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Hop proanthocyanidins induce apoptosis, protein carbonylation, and cytoskeleton disorganization in human colorectal adenocarcinoma cells via reactive oxygen species

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Abstract

Proanthocyanidins (PCs) have been shown to suppress the growth of diverse human cancer cells and are considered as promising additions to the arsenal of chemopreventive phytochemicals. An oligomeric mixture of PCs from hops (*Humulus lupulus*) significantly decreased cell viability of human colon cancer HT-29 cells in a dose-dependent manner. Hop PCs, at 50 or 100 µg/ml, exhibited apoptosis-inducing properties as shown by the increase in caspase-3 activity. Increased levels of intracellular reactive oxygen species (ROS) was accompanied by an augmented accumulation of protein carbonyls. Mass spectrometry-based proteomic analysis in combination with 2-alkenal-specific immunochemical detection identified β-actin and protein disulfide isomerase as major putative targets of acrolein adduction. Incubation of HT-29 cells with hop PCs resulted in morphological changes that indicated disruption of the actin cytoskeleton. PC-mediated hydrogen peroxide (H₂O₂) formation in the cell culture media was also quantified; but, the measured H₂O₂ levels would not explain the observed changes in the oxidative modifications of actin. These findings suggest new modes of action for proanthocyanidins as antitumorigenic agents in human colon cancer cells, namely, promotion of protein oxidative modifications and cytoskeleton derangement.

Keywords

Acrolein; actin; apoptosis; proanthocyanidins; protein carbonyls; HT-29 cells

1. Introduction

Proanthocyanidins (PCs) or condensed tannins are mixtures of oligomers and polymers composed of flavan-3-ol units found in fruits, vegetables, cereals, seeds, nuts, bark, spices, cocoa, and beverages (Rasmussen et al., 2005). PCs are of increasing interest because of their potential health benefits and cancer chemopreventive properties (Dixon et al., 2005; Prior and Gu, 2005). The potential chemopreventive properties of PCs against colon cancer has been

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demonstrated in mice dosed with 1,2-dimethylhydrazine (Gali-Muhtasib et al., 2001) and in rats treated with azoxymethane (Singletary and Meline, 2001). PCs isolated from various fruits have been shown to suppress growth and to induce apoptosis in colon cancer cells (Engelbrecht et al., 2007; Hostanska et al., 2007; Lizarraga et al., 2007; Seeram et al., 2006).

The anticarcinogenic effects of PCs may be explained by various mechanisms such as free radical scavenging activity, regulation of signal transduction pathways by altered expression of key enzymes such as protein kinases, cell cycle arrest, suppression of oncogenes, induction of apoptosis, modulation of enzyme activity related to carcinogen detoxification, inhibition of DNA synthesis, and stimulation of DNA repair (Aron and Kennedy, 2008). The induction of apoptosis in colon cancer cells by grape seed PCs was correlated with the inactivation of the PI3-kinase/PKB pathway (Engelbrecht et al., 2007).

Hops (*Humulus lupulus*) may be tapped as a rich source of PCs for the chemoprevention of colon cancer. Hop PCs are mixtures of oligomers that consist of monomer units such as catechin, epicatechin, galocatechin, epigallocatechin, afzelechin, and epiafzelechin (Li and Deinzer, 2006). These PCs also contain unique dimers such as prodelphinidins with galocatechin units and the propelargonidin dimer, afzelechin-(4 → 8)-catechin (Li and Deinzer, 2006; Taylor et al., 2003).

One of the properties that makes PCs particularly attractive in the chemoprevention of colon cancer is that the PC concentrations may reach low mM (< 3) concentrations in the colon (Scalbert and Williamson, 2000) and retention in the colon may give these polyphenolics the potential to function as local anti-colon cancer agents. In the colon, the microbial flora may convert the PCs into products that may have improved cell membrane permeability and anti-cancer activities. We have examined the growth inhibitory effect of PCs from hops in the human colon cancer cell line, HT-29, and investigated the possible molecular mechanisms underlying these effects. The present study shows that hop PCs are cytotoxic to HT-29 colorectal adenocarcinoma cells through formation of ROS, leading to protein carbonylation and to cytoskeleton disorganization.

