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Research Article

Formation of hydrogen peroxide in cell culture media by apple polyphenols and its effect on antioxidant biomarkers in the colon cell line HT-29

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Beneficial health effects of diets containing fruits have partly been attributed to polyphenols which display a spectrum of bioactive effects, including antioxidant activity. However, polyphenols can also exert prooxidative effects *in vitro*. In this study, polyphenol-mediated hydrogen peroxide (H_2O_2) formation was determined after incubation of apple juice extracts (AEs) and polyphenols in cell culture media. Effects of extracellular H_2O_2 on total glutathione (tGSH; =GSH + GSSG) and cellular reactive oxygen species (ROS) level of HT-29 cells were studied by coincubation ± catalase (CAT). AEs (≥30 μg/mL) significantly generated H_2O_2 in DMEM, depending on their composition. Similarly, H_2O_2 was measured for individual apple polyphenols/degradation products (phenolic acids > epicatechin, flavonols > dihydrochalcones). Highest concentrations were generated by compounds bearing the *o*-catechol moiety. H_2O_2 formation was found to be pH dependent; addition of CAT caused a complete decomposition of H_2O_2 whereas superoxide dismutase was less/not effective. At incubation of HT-29 cells with quercetin (1 – 100 μM), generated H_2O_2 slightly contributed to antioxidant cell protection by modulation of tGSH- and ROS-level. In conclusion, H_2O_2 generation *in vitro* by polyphenols has to be taken into consideration when interpreting results of such cell culture experiments. Unphysiologically high polyphenol concentrations, favoring substantial H_2O_2 formation, are not expected to be met *in vivo*, even under conditions of high end nutritional uptake.

Keywords: Apple juice polyphenols / Cell culture media / Glutathione / HT-29 cells / Hydrogen peroxide generation

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1 Introduction

Polyphenol-rich diets have found considerable interest with respect to their cell protective effects, potentially mitigating major degenerative diseases such as cancer or arteriosclerosis [1, 2]. In the Western diet, apples and apple juice represent a major source of polyphenols such as hydroxycinnamic acids, dihydrochalcones, monomeric and dimeric catechins [3 – 6]. Most of these polyphenols exhibit powerful antioxidant activity by acting as free radical scavengers, hydrogen donating compounds, singlet oxygen quenchers, and metal ion chelators [7]. Furthermore, they can induce cellular antioxidant defense by modulation of redox-sensitive gene expression [8].

However, prooxidant activities of polyphenols have also been reported under *in vitro* conditions [9]. Since cells in culture are exposed to a higher O_2 concentration (approx. 150 mmHg) compared to the *in vivo* situation (1 – 10 mmHg), more reactive oxygen species (ROS) may be

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Abbreviations: 3,4DHBA, 3,4-dihydroxybenzoic acid; **AE,** apple juice extract; **APE,** extract from pomace extraction juice; **CaA,** caffeic acid; **CAT,** catalase; **ChA,** chlorogenic acid; **DCF,** dichlorofluorescein; **DHCaA,** dihydrocaffeic acid; **DMEM/F12,** mixture of DMEM with Ham's Nutrient Mix F12 (1:1); **FCS,** fetal calf serum; **FI,** fluorescence increase; **FOX1,** ferrous oxidation xylenol orange; **GSH,** (reduced) glutathione; **H_2O_2,** hydrogen peroxide; **Pt,** phloretin; **Pxg,** phloretin-2′-*O*-xyloglucoside; **Pz,** phloridzin (phloretin-2′-*O*-glucoside); **Que,** quercetin; **ROS,** reactive oxygen species; **Rut,** rutin; **SOD,** superoxide dismutase; **TBH,** *tert*-butylhydroperoxide; **tGSH,** total glutathione (=GSH + GSSG)

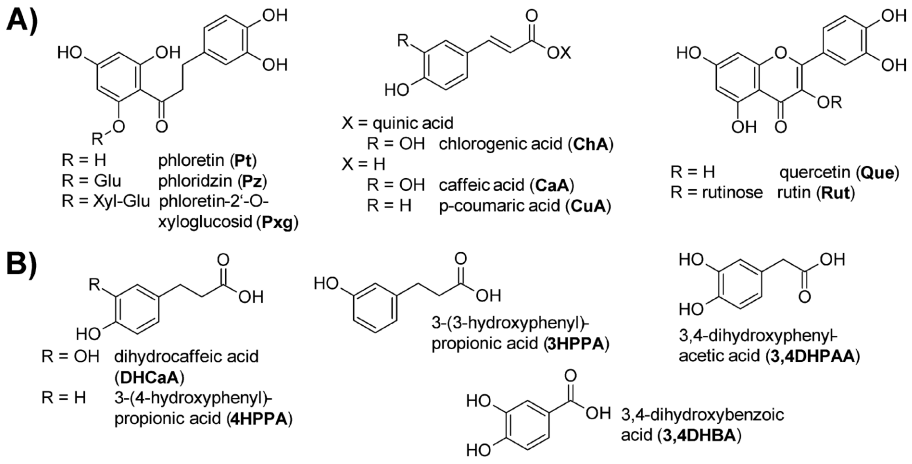

Figure 1. Structures of (A) major apple juice polyphenols/aglyca and (B) major intestinal phenolic degradation products.

produced in the medium under standard incubation conditions [10]. Polyphenols can be oxidized in cell culture medium producing significant amounts of ROS such as $O_2^{\bullet -}$ and H_2O_2 (hydrogen peroxide), which can lead to the artifactual modulation of cellular gene expression, apoptosis, or proliferation [10, 11].

The aim of the present study was to clarify, to which extent the biological effectiveness of apple phenolics in cell culture experiments is affected by polyphenol-mediated ROS formation. To this end, we studied the generation of H_2O_2 during incubation with extracts from apple and pomace extraction juice (AEs, APEs), and with major extract constituents (Fig. 1) in media. Structurally related phenolic acids, known to be generated by intestinal degradation of polyphenols [12], were also included. Structure–activity relationship of phenolic compounds and influence of medium composition on H_2O_2 generation were assessed. To study whether extracellularly generated H_2O_2 affects the cellular antioxidant defense, total glutathione level (tGSH; =GSH + GSSG) and modulation of ROS-level were monitored at incubation of HT-29 human colon carcinoma cells with selected apple polyphenols with/without catalase (CAT).

2 Materials and methods

2.1 Chemicals, cells, and media

All reagents were purchased from Sigma–Aldrich/Fluka (Taufkirchen, Germany) except for Phloretin (Pt) and HEPES, provided from Carl Roth (Karlsruhe, Germany); H_2O_2, from Merck (Darmstadt, Germany); 3-(3-hydroxyphenyl)-propionic acid (3HPPA), 3-(4-hydroxyphenyl)-propionic acid (4HPPA), and 3,4-dihydroxybenzoic acid (3,4DHBA) from Lancaster (Karlsruhe, Germany). Phloretin-2′-*O*-xyloglucoside (Pxg) was kindly provided by Junior Prof. Dr. E. Richling. BCA protein quantification kit was acquired from Uptima (Montluçon, France). All solvents and chemicals were of analytical grade or complied with the standards needed for cell culture experiments. HT-29 cells were obtained from Deutsche Sammlung fuer Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and media, fetal calf serum (FCS) and penicillin/streptomycin from Invitrogen (Karlsruhe, Germany). Media in use were DMEM [13, 14] and a 1:1 mixture of DMEM with Ham's Nutrient Mix F12 (DMEM/F12) [13, 14]. Cell culture consumable material (cell culture flasks, petri dishes, well plates, *etc*.) were purchased from Greiner Bio-One (Essen, Germany).

