive at 1 – 10 µmol concentrations that had been found in human plasma after consumption of polyphenol-rich food [55, 56]. In the intestinal lumen and in the colon, higher levels of unabsorbed phenolics, including degradation products and metabolites from both, intestinal flora and enterocytes, might well occur and play an important role in the antioxidant defence of the gastrointestinal tract [57].

The higher concentration range of the extracts required for the reduction of oxidative cell damage can be explained by the presence of weakly or not effective components, such as Plz (all endpoints) and ChA (DNA damage, GSH induction). Among the extracts, characteristic differences in the preventive effectiveness were observed. Modulation of (oxidative) DNA damage might be due to both, preventive capacity and concentration of identified major constituents:

AE01, the most active extract in both cell lines, was especially rich in CaA. The "pomace extract" APE03, which contains high concentrations of Plt and Que glycosides, was protective in Caco-2 cells. The observed effectiveness of the Que representative Rut suggests that also such intact glycosides contribute to the reduction of DNA damage. The weak effectiveness of AE04 and AE02 matches with the findings that preventive Que glycosides and CaA are minor constituents in these extracts, whereas the ineffective ChA was the main compound identified.

Extract-specific modulation of the cellular redox status can at best be explained in part by concentration and antioxidative activity of the individual constituents tested. For example, ChA, the only effective constituent in both cell lines, was present in high concentrations in AE02 as well as in AE01 and AE04. APE03, also quite effective, contained only low amounts of ChA. CaA, however, which was antioxidative in Caco-2 cells, might contribute to the high activity of AE01.

The (chemical) antioxidative capacity (TEAC) was rather similar for all extracts and a correlation with the cell-based endpoints was not observed for both, A(P)E and individual constituents. This supports the relevance of cellular response mechanisms and degradation/biotransformation of parent juice compounds for the preventive effectiveness of AE/constituents.

The aglyca Que and Plt that can be released from respective glycosides in the lumen of the intestinal tract and absorbed as such into enterocytes, exhibited the highest antioxidative/protective potential in all test systems, including tGSH induction. This might be due to a higher reactivity/antioxidative capacity, and a better uptake into the colon cells as compared to respective glycosides.

In conclusion, the polyphenolic apple juice extracts exhibit preventive effectiveness in colon cell lines, which in part can be related to individual identified constituents such as Que glycosides, CaA and ChA. Our cell-based endpoints, giving an aggregated measure of various parameters, including degradation/metabolism and modulation of cell response, provide additional information on the complex mode of action of AE. The identification of bioactive structures/extracts by these markers in colon cell lines combined with analytical data offers a concerted approach to develop juices and extracts with capacity to reduce oxidative cell damage by selection/mixture of fruit varieties and by juice technology.

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