2. Materials and methods

2.1. Materials

5-(and -6)-Chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂ DCFDA), rhodamine phalloidin and biotinylated anti-DNPH rabbit polyclonal antibody were obtained from Molecular Probes (Eugene, OR). The fluorogenic caspase-3 substrate, Ac-DEVD-AMC [N-acetyl-Asp-Glu-Val-Asp-AMC (7-amino-4-methylcoumarin)], was purchased from EMD Biosciences, Inc. (San Diego, CA, USA). 2,4-Dinitrophenylhydrazine (DNPH), epigallocatechin gallate (EGCG), and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were obtained from Sigma-Aldrich (St. Louis, MO). Coomassie Plus Protein assay kit, Laemmli sample buffer, pre-cast 10-well 12% Tris-HCl gels, prestained SDS-PAGE standards, Coomassie Blue G250 solution, and goat anti-mouse IgG-HRP conjugate were obtained from Bio-Rad Laboratories (Hercules, CA). Monoclonal anti-acrolein IgG and monoclonal anti-HNE IgG were purchased from COSMO BIO CO., LTD. (Carlsbad, CA) and Oxis International, Inc. (Foster City, CA), respectively. Monoclonal anti-β actin IgG and anti-PDI IgG were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). SuperSignal West Pico Chemiluminescent Substrate was obtained from Pierce Biotechnology, Inc. (Rockford, IL). All other test chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Cell culture

HT-29 cells were obtained from the American Type Culture Collection (Manassas, VA) and were grown in 75 cm² tissue culture flasks in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. The cell cultures were incubated at 37 °C in a humidified atmosphere of 5% CO₂.

2.3. Cytotoxicity assay

The cells were harvested from the culture flasks and plated on a 96-well microtiter plate, with each well containing 0.2 ml of complete phenol-red free RPMI 1640 medium per well. After 24 h of incubation at 37 °C in 5% CO₂, the medium was replaced with fresh phenol-red free and serum-free RPMI 1640 medium containing hop PC mixture at increasing concentrations (10, 50, and 100 µg/ml). The composition of this PC mixture was described in previous work (Stevens et al., 2002). These concentrations of the PC mixture were chosen on the basis of previous cytotoxicity studies of grape seed proanthocyanidins conducted in HT-29 cells (Kaur et al., 2006). Vehicle-treated control cells were fed RPMI medium containing methanol (used for dissolving the hop PC mixture) not to exceed 0.1%. After 24 h, cell viability was measured using the MTT assay as described by Chung et al. (2007). This assay was based on the reduction of a tetrazolium dye to a blue formazan by functioning mitochondria.

2.4. Caspase-3 activity

Caspase-3 activity was used to examine the effect of PCs on apoptosis in HT-29 cells using the method described by Chung et al. (2007). HT-29 cells were seeded at a density of 5×10^5 per well onto 6-well plates. After 24 h of incubation, the cells were fed serum-free RPMI media containing 0.1% methanol or hop PC mixture at various concentrations. The cells were incubated for another 24 h incubation before harvesting in caspase-3 buffer (25 mM HEPES, pH 7.4, 10% glycerol, and 1 mM EDTA) using a cell scraper. The cells were lysed by sonication and then centrifuged at $10,000 \times g$ for 5 min. The resulting supernatant was used to measure caspase-3 activity with the fluorogenic caspase-3 tetrapeptide-substrate, Ac-DEVD-AMC, at a final concentration of 30 µM, according to the manufacturer's instructions. Fluorescence intensity of control and treated wells was measured by a microplate fluorescence reader (SpectraMax Gemini, Molecular Devices, Sunnyvale, CA) with excitation wavelength at 380 nm and emission wavelength at 460 nm.

2.5. Detection of ROS in HT-29 cells

The formation of intracellular ROS in HT-29 cells was detected by the use of CM-H₂DCFDA. This dye is cell membrane-permeable and is cleaved by non-specific esterases to remove the acetyl groups inside the cell to form CM-H₂DCF which is then oxidized to a fluorescent product by ROS. Cells were cultured in serum-free RPMI medium in the presence and absence of increasing concentrations of hop PC mixture. After a 24-h incubation at 37 °C, the cells were washed twice with phenol red-free and serum-free RPMI medium and then incubated for 20 min at 37 °C in phenol red-free RPMI with 10 µM CM-H₂DCFDA. The cells were then washed of excess extracellular dye, trypsinized and washed with PBS before examination by fluorescence microscopy.