2.2 Preparation and analysis of phenolic apple juice extracts

Phenolic extracts (AEs and APEs) were produced from juices of different, mainly cider apple varieties, harvested at Geisenheim Research Center and from local orchards as described [15–17]. AEs (AE01, 02, 04, 05, 06, 07) were obtained from apple juices and APEs (APE03, 06) from pomace extraction juices. The juices differed with respect to the selection of apple cultivars and production year, resulting in different polyphenol patterns.

Briefly, after crushing and extraction of the apples, juices were separated and filtered. Polyphenols were adsorbed on adsorber resins and rinsed with water to eliminate sugars, organic acids, and minerals. Thereafter, the polyphenol fraction was eluted with ethanol, concentrated, freeze dried, and stored at 4°C, excluding light and moisture [15, 17]. For APEs, after the first crushing, pomace was treated with pectinases and cellulase before extraction as described [16, 17].

Polyphenols were determined on a Surveyor HPLC/DAD system (ThermoFinnigan, Dreieich, Germany). Chromatographic separation was achieved on a 150×2 mm^2, 3 µm RP-Reprosil-Pur C18-AQ column (Dr. Maisch, Ammerbuch, Germany) protected with a guard column of the same material in a cartridge holder. Injection volume was 3 µL, elution conditions were 200 µL/min flow rate at 40°C; solvent A was 2% acetic acid; solvent B was ACN/water/acetic

acid (50/49.5/0.5 v/v/v). Gradient elution was applied: 0–31 min from 10–55% B, 31–37.5 min to 100% B; washing with 100% B for 4.5 min before reequilibrating the column. Detection wavelengths were 280 nm for flavanols and dihydrochalcones, 320 nm for phenolic acids, and 360 nm for flavonols. Methanolic extracts were injected after centrifugation and 0.45 μm membrane filtration. Quantification was carried out using peak areas from external calibration curves. HPLC analysis was performed in duplicate.

Total proanthocyanidins were determined using an acid/butanol assay according to [15]. Briefly, A(P)Es were subjected to acid catalyzed depolymerization of oligomeric proanthocyanidins to yield colored anthocyanidins, which were detected photometrically. Methanolic extract solutions were mixed with 1-butanol/hydrochloric acid (95:5 v/v), followed by heating at 95°C for 2 h, cooling to room temperature, and absorbance reading at 555 nm. Quantification was performed using a purified standard of proanthocyanidins as described [15].

Sugar composition was analyzed as follows: Neutral and acidic sugars were determined after Saeman hydrolysis [18] of the polysaccharides followed by HPAEC on a Dionex Bio-LC system (Dionex Softron, Germering, Germany). In prehydrolysis, 10–15 mg of dried material was exactly weighed into glass vials and 125 μL of sulfuric acid (72% w/w) was added. Samples were sonicated for 45 min at ambient temperature. For the main hydrolysis, 1.35 mL of water was added, and the vials were placed in a heating block for 60 min at 120°C. After cooling to room temperature, the vials were transferred completely into 50 mL flasks and made up to volume with bidistilled water. To save time in larger test series, neutral sugars and D-galacturonic acid were determined in separate runs. Filtered (0.2 μm) samples (20 μL) were injected onto a 4×250 mm^2 Carbo Pac PA-1 column, guarded with a 4×50 mm^2 Carbo Pac PA-1 precolumn (Dionex Softron, Germering, Germany), both at 15°C in a Jasco column thermostat. Elution of neutral sugars was performed during 0–24 min with 12 mM NaOH, followed by flushing from 24.1 to 34 min with 400 mM NaOH and then equilibration to 12 mM NaOH. For D-galacturonic acid, the eluent was 400 mM NaOH. In both cases, the flow rate was 1.0 mL/min and the detection was electrochemical with pulsed amperometry. Quantitation was carried out using peak areas from external calibration with standard solutions. Analysis was performed in duplicate.

2.3 Incubation of AEs/polyphenols in cell culture media

AEs and polyphenols were dissolved in DMSO, diluted in cell culture medium (DMEM or DMEM/F12, supplemented with 10 and 20% FCS, respectively, and 100 U/mL penicillin, 100 μg/mL streptomycin) to reach final concentrations of 1–300 μg/mL and 1–300 μM, respectively, and incubated in 24-well tissue culture plates (final DMSO concentration 1%) for 1, 3, 6, and 24 h in a humified incubator at 37°C, 5% CO_2, and 95% relative humidity. Some incubations were performed in the presence of CAT (100 U/mL, from bovine liver) or superoxide dismutase (SOD, 1–100 U/mL, from bovine erythrocytes) to elucidate a potential influence on extracellular H_2O_2. The use of such antioxidant enzymes in the cell medium provides a tool to exclude effects originating from exogenous H_2O_2 generated during incubation. Addition of these enzymes does not affect the cellular markers, since they do not enter the cells and are removed after incubation.

To monitor the stability of H_2O_2 under the applied conditions, H_2O_2 (10, 100 μM) was added to the incubation mixture instead of polyphenols.

2.4 Determination of H_2O_2 (FOX1-assay)

H_2O_2 in cell culture medium was determined using the ferrous oxidation xylenol orange (FOX1) assay according to Wolff [19] with modifications [20]. After oxidation of Fe(II) to Fe(III) by H_2O_2, the resulting xylenol orange–Fe(III) complex was quantified photometrically (595 nm). Briefly, after 24 h incubation of medium under cell culture conditions (24-well plates, without cells), aliquots of medium were added to a solution of xylenol orange, sorbitol and Fe(II) in perchloric acid. After 20 min at 25°C, absorbance at 595 nm was monitored in a microplate reader (Synergy HT, Bio-Tek, Bad Friedrichshall, Germany). Peroxides were quantified by comparing the absorbance to a H_2O_2 standard curve (0–200 μM). CV (interassay) was 13%.

2.5 Cell culture

HT-29 cells were maintained in 175 cm^2 flasks in DMEM supplemented with 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin, in a humified incubator as described above [17]. Cells were harvested using trypsin/EDTA (0.5% v/v).

2.6 Incubation of HT-29 cells with AEs and polyphenols

When H_2O_2 formation was studied in the presence of HT-29 cells, these were seeded in 24-well plates (4.5×10^4 cells/well) allowed to grow for 24 h, washed with PBS and incubated with AEs or phenolic constituents (dissolved in DMSO with a final solvent concentration of 1%) for another 24 h in incubation medium containing 5% FCS.

For biomarker experiments, HT-29 cells were seeded either in petri dishes (tGSH determination: 10^6 cells/9.6 cm dish) or in 96-well plates [DCF (dichlorofluorescein) assay: 32 000 cells/well] and processed as described above.

