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Modulation of oxidative cell damage by reconstituted mixtures of phenolic apple juice extracts in human colon cell lines

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Diets rich in fruits and vegetables are associated with a lower risk of tumour induction in the intestine and other sites. Apple juice with high amounts of antioxidative phenolics might protect the intestine against reactive oxygen species-mediated cell damage. We investigated to which extent the preventive effectiveness of polyphenolic juice extracts is governed by the amounts of five major constituents (rutin, phloridzin, chlorogenic acid, caffeic acid and epicatechin). In human colon cell lines (Caco-2, HT29), reconstituted mixtures of these phenolics were investigated in comparison to the original juice extracts, originating from cider and table apples. Parameters studied were (oxidative) DNA damage (Comet assay), cellular redox status (dichlorofluorescein assay) and Trolox equivalent antioxidant capacity (TEAC). The TEAC of the reconstituted mixtures was higher compared to the respective original extracts (4.7 – 7.3 mM *vs.* 3.6 – 4.2 mM Trolox). After 24 h cell incubation, menadione-induced (oxidative) DNA damage was more effectively reduced by the reconstituted mixtures (1 – 100 μg/mL, 24 h), as compared to the original extracts. In contrast, the cellular ROS level was reduced to a rather similar extent by original extracts and reconstituted mixtures. The results lead to the conclusion that the selected constituents in their authentic proportions substantially account for the antioxidative effectiveness of phenolic apple juice extracts.

Keywords: Apple juice phenolics / Antioxidative effectiveness / Cellular redox status / Oxidative DNA damage / Trolox equivalent antioxidant capacity

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1 Introduction

Epidemiological studies as well as animal experiments have implicated that many dietary micronutrients may have anticarcinogenic and anti-inflammatory properties [1, 2]. Plant phenolics are a large and widely distributed group of phytochemicals whose beneficial effects have been attributed to multiple mechanisms, including antioxidative action, induction of detoxification enzymes and modulation of signal transduction pathways which may act concordantly [3, 4]. Human diet consists of complex mixtures of such phenolics, whose biological mode of action can not only be exclusively ascribed to a single structure but also to potential combined or synergistic effects. Thus, mixtures of several phenolic compounds offer a better tool to simulate the effects of phenolics in complex fruit and fruit preparations [4, 5].

The aim of the present study was to elucidate to what extent selected phenolics in authentic composition contribute to the antioxidative effectiveness of three apple juices, prepared from different cider and table apples and with different technology (AE01: Boskoop apples; AE02: table and cider fruit varieties; APE03: pomace of table apples, AE04: mixture of table and cider fruit varieties where AE stands for apple juice extract) protecting colon cells against oxidative damage. To this end, reconstituted mixtures of the extract (rAPEs) of five major apple-derived phenolics [6] rutin, phloridzin, caffeic acid, chlorogenic acid and epicatechin were studied in the human colon cell lines Caco-2 and HT29. To simulate pathological situations with increased oxidative stress, cells were additionally treated with oxi-

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Abbreviations: **ABTS**, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); **AE**, apple juice extract; **APE**, apple pomace extract; **DCF**, 2′7′-dichlorofluorescein; **FCS**, fetal calf serum; **FI**, fluorescence increase; **FPG**, formamidopyrimidine-DNA-glycosylase; **Md**, menadione; **ROS**, reactive oxygen species; **rAE**, reconstituted mixture of the juice extract; **rAPE**, reconstituted mixture of the pomace extract; **TEAC**, Trolox equivalent antioxidant capacity; **TBH**, *tert*-butylhydroperoxide

dants to generate radicals, previously found appropriate to induce specific oxidative events [7]: Modulation of (oxidative) DNA damage (induced by the redox cycler menadione (Md), generating superoxide), cellular redox status (induced by *tert*-butylhydroperoxide (TBH), generating the respective alkoxyl/peroxyl radical) and total antioxidant capacity of mixtures were monitored to compare with the effects of respective original APEs.

2 Materials and methods

2.1 Chemicals, cells and media

Rutin (quercetin-3-rutinoside), chlorogenic acid, phloridzin (Plt-2′-glucoside, hydrate), caffeic acid and epicatechin were purchased from Sigma-Aldrich (Taufkirchen, Germany). Md, TBH, 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. 2′,7′-Dichlorofluorescin-diacetate was purchased from Fluka (Deisenhofen, Germany). Agarose, low and normal melting, was obtained from BioRad (Munich, Germany). Formamidopyrimidine-DNA-glycosylase (FPG) was provided by A. R. Collins (Oslo, Norway).

All organic solvents and other chemicals were of analytical grade or compiled with the standards needed for cell culture experiments.