2.6. Measurement of hydrogen peroxide in cell culture media

The PC mixture (10, 50 or 100 µg/ml) was added to phenol red-free and serum-free RPMI 1640 media with or without HT-29 cells. After a 24-h incubation at 37 °C in 5% CO₂, the media were analyzed for H₂O₂ content by a ferrous ion oxidation-xylenol orange method using the PeroXOquantTM Quantitative Peroxide Assay kit (Pierce) according to the manufacturer's

directions. The samples were transferred to 96-well plates and the absorbance of the samples was read at 595 nm using a Microplate reader (SpectraMax 190).

2.7. Determination of protein carbonyls by ELISA

Protein carbonylation of control and PC-treated HT-29 cells was determined by an ELISA according to the method of Buss and Winterbourn (2002). Briefly, 96-well high-binding ELISA plates were coated overnight at 4 °C with aliquots (200 µl/well, containing 1 µg of protein) of test samples or of increasing concentrations of oxidized BSA standards (Zenith PC Test Kits). Prior to loading, samples and BSA standards were first reacted with 3 volumes of 10 mM DNPH in 6 M guanidine hydrochloride in 0.5 M phosphate buffer, pH 2.5, for 45 min at room temperature. The plates were washed with PBS and then incubated with PBS containing 0.1% Tween 20 (PBS-T). The plates were extensively washed with PBS and then incubated with 200 µl of biotinylated anti-DNPH rabbit polyclonal antibody per well. Following a 30-min incubation at room temperature, the wells were washed with PBS and then 200 µl of Streptavidin-HRP was added to each well. After 1 h of incubation, the wells were washed and 200 µl of TMB + Substrate-chromogen (Dako, Carpinteria, CA) was added to each well. After a 5-min incubation, the reaction was stopped by adding 100 µl of 2.5 M sulfuric acid. The absorbance was read at 450 nm using a Microplate reader (SpectraMax 190, Molecular Devices, Sunnyvale, CA).

2.8. SDS-PAGE and Western blots

HT-29 cells were treated with PC mixture (0, 10, 50, or 100 µg/ml) and after 24 h, the cells were harvested and collected by centrifugation at $200 \times g$ for 3 min. The cell pellet was resuspended in PBS containing 0.5% Triton X-100, vortexed vigorously and stored at -80 °C for 20 min. The cells were thawed, vortexed, and then centrifuged at $10,000 \times g$ for 10 min. The resulting supernatant was analyzed for protein content by using the Coomassie Plus Protein assay kit. Aliquots (20 µg) of the samples were mixed with Laemmli sample buffer prior to loading onto a 10-well 12% Tris-HCl gel with prestained SDS-PAGE protein standards. After electrophoresis, the proteins were transferred to a nitrocellulose membrane for Western blot analysis as described by Chung et al. (2007) using anti-acrolein IgG, anti-HNE IgG, anti-β actin IgG, or anti-PDI IgG as probes. Proteins were detected using the chemiluminescence reagent, SuperSignal West Pico Chemiluminescent Substrate, and the resulting immunoreactive protein bands were visualized on X-ray film.

To identify the proteins recognized by these probes, parallel gels were stained with IEF gel staining solution. The bands of interest were excised and the protein bands were digested with trypsin and desalted with Ziptip C18. The extracted peptides were analyzed by tandem mass spectrometry using a MALDI-TOF/TOF instrument.

2.9. Two-dimensional (2D) Electrophoresis

Proteins (100 µg) were dissolved in rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.2% (v/v) biolytes, 100 mM dithiothreitol (DTT), and 0.001% bromophenol blue) and sonicated for 10 min before applying to a Ready Strip IPG (pH 3–10). The strips were then actively rehydrated at 50 V for 15 h in a protean IEF cell (Bio-Rad). Isoelectric focusing (IEF) was performed at 20 °C as follows: 250V for 15 min, 8,000 V for 2.5 h rapid gradient and 35,000 V·h. Prior to second dimension separation, gel strips were equilibrated for 10 min in 375 mM Tris–HCl (pH 8.8) containing 6 M urea, 2% (w/v) sodium dodecyl sulfate, 20% (v/v) glycerol, and 2% DTT. The strips then were equilibrated for 10 min in the same buffer containing 2.5% iodoacetamide in place of DTT. Gradient criterion Tris–HCl gels (8–16%, Bio-Rad) were used to perform second dimension electrophoresis. Prestained protein standards were run along with the sample at 130 V for 115 min. After electrophoresis, the proteins were transferred to a nitrocellulose membrane and blocked with 5% non-fat milk in TBS (Tris-

buffered saline) for 2 h at room temperature. The membrane was probed with anti-acrolein IgG. The membrane was washed 6 times with TBS-T (TBS with 0.1% tween-20) and then incubated with goat anti-mouse IgG-HRP conjugate. After washing, the membrane was incubated with SuperSignal West Pico Chemiluminescent Substrate for 5 min. The acrolein-positive bands were then visualized on X-ray film.