2.7 Cellular ROS-level (DCF-assay)

Oxidative stress in cells was quantified using the DCF-assay according to Wang and Joseph [21], with slight modifications [12]. Briefly, after incubation with A(P)E/polyphenols, cells were washed and treated for 30 min with dichlorofluorescin-diacetate (final concentration, 50 μM in PBS pH 7.0; 0.5% DMSO v/v), washed and treated with *tert*-butylhydroperoxide (TBH, 250 μM in PBS) for 30 min at 37°C. The increase of fluorescence (FI), resulting from oxidation of the nonfluorescent probe dichlorofluorescin to fluorescent DCF by intracellular ROS, was determined from measurements at 0 and 30 min after TBH addition in a microplate reader (ex/em: 485/528 nm). All treatments and fluorimetric determination were performed in the dark. FI was calculated as described [21] and expressed as rel. FI in % of TBH-treated control. CV (intraassay) was 13%.

2.8 GSH-level (photometric kinetic assay)

Total glutathione (tGSH = GSH + GSSG) was measured by photometric determination of 5-thio-2-nitrobenzoate, formed from 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) by (reduced) glutathione (GSH), as described previously [17]. Briefly, after the incubation, cells were isolated by trypsin treatment (0.5% w/v), washed and resuspended in phosphate buffer. Aliquots were used for protein quantification. In the remaining suspension, cells were lysed by protein precipitation with 5-sulfosalicylic acid, followed by centrifugation and photometric determination of tGSH in the supernatant. A freshly prepared reaction mixture (containing DTNB, NADPH, and glutathione reductase in phosphate buffer) was added to the centrifugation supernatant and GSH-dependent formation of 5-thio-2-nitrobenzoate was monitored in a microplate reader at 412 nm. tGSH was calculated as nmol/mg protein and expressed as % of untreated solvent control. CV (intraassay) was 12%.

2.9 Statistics

Results of cell assays are presented as mean ± SD of 3 – 5 independent experiments, each performed at least in duplicate. Data were analyzed for significant difference ($p < 0.05$) to either oxidant-treated control (DCF-assay) or respective solvent control (FOX1, tGSH determination) by one-sided *t*-test.

Linear regression analysis (Microcal Origin 7.5) was used to assess correlations between H_2O_2 formation and concentration of AE composition.

3 Results and discussion

3.1 Composition of AEs

Table 1 illustrates the composition of the AEs/APEs under investigation. The amount of identified compounds in the AE01, 02, 04, 05, and APE03, reported previously [15, 17, 22], was included for comparison with the composition of AE06, AE07, and APE06. Polyphenols were found to be the major extract constituents, consisting mainly of procyanidins (including (+)-catechin and (−)-epicatechin) and phenolic acids (together representing more than 50% of the extracts), followed by dihydrochalcones (3 – 11%) and quercetin (Que) glycosides (0.3 – 11.6%). After hydrolysis, substantial amounts of sugars (up to 38%), originating mainly from cell wall oligosaccharides (such as pectins and hemicelluloses) associated to AE polyphenols [23], were detected in the AE hydrolysates. As expected, highest amounts of oligosaccharides were present in the two APEs, as a consequence of enzymatic pomace liquefaction [16]. Monomeric sugars, present as constituents in the apple juices, were found at best in trace amounts (data not shown).

Extract specific differences of composition were observed between AEs and APEs in the amount of phenolic acids (AEs > APE06 > APE03), in the amount of Que glycosides (APE03 >> other A(P)Es), and sugar oligomers (APEs > AEs).

3.2 H_2O_2 generation by polyphenolic AEs

All tested extracts (AEs, APEs) generated H_2O_2 in a concentration-dependent manner (Fig. 2) under conditions routinely used for experiments with HT-29 cells (DMEM + 5%FCS, 24 h incubation, 37°C, 5% CO_2, without cells). Amounts of H_2O_2 significantly exceeding solvent control were found at AE concentrations ≥30 μg/mL. AEs showed distinctly higher H_2O_2 concentrations than both APEs at 24 h incubation of 100 μg/mL extract. H_2O_2 generation was linearly increasing with AE concentration ($R^2 > 0.983$). Time-dependent increase of H_2O_2 was also found to be linear at incubation with 100 μg/mL AE07, which resulted in H_2O_2 concentrations of 6.5, 9.4, 12.5, and 44.4 μM after 1, 3, 6, and 24 h incubation ($R^2 = 0.997$).

3.3 Correlation between AE/APE composition and peroxide concentration

Linear regression analysis showed a direct correlation for the sum of polyphenols (coefficient of correlation $R = 0.707$; without oligomeric procyanidins) with H_2O_2 concentration in the medium after 24 h incubation of 100 μg/mL A(P)E, suggesting a marked contribution of these AE constituents to H_2O_2 formation. For the total amount of sugar oligomers, an inverse correlation was found ($R = -0.842$), indicating that higher concentrations of, *e.g.*, pectins or hemicelluloses (corresponding to lower polyphenol concentrations) in the extract are associated with lower H_2O_2 concentrations in the medium. Regarding the oligomeric procyanidins, no correlation was found ($R = 0.044$). Within the group of polyphenols, the most distinct correlation was observed for the class of phenolic

Table 1. Amounts of identified compounds in the extracts from AEs and APEs in mg/g (polyphenols) or mass% (sugars, proanthocyanidins, total recovery)