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

2.2 Reconstituted mixtures rAPEs of APEs

Reconstituted mixtures were prepared using five major constituents (see Table 1), reflecting the composition of original AEs (AE01, AE02) and a pomace juice extract (APE03) produced from different (mainly cider) apple varieties, harvested in the Research Institute Geisenheim and from local orchards and characterised analytically as described elsewhere [7–9].

2.3 Trolox equivalent antioxidant capacity (TEAC) of mixtures

Antioxidant capacity of extracts was determined with the ABTS radical cation decolourisation assay, according to Re *et al.* [10], with slight modifications. The water-soluble vitamin E analog Trolox was used as antioxidant standard.

Briefly, reconstituted mixtures, dissolved in DMSO, were added to a preactivated ABTS solution (extinction of 0.7 ± 0.02) reaching final concentrations of 0–10 μg/mL and 0–15 μM Trolox, respectively (1% DMSO v/v) in the reaction mixture. After 6 min at 30°C, absorbance at 734 nm was measured. TEAC values are expressed in millimolar Trolox equivalent to extract concentration of 1 mg/mL [11]. Additionally, the TEAC was monitored during 24 h incubation of mixtures under physiological conditions (PBS pH 7.4, 37°C, 5% CO_2) and expressed as relative TEAC in percent of initial value (% of t_{0h}).

2.4 Cell culture

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

2.5 Cellular redox status 2′7′-dichlorofluorescein (DCF) assay

Cellular ROS level was quantified by the DCF assay according to Wang and Joseph [12], with slight modifications [7]. Briefly, cells were seeded (2×10^4 Caco-2/well, 48-well plate; 3×10^4 HT29/well, black/clear bottom 96-well plate), cultivated for 24 h and incubated for 24 h with rAPEs. After washing, cells were incubated for 30 min with 50 μM dichlorofluroescin-diacetate, washed and incubated with

Table 1. Proportion of the selected phenolics (% of total) in the reconstituted mixtures

Compound	rAE01 (%)	rAE02 (%)	rAPE03 (%)
Σ phloretin glycosides (as phloridzin)	26.1	37.3	44.9
Σ quercetin-glycosides (as rutin)	1.8	2.6	47.2
Chlorogenic acid	43.3	53.0	7.8
Caffeic acid	25.9	1.4	1.6
Epicatechin	2.9	5.6	7.2
Total[a)]	100	100	100

a) Proportion of total selected phenolics in the original AEs: AE01: 39.9%, AE02: 34.2%; APE03: 24.6%.

Table 2. TEAC of the reconstituted mixtures, rAPE, in comparison to original APEs[a)] and to theoretical TEAC value[b)]: (a) TEAC, calculated according to [10]; (b) relative TEAC after 24 h incubation in PBS (37°C), in percentage of the initial value (t_{0h})

	(a) TEAC (mM)			(b) Relative TEAC (% of t_{0h})	
Mixtures/extracts	Mixture	Extract[a]	Theoretical[b)]	Mixture	Extract[a]
rAE01	5.0 ± 0.1	3.4 ± 0.1	5.2	100 ± 12	74 ± 4
rAE02	5.3 ± 0.0	3.7 ± 0.0	5.2	93 ± 9	76 ± 4
rAPE03	7.9 ± 0.2	4.2 ± 0.0	7.6	90 ± 10	73 ± 9

a) Taken from Schaefer *et al.* [7].
b) Sum, calculated from TEACs of the individual constituents.
TEAC and relative TEAC values are mean ±SD obtained from three to four independent experiments.

250 μM TBH in PBS for 40 min. The increase of fluorescence (FI) was measured at 0 and 40 min in a microplate reader. FI was calculated as $(F_{40\,min}-F_{0\,min})/F_{0\,min} \times 100$ as described [12]. Results are expressed as relative FI (in percentage of TBH control).

2.6 (Oxidative) DNA damage (comet assay)

Alkaline single cell gel electrophoresis was performed according to Collins *et al.* [13], with slight modifications [7, 14].

Briefly, 2.5×10^5 Caco-2 and 1×10^6 HT29 cells were cultivated in petri dishes in growth medium for 24 h, washed and incubated 24 h with rAPEs (1–250 μg/mL), dissolved in DMSO (final concentration 0.1% v/v) in medium with reduced FCS (HT29: 5% and Caco-2: 10%). After washing, cells were treated with Md (5 μM for Caco-2 and 20 μM for HT29 cells, 1 h in serum-free medium). After incubation, cells were washed and isolated by trypsin (0.5% w/v) treatment. For the comet assay, $4 \times 60\,000$ cells were centrifuged and the pellet was mixed with low-melting agarose, distributed onto a microscope slide, submitted to lysis and treated with FPG enzyme [13]. 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 and analysed by computerised image analysis (Perceptive Instruments, Suffolk, Great Britain), 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 = 100%).