2.10. Identification of immunoreactive proteins by tandem mass spectrometry

To identify the proteins recognized by anti-acrolein antibody, a parallel gel was stained with Coomassie Blue G250 solution for 2 h at room temperature. The gel was destained with water. The bands or protein spots of interest were excised. The protein bands or spots were digested with trypsin and desalted with Ziptip C18 (Millipore, ZTC18S096). The peptides were analyzed by MALDI tandem mass spectrometry as described by Chavez et al. (2006).

2.11. Visualization of actin cytoskeleton by fluorescence microscopy

HT-29 cells were grown in a 35 mm petri dish with a 14 mm microwell coverglass (Mat Tek Corporation, Ashland, MA) and treated with 0, 10, 50 or 100 $\mu\text{g/ml}$ PC mixture for 24 h. After treatment, the cells were washed twice with PBS and then fixed with 3.7% formaldehyde solution in PBS for 10 min. After washing with PBS, the cells were incubated with 0.1% Triton X-100 for 5 min. The cells were again washed with PBS and then stained with rhodamine phalloidin (5 units/ml) in PBS. The cells were washed with PBS and then examined by using a fluorescence microscope with a 40 $\times$ objective and a video camera for recording the images.

2.12. Statistics

Unpaired Student's t-test was performed to assess the differences between groups. Differences in means between treatment groups were considered statistically significant if $P < 0.05$. All values are given as mean $\pm$ SE.

3. Results

3.1. Effect of hop PCs on HT-29 cell viability

Treatment of HT-29 cells with the mixture of PCs isolated from hops produced a significant dose-dependent decrease in cell viability as measured by the MTT assay (Fig. 1). Cell viability of HT-29 cells treated with 10, 50, and 100 $\mu\text{g/ml}$ PC mixture were 104, 75.2, and 47.2 of control value. The cytotoxicity of the PC mixture was not significantly affected by the addition of catalase (100 units/ml) to the culture medium (Fig. 1), indicating that the observed effect was not due to H_2O_2 generated by autooxidation of the polyphenols in the cell culture medium.

3.2. H_2O_2 content of RPMI media

PCs as many other antioxidants are prone to oxidation in diverse cell culture media resulting in the formation of H_2O_2 and semiquinones/quinones (Long et al., 2000). The chemical composition of PCs varies from source to source. Hop PCs differ from grapeseed or tea PCs by the absence of gallate esters and the presence of PC units with one OH group at the B-rings. The effect of hop PCs on H_2O_2 formation and acrolein adduction to cellular proteins has not previously been studied. Because H_2O_2 formation was found to be associated with higher levels of acrolein adduction to proteins, we determined the levels of H_2O_2 in the culture media in the presence and absence of HT-29 cells as well as the intracellular levels of H_2O_2 .

Both fresh and incubated phenol red-free RPMI 1640 media contained 1.1 μM H_2O_2 (Fig. 2A). Incubation of RPMI 1640 with the PC mixture in the absence of cells for 24 h at 37 $^\circ\text{C}$ caused a dose-dependent increase in the levels of H_2O_2 , reaching up to 50 μM in the culture media treated with 100 $\mu\text{g/ml}$ PCs. When the RPMI 1640 medium was added to cells and incubated

for 24 h at 37 °C in the absence of PCs, the amount of H₂O₂ in the culture medium was 0.56 μM. In the presence of cells, the H₂O₂ concentration determined after 24 h was 2.94 μM for the media containing 100 μg/ml PCs. These findings suggest that the hop PCs induced the formation of H₂O₂ in the medium and that the cells' antioxidant enzymes (i.e. catalase, glutathione peroxidase and peroxiredoxins) are capable of efficiently removing the PC-generated H₂O₂ in concordance with a study of Antunes and Cadenas (2000). The addition of H₂O₂ (3 -100 μM) to the culture medium with cells did not cause a significant increase in H₂O₂ content of the culture medium after 24 h of incubation (Fig. 2B).