Compound	AE01 [17]	AE02 [42]	AE04 [17]	AE05 [15]	AE06	AE07	APE03 [17]	APE06
Procyanidin B_1	2.9	7.0	n.d.	2.4	2.1	2.5	6.2	2.4
(+)-Catechin	n.d.	n.d.	n.d.	5.9	4.2	4.8	2.7	4.2
Procyanidin B_2	16.0	15.1	12.1	n.d.	28.6	20.6	18.4	17.1
● (−)-Epicatechin	11.8	19.2	12.5	5.9	30.4	12.8	17.7	14.3
Procyanidin C_1	4.7	n.d.	2.0	2.5	16.0	3.9	3.4	13.5
∑ Flavan-3-ols	**35.4**	**41.3**	**26.6**	**16.7**	**81.3**	**44.5**	**48.4**	**51.5**
● Phloretin-2′-*O*-xyloglucoside	42.7	66.2	68.9	28.2	29.1	54.2	31.7	9.6
● Phloridzin	34.7	27.9	48.0	28.7	9.2	23.6	78.9	24.4
∑ Dihydrochalcones	**77.4**	**94.1**	**116.9**	**56.9**	**38.4**	**77.7**	**110.6**	**34.0**
● 5-Caffeoylquinic acid	171.8	181.5	183.2	183.9	140.3	124.9	19.2	76.6
4-Caffeoylquinic acid	10.2	n.d.	9.2	20.9	4.1	16.3	1.2	5.0
Coumaroylglucose	0.8	n.d.	11.9	2.9	0.1	1.2	n.d.	0.2
● Caffeic acid	5.5	4.8	7.5	3.9	0.1	0.0	4.0	0.7
3-Coumaroylquinic acid	16.0	9.5	9.4	2.0	3.1	5.5	3.0	0.7
4-Coumaroylquinic acid	72.4	77.3	66.0	84.9	37.3	98.2	5.0	52.1
5-Coumaroylquinic acid	7.0	10.4	39.8	n.d.	2.0	0.0	3.8	0.2
● *p*-Coumaric acid	1.8	n.d.	2.6	1.3	0.2	1.0	4.2	0.6
∑ Phenolic acids	**285.5**	**283.5**	**329.6**	**299.8**	**187.3**	**247.1**	**40.4**	**136.0**
● Quercetin-3-rutinoside	1.8	2.6	4.5	n.d.	n.d.	n.d.	49.1	n.d.
Quercetin-3-galactoside	0.9	0.8	1.8	1.5	1.0	1.1	8.1	2.8
Quercetin-3-glucoside	1.4	1.4	1.5	0.7	0.5	0.4	12.3	0.6
Quercetin-3-xyloside	0.5	n.d.	n.d.	n.d.	0.3	0.6	18.1	1.4
Quercetin-3-arabinoside	n.d.	n.d.	n.d.	n.d.	0.2	0.9	3.5	0.0
Quercetin-3-rhamnoside	3.2	4.1	4.3	1.3	1.4	1.5	25.1	0.9
∑ Flavonols	**7.8**	**8.9**	**12.1**	**3.5**	**3.4**	**4.4**	**116.2**	**5.7**
∑ Polyphenols	**406.1**	**427.8**	**485.2**	**376.9**	**310.3**	**373.7**	**315.6**	**227.3**
% Polyphenols	**40.6**	**42.8**	**48.5**	**37.7**	**31.0**	**37.4**	**31.6**	**22.7**
Fucose	0.3	0.2	0.0	0.1	0.1	0.0	0.1	0.6
Rhamnose	0.3	0.3	0.5	0.6	0.3	0.5	1.8	2.2
Arabinose	0.8	1.0	0.3	1.8	0.7	0.6	5.0	17.9
Galactose	0.8	0.7	0.3	0.9	0.8	0.4	2.5	2.9
Glucose	12.7	11.2	5.6	7.5	6.1	7.0	11.2	9.9
Xylose	2.0	2.7	0.6	1.0	0.4	1.1	1.3	2.9
Galacturonic acid	0.1	0.0	0.2	0.2	0.4	0.1	0.6	1.6
Glucuronic acid	0.8	0.2	0.1	1.0	0.1	0.6	0.2	0.2
∑ Sugars[a)]	**17.7**	**16.3**	**7.5**	**13.0**	**8.7**	**10.3**	**22.8**	**38.2**
Total Procyanidins[b)]	25	36	52	24	57	48.3	46	35
Total	**83.3**	**95.1**	**108.1**	**74.7**	**96.7**	**96.0**	**100.3**	**96.0**

Polyphenols marked with a ● were studied for H_2O_2 generation in medium.
Values given are means of two independent determinations.
a) Total content of sugars after extract hydrolysis, resulting mainly from oligo-/polysaccharides.
b) Total content of procyanidins (photometrically, including the amount of flavan-3-ols quantified by HPLC-DAD).

acids ($R = 0.776$), whereas for flavan-3-ols ($R = 0.098$) and dihydrochalcones ($R = 0.163$), almost no correlation and for Que glycosides ($R = -0.590$) an inverse correlation was found. Strongest direct correlations of individual polyphenols were seen for chlorogenic acid (ChA, $R = 0.758$), Pxg ($R = 0.725$), and 4-coumaroylquinic acid ($R = 0.684$). Similar to the group of flavonol glycosides, quercetin-3-galactoside and quercetin-3-xyloside showed an inverse correlation ($R = -0.754$ and -0.641, respectively). All other polyphenols exhibited less distinct correlations with absolute R values <0.6.

3.4 Peroxide generation by individual apple juice phenolics and their intestinal degradation products

ChA, caffeic acid (CaA); Pz, phloridzin (phloretin-2′-*O*-glucoside); Pxg; (−)-epicatechin; and rutin (Rut), representing major extract polyphenols, were comparatively tested at the described conditions to elucidate whether the correlations were supported by experimental results on H_2O_2 generation from individual phenolic constituents. The aglyca Que and Pt, representing the functional structures of many widespread extract glycosides, were included. Results are sum-

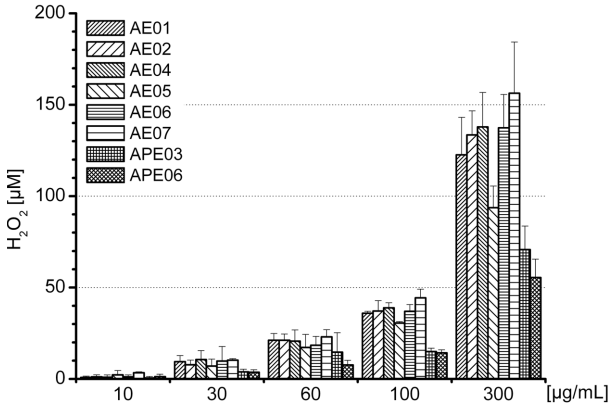

Figure 2. H_2O_2 concentration in cell culture medium (DMEM + 5%FCS, 100 U/mL penicillin, 100 µg/mL streptomycin) after 24 h incubation with extracts (10 – 300 µg/mL) from AE and APE. Mean and SD from $n = 3 – 5$ independent experiments. Solvent control (1% DMSO): <1 µM H_2O_2

marized in Table 2. After 24 h incubation, the dihydrochalcones, Pz and Pxg, as well as their aglycon Pt (each 100 µM) showed no H_2O_2 formation significantly exceeding the level of solvent control. Under these conditions, the flavonol Rut produced a small, yet significant amount of H_2O_2, whereas Que, (–)-epicatechin, and ChA were more potent H_2O_2 generators. The highest H_2O_2 level was observed for incubation with CaA. At 10 µM concentration, only Que, ChA, and CaA significantly generated H_2O_2 (exceeding solvent control). These results agree with findings of Long *et al*. [24], who reported generation of H_2O_2 by catechin, Que, and other polyphenols (100 µM each, 1 h incubation) in DMEM and other cell culture media.

ChA- and CaA-mediated H_2O_2 level was linearly elevated with incubation time (100 µM, $R^2 = 0.9995$ and 0.955, respectively, data not shown), as already observed for AE07 (see Section 2). In contrast, incubation with 100 µM Que resulted in rather similar H_2O_2 concentrations at 1, 3, 6, and 24 h (25, 28, 28, and 21 µM H_2O_2, respectively). This might be due to fast oxidation of Que [25] which is in line with the observed decay of Que in DMEM + 5% FCS [17, 26], whereas ChA and CaA were much more stable under these conditions [17].

Phenolic acids, known as intestinal degradation products of above polyphenols [12], were comparatively studied (DMEM + 5% FCS, 24 h incubation). The selected compounds differ in extent of ring hydroxylation and structure of the aliphatic side chain. Distinct H_2O_2 formation was found for substances bearing an *o*-catechol (3,4-dihydroxyphenyl) moiety but not for the respective monohydroxylated phenols: CaA > *p*-coumaric acid (CuA) and dihydrocaffeic acid (DHCaA) > 3HPPA ≈ 4HPPA. This agrees well with our findings for dihydrochalcones, lacking the catechol structure and with Miura *et al*. [27], who stated that polyphenols which possess pyrogallol or catechol moieties show strong H_2O_2-generating activity. For phenolic acids with an *o*-catechol group, saturation of the aliphatic side chain resulted in elevated H_2O_2 formation: CaA < DHCaA, whereas no influence of chain length was observed [3,4-dihydroxyphenylacetic acid (3,4DHPAA) ≈ DHCaA]. The low H_2O_2 generation from 3,4DHBA might be due to the COOH group, enhancing the bond dissociation energy for the phenolic O-H bond in *para*-position [28]. In the case of ChA, hydrolysis of the ester bond was found to increase H_2O_2 formation: ChA < CaA.