2.7 Statistics

Results are reported as mean ±SD of three to six independent experiments. Data of samples treated with rAPE plus oxidant and oxidant controls were analysed for significant difference ($p < 0.05$) by one sample *t*-test (one sided).

3 Results and discussion

3.1 TEAC

The antioxidative capacity of reconstituted mixtures, rAPEs, is shown in Table 2, in comparison to the TEAC of the original juice (pomace) extracts APEs, and to the sum, calculated from TEACs of the individual constituents [7]. The TEAC values of all rAPEs were distinctly higher than those of the respective original extracts. The rAPEs, which represent 25–40 mass percent of total phenolics in the original extracts (Table 1), contribute with a 46–58% proportion to the antioxidant capacity. This emphasises a major role of the selected five constituents in the radical-scavenging capacity of the APEs. It may thus be concluded that other, not yet identified, phenols in the original extracts contribute to a minor extent to the total antioxidative potential.

Both the pomace extract APE03 and its reconstituted mixture, exhibit a higher antioxidative capacity than the juice extracts AE01 and AE02 (original and reconstituted). This might be due to the high amounts of quercetin- and phloretin glycosides, mainly occurring in the apple peel and core [15] which are released by enzyme treatment of the apple pomace [16].

Comparing TEAC values of the mixtures with the corresponding theoretical sum values of the five constituents shows almost identical antioxidative capacities. This does not indicate synergistic interactions among the individual compounds of the mixture; antagonistic effects of the phenolics which have been described for a mix of resveratrol, quercetin and catechin [17] were not observed either.

During 24 h incubation under physiological conditions, the antioxidative capacity of all rAPEs was found to slightly decrease down to 90% of the initial value (Table 2b), independent from the phenolic composition (Table 1). Since the antioxidant capacity of the original extracts was diminished down to 75% within 24 h incubation, the portion of unknown phenolics (60–75%) is supposed to transform

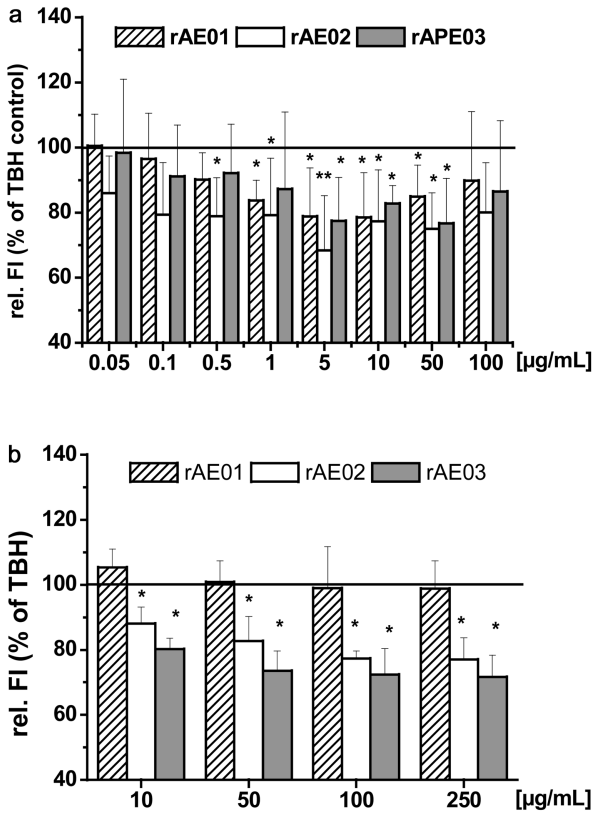

Figure 1. (a) Effects of reconstituted mixtures rAPEs on TBH-induced ROS in Caco-2 cells, measured by 2′,7′-dichlorofluorescin oxidation. Values are expressed as increase of fluorescence (rel. FI, in% of TBH control), as mean ± SD of three to six independent experiments, each performed in duplicate. */**, Significantly lower than TBH control, * $p < 0.05$, **, $p < 0.01$. (b) Effects of reconstituted mixtures rAPEs on TBH-induced ROS in HT29 cells, measured by 2′,7′-dichlorofluorescin oxidation. Values are expressed FI (rel. FI, in% of TBH control), as mean ± SD of three independent experiments, each performed in quadruplicate. */**, significantly lower than TBH control, *, $p < 0.05$.

into products exhibiting lower antioxidative capacity or even antagonistic effects [17].