3.3. Effect of hop PCs on caspase-3 activity

Caspase-3-like activity of the cytosol fraction of HT-29 cells pretreated with 50 or 100 μg/ml PCs was significantly increased by 3- and 4-fold, respectively, compared to control cells (Fig. 3). No significant increase in caspase-3 activity was observed at 10 μg/ml PCs.

3.4. Effect of hop PC mixture on intracellular ROS levels

Intracellular ROS was detected in HT-29 cells using CM-H₂ DCFDA which is oxidized to the highly fluorescent DCF. Exposure to 50 or 100 μg/ml PCs caused an increase of ROS levels in treated cells as reflected by increased fluorescence intensity of the treated as compared to untreated control cells (Fig. 4). However, this assay does not allow deciphering if the ROS detected in cells were generated intracellularly or extracellularly. The results reported in Figure 2 show that there is a PC dose-dependent increase of H₂O₂ levels in the medium and that the extracellular H₂O₂ levels are lower in the presence of cells. These data clearly indicate that the medium is a prominent source of H₂O₂ formation and that extracellular H₂O₂ may migrate into cells across the cell membrane (Antunes and Cadenas, 2000). However, intracellular H₂O₂ formation due to PCs taken up by the cells cannot be excluded.

3.5. Effect of hop PCs on protein carbonyl content

Whole cell homogenates of control and PC-treated HT-29 cells were examined for protein oxidative damage using anti-DNP antibody in ELISA. Fig. 5A shows a significant increase in protein carbonyls of HT-29 cells with increasing concentrations of hop PC mixture over control levels. Treatment of HT-29 cells with 10, 50 or 100 μg/ml PCs resulted in 14-, 20- and 22-times higher protein carbonyl levels with respect to the control cells. Exposing the HT-29 cells to bolus additions of 15 and 50 μM H₂O₂ resulted in no significant elevation of protein carbonyl levels; whereas exposure to 100 μM H₂O₂ resulted in marked increase in protein carbonyl levels (Fig. 5B).

3.6. Identification of β-actin and PDI as the major targets of PC-induced oxidative stress in HT-29 cells

To determine if PCs treatments of HT-29 cells affect protein levels, we separated whole protein cell lysates on SDS-PAGE (Fig. 6A), excised protein bands and subjected the gel pieces to trypsin digestion. Resulting peptide extracts were subjected to MALDI-TOF/TOF tandem mass spectrometry for obtaining peptide sequencing data and the proteins were identified using the MASCOT search engine. The levels of the following proteins appeared to be lower in the PC-treated cells (lanes 3, 50 μg/ml PC mixture; and lane 4, 100 μg/ml PC mixture, Fig. 6A) than in the controls (lane 1): myosin-9, heat shock protein 90-α and β, α-enolase, 14-3-3 protein gamma, and triosephosphate isomerase. In contrast, the levels of α-actin 1 (band no. 2) were higher in cells treated with 50 (lane 3) or 100 μg/ml (lane 4) PC mixture than in the controls (lane 1). Other proteins showed no obvious change in their cellular concentration in the SDS-PAGE and they were identified as follows: 78 kDa glucose regulatory protein; heat shock protein 71; protein disulfide isomerase (PDIA3); cytoplasmic 1 actin (β-actin; Fig. 6A, band no.8.); keratin type I; keratin type II; glyceraldehyde 3-phosphate dehydrogenase; 14-3-3

protein gamma; and glutathione S-transferase. The mass spectrometry data of the identified proteins are summarized in Table 1.

To further examine if the elevated protein carbonyls levels are, at least in part, related to oxidative modifications caused by reactive lipid peroxidation products, we used 2-alkenal specific antibodies in Western blot analyses. Proteins that were recognized by anti-acrolein antibody in Western blots were protein disulfide isomerase, β -actin, and keratin type I (Fig. 6B). The levels of immunoreactive β -actin detected by anti-acrolein antibody were markedly increased by treatment of the cells with 50 or 100 μ g/ml PC mixture. PDI, with a monomeric molecular weight of 55 kDa, was also detected by anti-acrolein IgG in these PC-treated cells but not in control cells (Fig. 6B). The levels of immunoreactive keratin type I were not altered by treatment of the cells with the PC mixture.