Table 2. H_2O_2 concentrations in cell culture medium (DMEM + 5%FCS, 100 U/mL penicillin, 100 µg/mL streptomycin) after 24 h incubation with selected apple polyphenols (1 – 100 µmol/L) and major degradation products (100 µmol/L)

	1 µmol/L	10 µmol/L	100 µmol/L
Solvent control[a)]	n.d.[b)]	n.d.[b)]	n.d.[b)]
Phloridzin	1.7	1.8	1.9
Phloretin	1.8	2.0	2.4
Phloretin-2′-*O*-xyloglucoside	–[c)]	–	1.7
Rutin	1.4	1.4	4.8
Quercetin	1.6	2.6	20.9
(–)-Epicatechin	n.d.	1.5	32.3
Chlorogenic acid	1.7	2.8	32.5
Caffeic acid	1.4	3.6	50.7
p-Coumaric acid	–	–	n.d.
3-(3-Hydroxyphenyl)-propionic acid	–	–	n.d.
3-(4-Hydroxyphenyl)-propionic acid	–	–	n.d.
Dihydrocaffeic acid	–	–	89.0
3.4-Dihydroxyphenylacetic acid	–	–	83.7
3.4-Dihydroxybenzoic acid	–	–	3.8
4-Methylcatechol	–	–	95.0
(–)-Quinic acid	–	–	n.d.

a) DMSO at a concentration of 1% in cell culture medium.
b) n.d. not detectable: H_2O_2 <1 µmol/L.
c) not analyzed.

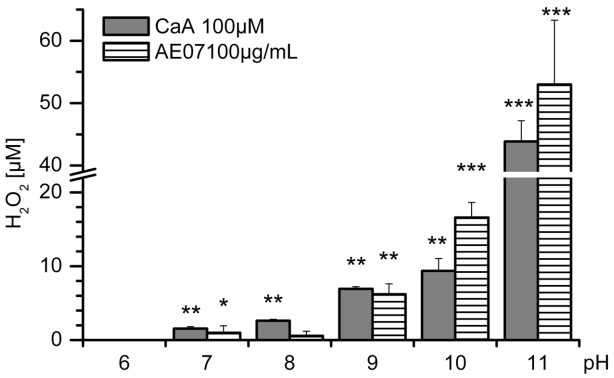

Figure 3. H_2O_2 concentration after 24 h incubation of PBS (adjusted to pH values of 6 – 11) with AE07 (100 µg/mL) or CaA (100 µM). Solvent control 1% DMSO: H_2O_2 formation for all pH values <1 µM. Mean and SD from $n = 3 – 4$ independent experiments. Significantly higher than solvent control: $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

3.5 Influence of cell culture medium composition on H_2O_2 concentration

AE07 and CaA, both potent H_2O_2 generators in DMEM (see Sections 2 and 4, respectively), were comparatively studied in DMEM/F12 and selected medium constituents (24 h, cell incubator with 5% CO_2) in the absence of cells. In addition, some experiments were performed with H_2O_2 (100 µM) instead of polyphenols to gain information on the stability of H_2O_2 in the medium. After 24 h incubation with CaA or AE07, practically no H_2O_2 could be detected using DMEM/F12 ($H_2O_2 < 2$ µM), in contrast to DMEM. Artifactual H_2O_2 formation in DMEM, but not in F12, has also been described for epigallocatechin gallate (2 h incubation) [29]. When H_2O_2 was added to the medium instead of polyphenols, its concentration substantially decreased in DMEM and even more so in DMEM/F12 (down to 14 and <1%, respectively), whereas in water H_2O_2 remained stable (>95% recovery after 24 h; data not shown). This suggests that in DMEM/F12, decomposition of H_2O_2 substantially contributes to the observed low H_2O_2 concentration after polyphenol incubation.

Among the DMEM constituents tested, only sodium bicarbonate was found to clearly accelerate H_2O_2 generation by polyphenols, *e.g.*, after 24 h incubation of 100 µM CaA in 44 mM $NaHCO_3$, 47.9 ± 1.8 µM H_2O_2 were measured. Incubation of CaA and AE07 in PBS at pH 4 – 11 showed that significant H_2O_2 formation occurred only at pH values ≥7 (Fig. 3). This supports the relevance of alkaline pH for H_2O_2 formation, since higher reactivity of the phenolate anion results in facilitated generation of the semiquinone radical anion [30] (Fig. 4). This is in line with the reduced stability of apple polyphenols in DMEM, compared to DMEM/F12 [17, 31], and favors the use of HEPES buffer (as in DMEM/F12) over HCO_3^-, whose pH adjustment is dependent on the CO_2 atmosphere in the incubator.

The observed ineffectiveness of redox active Fe (*e.g.*, 0.248 µM Fe(III) in DMEM) on H_2O_2 formation agrees well with earlier findings of Lapidot *et al*. [32], incubating gallic acid and $FeCl_3$ in DMEM. Supplementation of DMEM with 5% FCS diminished H_2O_2 concentration by 49% (AE07) and 12% (CaA) as compared to the incubation in DMEM without FCS. Since decomposition of 100 µM H_2O_2, added to DMEM, was not significantly affected by the presence of FCS (from 100 µM to 18 and 14 µM in DMEM with/without FCS, respectively), an influence of serum enzymatic activity on the degradation of H_2O_2, as stated [32], does not seem to play a major role here. Rather, polyphenol-mediated H_2O_2 generation appears to be diminished, probably due to covalent or noncovalent binding of polyphenols to serum protein [25, 33].

3.6 Effects of extracellular antioxidant enzyme (CAT, SOD) supplementation on H_2O_2 concentration

Supplementing cell culture medium with 100 U/mL CAT resulted in almost complete decomposition of polyphenol-generated H_2O_2 (Rut, Que, ChA, and CaA, 100 µM each, 24 h incubation) down to concentrations of <1 µM, as described for DMEM after incubation with delphinidin or gallic acid [22]. Accordingly, Chai *et al*. [34] reported that H_2O_2-mediated cytotoxicity, resulting from incubation of PC12 cells with green tea or red wine, was completely prevented by the addition of bovine liver CAT.

To elucidate the contribution of superoxide radical anions ($O_2^{\bullet -}$) in polyphenol-mediated H_2O_2 formation (Fig. 4), we used SOD (catalyzing the dismutation of two molecules of $O_2^{\bullet -}$ to H_2O_2 and O_2) instead of CAT. Incubations were performed with 100 µM Cha, CaA, Que, and 100 µg/mL AE07 for 1 and 24 h. With raising SOD concentrations (1, 10, and 100 U/mL), a decrease of H_2O_2 could be observed for AE07 and Que (Fig. 5). This is in line with the observation that oxygen consumption of myricetin could be inhibited by SOD, suggesting that $O_2^{\bullet -}$ might be involved in the reaction [35]. For ChA and CaA, however, H_2O_2 concentration was increased mainly after 24 h, reaching its maximum by use of 10 U/mL SOD (Fig. 5).