3.2 Modulation of cellular redox status

The rAPEs (>0.5 µg/mL) reduced TBH-induced ROS production at 24 h incubation of Caco-2 cells in a composition- and concentration-dependent manner (Fig. 1a). The decrease of ROS level by the rAPEs corresponded largely to that of the original juice/pomace extracts. The phenolic acids chlorogenic acid and caffeic acid, which previously had been found highly effective [7], are supposed to add to this decrease of ROS level. In HT29 cells, rAE02 and rAPE03 were also effective (Fig. 1b); in comparison to the original extracts [7], the following ranking was obtained:

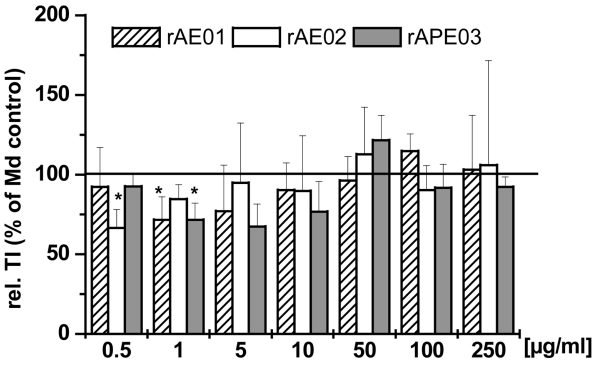

Figure 2. Effects of reconstituted mixtures rAPEs on Md-induced DNA damage (+FPG) in Caco-2 cells. Values are expressed as rel. TI% (in% of Md control) as mean ± SD of three to six independent experiments. *, significantly lower than Md control, *, $p < 0.05$.

AE02 > rAPE03, rAE02 > APE03 > AE01 ⩾ rAE01. The results with both cell lines suggest that the high proportion of other, mostly unidentified compounds in the original extracts (60–75%, see Table 1) contributes to diminish the cellular ROS level.

3.3 Modulation of Md-induced (oxidative) DNA damage

The modulation of Md-induced total DNA damage in Caco-2 cells by the reconstituted mixtures (0.5–250 µg/mL) is shown in Fig. 2. rAE01 and rAPE03 reduced DNA damage at 1 µg/mL down to 70%. rAE02 exhibited optimal effectiveness already at 0.5 µg/mL; when tested at still lower concentration (0.1 µg/mL; rel. TI: 100 ± 19%, $n = 3$), reduction of DNA damage was no longer observed. The effectiveness of the rAPEs might be attributed in part to the type of compounds in the mixture. For example, caffeic acid, which was found in especially high concentration in our extract rAE01 (26%) from Boskoop apples, was markedly preventive (3–30 µM) [7]. The compounds chlorogenic acid and phloridzin (together >90% in rAE02) had no potential to reduce DNA damage (1–100 µM) under these conditions [7]. This suggests that the observed effect of this mixture cannot be ascribed to these two compounds, and that synergistic interaction of all constituents may be responsible.

All original extracts caused a similar reduction at concentrations >10 µg/mL, indicating a lower effectiveness which might be due to the presence of other, ineffective or even antagonistic constituents. The observed re-increase of DNA damage by the rAPEs at higher concentrations, also seen previously with original extracts and individual constituents [7], is attributed to prooxidative reactions, known to occur

with flavonoids at high concentrations under *in vitro* conditions [18].

Differences observed in the efficacy of the mixtures/extracts in Caco-2 cells to decrease DNA damage and ROS level might be due to the type of radical generated by Md and TBH. Additionally, the cell compartment, in which the preventive effects are monitored, might be a decisive factor, since the oxidation of dichlorofluorescin by intracellular ROS is mainly visualised in the cytoplasm. To clarify this aspect, HT29 cells were incubated with chlorogenic acid (24 h, 1 – 100 μM) to study TBH-induced ROS level and DNA damage. Similar to previous experiments with Md as oxidant [7] TBH-induced DNA damage was not reduced ($n = 2$), whereas ROS level was effectively diminished [7]. This suggests that the type of oxidant plays a minor role for the observed differences in the efficacy of phenolics to reduce (oxidative) cell/DNA damage.

4 Concluding remarks

In summary, cellular ROS level and (oxidative) DNA damage were reduced at 24 h incubation of colon cell lines with reconstituted apple juice/pomace model mixtures, consisting of five major constituents. In contrast to the reduction of cellular ROS level, which was similar for the reconstituted mixtures and original extracts, the decrease of (oxidative) DNA damage by the reconstituted mixtures rAE01 and rAPE03 was more prominent, compared to the original extracts; correspondingly, the (chemical) antioxidative capacity (TEAC) of the reconstituted mixtures was higher than that of the respective original extracts.

Taken together, our investigations on selected constituents in their authentic proportions compared to original juice extracts and individual components led to the identification of polyphenolics which contribute to a major extent to preventive effects of extracts such as rutin (decrease of DNA damage), chlorogenic acid (decrease of ROS level) and caffeic acid (decrease of both, DNA damage and of ROS level). The selected *in vitro* assay proceeding was highly useful for characterisation of antioxidative effectiveness of nutritional components in gut cells.

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