In Western blots probed with anti-HNE antibody, the levels of an immunoreactive band corresponding β -actin were highly increased in HT-29 cells treated with 50 or 100 μ g/ml PC mixture (Fig. 6C). No immunoreactive band was detected by anti-HNE in the region of the PDI (Fig. 6C).

The nitrocellulose membrane used in Figure 6B was re-probed with anti- β -actin monoclonal antibody, followed by anti-PDI IgG and the resulting blot is shown in Fig. 6D. Only one protein band corresponding to β -actin was detected by the anti- β -actin antibody in control and PC-treated cells. When the same blot was re-probed with anti-PDI, a second band appeared corresponding to PDI. The levels of β -actin and PDI proteins appeared to be unchanged after treating the cells with the PC mixture (Fig. 6D). HT-29 cells treated with 50 or 100 μ M H_2O_2 for 24 h did not show an increase in immunoreactive β -actin detected by anti-acrolein antibody (Fig. 6E).

To unequivocally confirm the formation of oxidatively modified β -actin, protein samples used in the 1D electrophoresis were separated by 2D electrophoresis (Fig. 7). Tandem mass spectrometry of the tryptic peptides extracted from the excised spots indicated that β -actin was present in multiple spots and that treatment of HT-29 cells with 100 μ g/ml hop PCs resulted in increased diversity and levels of post-translationally modified β -actin proteins.

Western blot analysis of the 2D gel with anti-acrolein antibody indicated that several of the modified β -actin proteins stained highly positive in HT-29 cells treated with 100 μ g/ml PCs (Fig. 7B) but were only appearing as faint spots in the control sample (Fig. 7A).

In addition, the 2D Western blot analysis confirmed unequivocally PDI as an additional target of acrolein adduction. In the center panel, two spots labeled as 4 were seen in control (Fig. 7A) and PC-treated cells (Fig. 7B) and these spots were identified as PDIA3. The two PDIA3 spots were recognized by anti-acrolein IgG in the PC-treated cells (right lower panel, Fig. 7B) but not in the control cells (right upper panel, Fig. 7A). The mass spectrometry data used for the identification of β -actin and PDIA3 in the 2D-gel are included in Table 1.

3.7. PC-induced disruption of the actin cytoskeleton

Chemical modification of β -actin has been previously linked to filament misfolding and cytoskeleton disorganization (Allingham et al., 2006). We therefore examined the organization of the HT-29 cytoskeleton before and after treatment with hop PCs. The actin filaments were stained with phalloidin covalently linked to rhodamine and visualized using a fluorescence microscope. In control cells, the actin filament showed a ring-like organization at the cell periphery whereas in PC-treated cells, actin appeared in clusters as shown by the increased fluorescence intensity in the affected cells (100 μ g/ml PC vs. control cells, Fig. 8). Cluster appearance of actin has also been reported by Feick et al. (2006) after treating HT-29 cells with

formaldehyde. The cells also appear to be aggregated but this is normal for HT-29 cells if these cells are grown on a petri dish or in a culture flask before becoming confluent. The aggregation of HT-29 cells has been described previously (Feick et al., 2006).

4. Discussion

The present study revealed that hop PCs are cytotoxic to HT-29 colon cancer cells and their toxicity was associated with elevated levels of intracellular ROS, increased formation of protein carbonyls and cytoskeletal disorganization.

Many earlier studies have shown that PCs from diverse polyphenols can act either as antioxidants or prooxidants depending on the cell type and concentrations used (Hadi et al., 2007; Oikawa et al., 2003; Sakano et al., 2005). For example, (epi)catechin caused oxidative DNA damage, as indicated by the formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) in the human leukemia cell line HL-60 but not in HP100, a hydrogen peroxide (H_2O_2)-resistant cell line derived from HL-60 (Oikawa et al., 2003). Similarly, procyanidin B2 (epicatechin-(4 β -8)-epicatechin) inhibited the formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) in HL-60 cells treated with an H_2O_2 -generating system, but high concentration of procyanidin B2 (> 500 μ g/ml) increased the formation of 8-oxodG in these cells (Sakano et al., 2005). Several studies indicate that at least some of the tumoricidal activities of EGCG can be attributed to the cytotoxicity of H_2O_2 generated during the oxidation of polyphenols to the semiquinone and quinone derivatives, a reaction promoted by the presence of superoxide radical anion (Chan et al., 2006; Yamamoto et al., 2004). For HT-29-18-C1 cells, the median lethal dose for one h exposure to H_2O_2 was approximately 0.1 mM (Watson et al., 1994). Whereas, a bolus addition of 30 μ M H_2O_2 directly to the culture media did not affect cell viability of HT-29 cells (Klenow et al., 2008).