These results give evidence that addition of CAT to cell culture medium more efficiently prevents extracellular H_2O_2 than SOD.

3.7 Interactions of HT-29 cells with polyphenol-mediated peroxides

In the presence of HT-29 cells (under standard conditions of cell incubation, without CAT), H_2O_2 concentration in the medium after 24 h-incubation of different polyphenols (Rut, Que, ChA, CaA: 1, 10 and 100 µM each did not significantly differ from solvent control. This is attributed to decomposition of H_2O_2 by cellular antioxidant enzymes

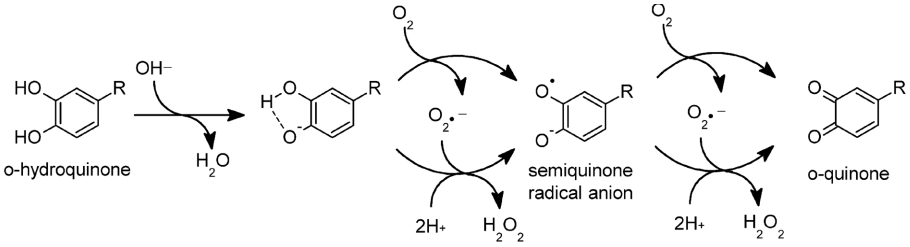

Figure 4. Possible mechanism for the autoxidation of polyphenols containing an *o*-hydroquinone moiety to the respective quinones with simultaneous generation of ROS, according to [30, 41], modified.

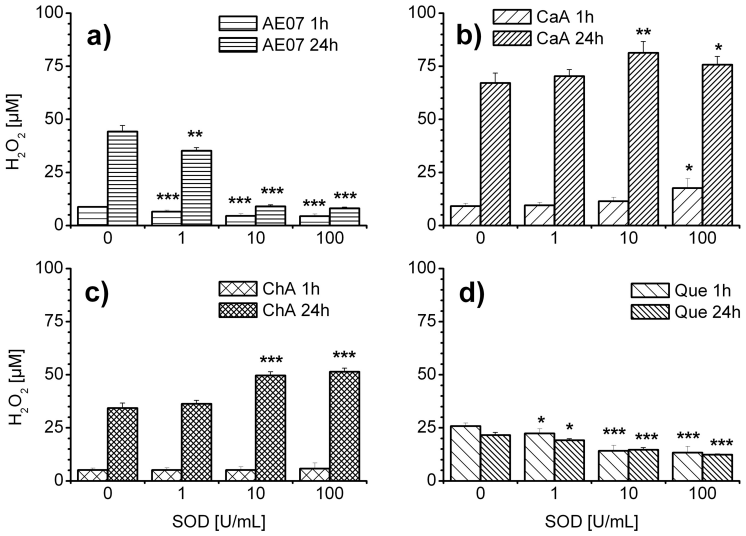

Figure 5. H_2O_2 concentration after 1 and 24 h incubation of cell culture medium (DMEM + 5%FCS), supplemented with SOD (1, 10, or 100 U/mL) and with (a) AE07 (100 µg/mL), (b) CaA, (c) ChA, or (d) Que (100 µM, each). Mean and SD from $n = 4$ independent experiments. Significantly different from control without SOD (SOD 0 U/mL): $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

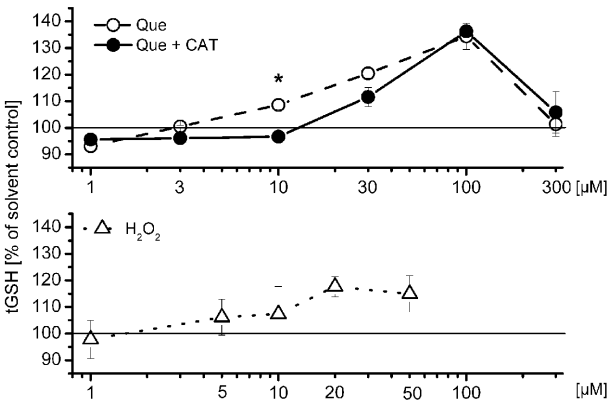

Figure 6. Total glutathione concentration (tGSH=GSH + GSSG) of HT-29 cells after 24 h incubation with Que (1 – 300 µM, with/without CAT 100 U/mL) or H_2O_2 (1 – 50 µM, without CAT). Mean and SD from $n = 3 – 5$ independent experiments. Significant differences between Que incubation with and without CAT: $^*p < 0.05$.

and/or metabolic acidification of the medium, as described [36].

Effects of polyphenol-mediated H_2O_2 generation on tGSH- and cellular ROS-level of HT-29 cells were studied by comparative incubation of polyphenols with/without addition of 100 U/mL CAT to the medium (DMEM + 5%FCS, 24 h incubation time). Without CAT, tGSH-level was raised by Que (10 – 100 µM) in a concentration-dependent manner (Fig. 6), as reported previously [17]. In the presence of CAT, however, the tGSH-level was not elevated until 30 µM Que. At ≥100 µM Que, such CAT-specific effect was no longer detectable. These results give strong evidence that moderate amounts of extracellularly generated H_2O_2 contribute to the Que-mediated induction of tGSH-level. For incubation of HT-29 cells with H_2O_2 (0.5 – 50 µM) instead of Que, a concentration-dependent increase of tGSH-level up to 115 – 120% of solvent control was observed (Fig. 6), which is in line with findings of Day and Suzuki [37], showing an increase of GSH-level in bovine artery endothelial cells by moderate H_2O_2 levels. By use of the more potent H_2O_2 generators ChA and CaA (10 – 100 µM), the increase of HT-29 tGSH-level was marginal and not modulated by CAT treatment (data not shown). Taken together, these results give evidence that extracellularly generated H_2O_2 moderately contributes to the observed (intracellular) effects of polyphenols on tGSH-level.

TBH-induced cellular ROS-level was concentration dependently decreased by Que pretreatment (0.3 – 100 µM; Fig. 7). Since only marginal differences between incubation of Que with/without CAT were observed we assume that the reduction of cellular ROS-level originates mainly from Que than from generated H_2O_2.

Preincubation with H_2O_2 (0.3 – 100 µM) instead of Que resulted in a distinct decrease of cellular ROS-level only at concentrations ≥30 µM H_2O_2 (Fig. 7), which can be ascribed to H_2O_2-mediated induction of antioxidant defense

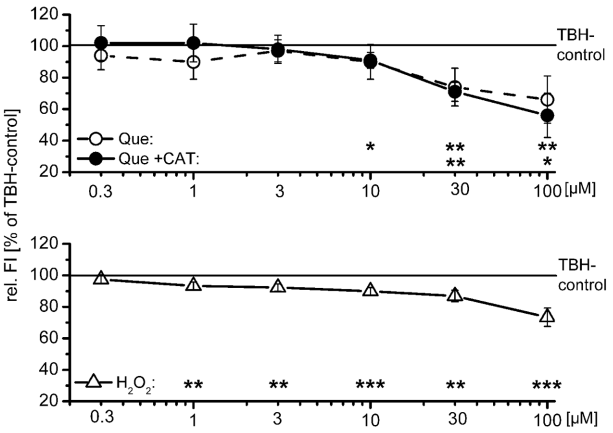

Figure 7. Modulation of TBH-induced ROS-level in HT-29 cells after 24 h incubation with Que (0.3 – 100 μM with/without CAT 100 U/mL) or H_2O_2 (0.3 – 100 μM). Mean and SD from $n = 3-4$ independent experiments. Significantly lower than TBH-treated control: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

[38]. No such effect was observed incubating the cells with Que without CAT; the generated H_2O_2 concentrations (see Table 2) were probably too low to induce ROS-decomposing enzymes.

4 Concluding remarks

Taken together, all AEs generated H_2O_2 in the commonly used cell culture medium DMEM (+5% FCS, without cells). Significant formation already occurred at extract concentrations ≥30 μg/mL, which are in the lower range of polyphenols present in the apple juice (10 – 400 μg/mL) [39]. H_2O_2 levels increased with incubation time and extract concentration and were found largely dependent on extract composition: highest values were generated by AEs (up to 44 μM for 100 μg/mL AE07, 24 h), whereas APEs were less efficient H_2O_2 generators (≤15 μM).

The direct correlation of total extract polyphenols with H_2O_2 concentration in the medium points to the relevance of these apple juice constituents, whereas oligosaccharide concentration in the extracts inversely correlated with H_2O_2 formation. Oligomeric procyanidins did not seem to contribute to H_2O_2 generation. Within the group of polyphenols, correlations with H_2O_2 levels were in the following order: phenolic acids ($R > 0$) > dihydrochalcones, flavan-3-ols ($R \approx 0$) > flavonol glycosides ($R < 0$). This ranking of H_2O_2-generating potency was confirmed by experiments with individual phenolic compounds: Phenolic acids (ChA, CaA) were the most potent H_2O_2 generators, whereas dihydrochalcones (Pt, Pxg, Pz) were practically ineffective. In contrast to the inverse correlation of total flavonol amount with H_2O_2 concentration, Rut and Que acted as moderate H_2O_2 generators. Comparing results of different apple phenolics confirms the paramount importance of the catechol moiety for H_2O_2 formation. Moreover, saturation of the aliphatic side chain (CaA *vs*. DHCaA) and its elongation (3,4DHBA *vs*. 3,4DHPAA) were also found to be enforcing factors.

Polyphenol-mediated H_2O_2 generation significantly varied in the media DMEM and DMEM/F12, largely due to differences in buffer composition and pH. Significant H_2O_2 levels were observed only at pH ≥ 7, implicating that the oxidation of the catechol to the (semi)quinone is facilitated for the phenolate anion. This is in line with our findings on enhanced H_2O_2 formation in the more alkaline DMEM, concomitant with diminished decomposition of H_2O_2. Other medium constituents, particularly redox active Fe(III), were not found to be relevant as H_2O_2 generators. FCS supplementation slightly reduced medium H_2O_2 level, probably due to binding of polyphenols to protein. Complete reduction of medium H_2O_2 was achieved by addition of CAT, whereas use of SOD resulted at best in partial decrease or even increased H_2O_2 level, suggesting that CAT is the enzyme of choice to remove H_2O_2, generated in cell culture medium during incubation.

In the presence of HT-29 cells (without CAT), polyphenol-generated H_2O_2 level in DMEM was hardly raised over solvent control, pointing to cellular uptake/decomposition of H_2O_2. Effects of extracellular H_2O_2 on cellular antioxidant defense were studied by incubation with polyphenols ± CAT or with H_2O_2. In both cases, moderate concentrations of extracellular H_2O_2 resulted in elevation of tGSH-level, probably by an adaptive response due to increased γ-glutamylcysteine ligase activity [40]. At variance to tGSH, Que-mediated decrease of cellular ROS-level was at best slightly intensified in the presence of CAT, pointing to a minor contribution of extracellular H_2O_2. Results of both biomarkers substantiate that moderate levels of extracellularly generated H_2O_2 can exert beneficial antioxidant effects in HT-29 cells which, however, contribute only to a minor extent to the observed induction of antioxidant defense by apple polyphenols in food relevant concentrations (up to 100 μM). It should be considered that higher polyphenol concentrations are known to result in H_2O_2-induced cytotoxicity/growth inhibition [22, 29].

In conclusion, H_2O_2 generation in cell culture media by individual polyphenols and by mixtures as AEs is governed by structure and concentration of phenolics, by incubation time, and by type of medium. Extracellular CAT efficiently scavenges H_2O_2. The latter was found to moderately contribute to the observed induction of tGSH-level by polyphenols.

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The authors have declared no conflict of interest.

5 References

[1] Khan, N., Afaq, F., Mukhtar, H., Cancer chemoprevention through dietary antioxidants: Progress and promise, *Antioxid. Redox Signal.* 2008, *10*, 475 – 510.

[2] Boyer, J., Liu, R. H., Apple phytochemicals and their health benefits, *Nutr. J.* 2004, *3*, 5.

[3] Brat, P., George, S., Bellamy, A., Du Chaffaut, L., *et al*., Daily polyphenol intake in France from fruit and vegetables, *J. Nutr.* 2006, *136*, 2368 – 2373.

[4] Manach, C., Scalbert, A., Morand, C., Remesy, C., Jimenez, L., Polyphenols: Food sources and bioavailability, *Am. J. Clin. Nutr.* 2004, *79*, 727 – 747.

[5] Ovaskainen, M. L., Torronen, R., Koponen, J. M., Sinkko, H. *et al*., Dietary intake and major food sources of polyphenols in Finnish adults, *J. Nutr.* 2008, *138*, 562 – 566.

[6] Scalbert, A., Williamson, G., Dietary intake and bioavailability of polyphenols, *J. Nutr.* 2000, *130 (8S Suppl.)*, 2073S – 2085S.

[7] Rice-Evans, C. A., Miller, N. J., Bolwell, P. G., Bramley, P. M., Pridham, J. B., The relative antioxidant activities of plant-derived polyphenolic flavonoids, *Free Radic. Res.* 1995, *22*, 375 – 383.

[8] Rahman, I., Biswas, S. K., Kirkham, P. A., Regulation of inflammation and redox signaling by dietary polyphenols, *Biochem. Pharmacol.* 2006, *72*, 1439 – 1452.

[9] Halliwell, B., Are polyphenols antioxidants or pro-oxidants? What do we learn from cell culture and *in vivo* studies? *Arch. Biochem. Biophys.* 2008, *476*, 107 – 112.

[10] Halliwell, B., Oxidative stress in cell culture: An underappreciated problem? *FEBS Lett*. 2003, *540*, 3 – 6.

[11] Lee-Hilz, Y. Y., Boerboom, A. M., Westphal, A. H., Berkel, W. J., *et al*., Pro-oxidant activity of flavonoids induces EpRE-mediated gene expression, *Chem. Res. Toxicol.* 2006, *19*, 1499 – 1505.

[12] Bellion, P., Hofmann, T., Pool-Zobel, B. L., Will, F., *et al*., Antioxidant effectiveness of phenolic apple juice extracts and their gut fermentation products in the human colon carcinoma cell line caco-2, *J. Agric. Food Chem.* 2008, *56*, 6310 – 6317.

[13] Dulbecco, R., Freeman, G., Plaque production by the polyoma virus, *Virology* 1959, *8*, 396 – 397.

[14] Barnes, D., Sato, G., Methods for growth of cultured cells in serum-free medium, *Anal. Biochem.* 1980, *102*, 255 – 270.

[15] Huemmer, W., Dietrich, H., Will, F., Schreier, P., Richling, E., Content and mean polymerization degree of procyanidins in extracts obtained from clear and cloudy apple juices, *Biotechnol. J.* 2008, *3*, 234 – 243.

[16] Will, F., Bauckhage, K., Dietrich, H., Apple pomace liquefaction with pectinases and cellulases: Analytical data of the corresponding juices, *Eur. Food Res. Technol.* 2000, *211*, 291 – 297.

[17] Schaefer, S., Baum, M., Eisenbrand, G., Dietrich, H., *et al*., Polyphenolic apple juice extracts and their major constituents reduce oxidative damage in human colon cell lines, *Mol. Nutr. Food Res.* 2006, *50*, 24 – 33.

[18] Saeman, J. F., Bubl, J. L., Harris, E. E., Quantitative saccharification of wood and cellulose, *Ind. Eng. Chem.* 1945, *17*, 35 – 37.

[19] Wolff, S. P., Ferrous ion oxidation in presence of ferric ion indicator xylenol orange for measurement of hydroperoxides, *Methods Enzymol.* 1994, *233*, 182 – 189.

[20] Gay, C. A., Gebicki, J. M., Perchloric acid enhances sensitivity and reproducibility of the ferric-xylenol orange peroxide assay, *Anal. Biochem.* 2002, *304*, 42 – 46.

[21] Wang, H., Joseph, J. A., Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader, *Free Radic. Biol. Med.* 1999, *27*, 612 – 616.

[22] Kern, M., Fridrich, D., Reichert, J., Skrbek, S., *et al*., Limited stability in cell culture medium and hydrogen peroxide formation affect the growth inhibitory properties of delphinidin and its degradation product gallic acid, *Mol. Nutr. Food Res.* 2007, *51*, 1163 – 1172.

[23] Will, F., Dietrich, H., Analysis of the monomer composition of wine polysaccharides, *Z. Lebensm. Unters. For.* 1990, *191*, 123 – 128.

[24] Long, L. H., Clement, M. V., Halliwell, B., Artifacts in cell culture: Rapid generation of hydrogen peroxide on addition of (–)-epigallocatechin, (–)-epigallocatechin gallate, (+)-catechin, and quercetin to commonly used cell culture media, *Biochem. Biophys. Res. Commun.* 2000, *273*, 50 – 53.

[25] Dangles, O., Fargeix, G., Dufour, C., One-electron oxidation of quercetin and quercetin derivatives in protic and non protic media, *J. Chem. Soc., Perkin Trans. 2* 1999, 1387 – 1395.

[26] Boulton, D. W., Walle, U. K.,Walle, T., Fate of the flavonoid quercetin in human cell lines: Chemical instability and metabolism, *J. Pharm. Pharmacol.* 1999, *51*, 353 – 359.

[27] Miura, Y. H., Tomita, I., Watanabe, T., Hirayama, T., Fukui, S., Active oxygens generation by flavonoids, *Biol. Pharm. Bull.* 1998, *21*, 93 – 96.

[28] Brigati, G., Lucarini, M., Mugnaini, V., Pedulli, G. F., Determination of the substituent effect on the O-H bond dissociation enthalpies of phenolic antioxidants by the EPR radical equilibration technique, *J. Org. Chem.* 2002, *67*, 4828 – 4832.

[29] Long, L. H., Kirkland, D., Whitwell, J., Halliwell, B., Different cytotoxic and clastogenic effects of epigallocatechin gallate in various cell-culture media due to variable rates of its oxidation in the culture medium, *Mutat. Res.* 2007, *634*, 177 – 183.

[30] Amorati, R., Pedulli, G. F., Cabrini, L., Zambonin, L., Landi, L., Solvent and pH effects on the antioxidant activity of caffeic and other phenolic acids, *J. Agric. Food Chem.* 2006, *54*, 2932 – 2937.

[31] Agullo, G., Gamet, L., Besson, C., Demigne, C., Remesy, C., Quercetin exerts a preferential cytotoxic effect on active dividing colon carcinoma HT29 and Caco-2 cells, *Cancer Lett*. 1994, *87*, 55 – 63.

[32] Lapidot, T., Walker, M. D., Kanner, J., Can apple antioxidants inhibit tumor cell proliferation? Generation of H(2)O(2) during interaction of phenolic compounds with cell culture media, *J. Agric. Food Chem.* 2002, *50*, 3156 – 3160.

[33] Rawel, H. M., Frey, S. K., Meidtner, K., Kroll, J., Schweigert, F. J., Determining the binding affinities of phenolic compounds to proteins by quenching of the intrinsic tryptophan fluorescence, *Mol. Nutr. Food Res.* 2006, *50*, 705 – 713.

[34] Chai, P. C., Long, L. H., Halliwell, B., Contribution of hydrogen peroxide to the cytotoxicity of green tea and red wines, *Biochem. Biophys. Res. Commun.* 2003, *304*, 650 – 654.

[35] Hodnick, W. F., Kung, F. S., Roettger, W. J., Bohmont, C. W., Pardini, R. S., Inhibition of mitochondrial respiration and production of toxic oxygen radicals by flavonoids. A structure-activity study, *Biochem. Pharmacol.* 1986, *35*, 2345 – 2357.

[36] Wee, L. M., Long, L. H., Whiteman, M., Halliwell, B., Factors affecting the ascorbate- and phenolic-dependent generation of hydrogen peroxide in Dulbecco's Modified Eagles Medium, *Free Radic. Res.* 2003, *37*, 1123 – 1130.

[37] Day, R. M., Suzuki, Y. J., Cell proliferation, reactive oxygen and cellular glutathione, *Dose Response* 2005, *3*, 425 – 442.

[38] Wijeratne, S. S., Cuppett, S. L., Schlegel, V., Hydrogen peroxide induced oxidative stress damage and antioxidant enzyme response in Caco-2 human colon cells, *J. Agric. Food Chem.* 2005, *53*, 8768 – 8774.

[39] Thielen, C., Will, F., Zacharlas, J., Dietrich, H., Jacob, H., Polyphenols in apples: Distribution of polyphenols in apple tissue and comparison of fruit and juice, *Deutsche Lebensmittel-Rundschau* 2004, *100*, 389 – 398.

[40] Seo, Y. J., Lee, J. W., Lee, E. H., Lee, H. K., *et al*., Role of glutathione in the adaptive tolerance to H_2O_2, *Free Radic. Biol. Med.* 2004, *37*, 1272 – 1281.

[41] Metodiewa, D., Jaiswal, A. K., Cenas, N., Dickancaite, E., Segura-Aguilar, J., Quercetin may act as a cytotoxic prooxidant after its metabolic activation to semiquinone and quinoidal product, *Free Radic. Biol. Med.* 1999, *26*, 107 – 116.

[42] Kern, M., Tjaden, Z., Ngiewih, Y., Puppel, N., *et al*., Inhibitors of the epidermal growth factor receptor in apple juice extract, *Mol. Nutr. Food Res.* 2005, *49*, 317 – 328.