Polyphenolic compounds get readily oxidized in cell culture media to semiquinones/quinones, whereby H_2O_2 is released into the cell medium (Halliwell et al., 2000; Halliwell, 2003; Lambert et al., 2007). It is therefore conceivable that H_2O_2 generated in the cell culture medium may at least partly contribute to the observed cytotoxicity and intracellular effects attributed to polyphenols exposure. In our study, the addition of the PC mixture (100 μ g/ml) and 24-h incubation at 37 °C resulted in H_2O_2 concentrations in RPMI 1640 media of 50 μ M and 2.9 μ M in the absence and presence of HT-29 cells, respectively. A previous study by Cadenas and colleagues (Antunes and Cadenas, 2000) demonstrates that the rapid enzymatic consumption of H_2O_2 inside the cell results in a steep H_2O_2 gradient across the plasma membrane after a bolus addition of H_2O_2 and low intracellular concentration of H_2O_2 . Therefore, the cells' arsenal of H_2O_2 -consuming enzymes and capability of regulating membrane permeability for H_2O_2 will affect the intracellular effects of polyphenolics. In our hands, the presence of catalase (100 units/ml) in the culture medium did not affect the cytotoxicity of the PC mixture on the HT-29 cells (Fig. 1). We also tested the impact of different concentrations of H_2O_2 on protein carbonyl levels. These experiments revealed that protein carbonyl levels (Fig. 5) as well as acrolein adduction to β -actin (Fig. 6E) were unaffected by bolus additions of H_2O_2 up to 50 μ M. The H_2O_2 content of culture media with cells was not significantly increased after a 24-h incubation with 100 μ M H_2O_2 . Therefore, we concluded that low amounts of H_2O_2 generated in the media in the presence of cells were unlikely responsible for the cytotoxic effects observed for the hop proanthocyanidin mixture tested.

Measurement of intracellular DCF fluorescence showed that treatment of HT-29 cells with hop PCs led to increased intracellular levels of ROS (Fig. 4). During condition of oxidative stress reduced glutathione (GSH) is converted into glutathione disulfide (GSSG) and, consequently, GSH may get depleted. Glutathione peroxidase converts H_2O_2 and lipid hydroperoxides to water and lipid alcohols, respectively, whereby GSH provides the reducing equivalents

(Doroshov et al., 1990). In addition, GSH readily forms adducts with a great variety of both endogenous and exogenous electrophiles. In our system, it is conceivable that reactive lipid peroxidation products, such as acrolein and HNE, as well as phenolic semiquinone/quinones contribute to GSH consumption. Maintenance of GSH/GSSG homeostasis is critical for cell survival. Depletion of GSH triggers apoptotic pathways and we demonstrate a dose-dependence of caspase-3 like activity. Disturbance of the cellular redox balance results in increased protein oxidative insults.

We measured total carbonyl levels using a DNPH-based ELISA and found that hop PCs induce the formation of protein carbonyls in HT-29 cells. We performed proteomic analysis (1D or 2D gel electrophoresis followed by mass spectrometry) to gain an insight into the nature of the proteins that were targets of oxidative stress-related modifications. Protein carbonylation reactions are chemically diverse; beside the direct oxidation of amino acid side chains to semialdehydes (Requena et al., 2001), the adduction of nucleophilic sites to α,β -unsaturated lipid peroxidation products contributes to elevated carbonyl levels (Aldini et al., 2007b). With the use of anti-acrolein IgG as a probe, we found that β -actin and PDI were the major targets of protein adduction by acrolein in HT-29 cells exposed to hop PCs (Figs. 6 and 7). The increase in β -actin-acrolein adduct formation is not the result of increased levels of β -actin protein because β -actin levels were not altered by PC treatment as shown in the Western blot probed with anti- β -actin antibody (Fig. 6D). HNE also contributed to the carbonylation of β -actin as shown by the Western blot probed with anti-HNE IgG (Fig. 6C). Our finding is in agreement with previous studies which showed that β -actin is a major target of protein carbonylation as in gerbil synaptosomes treated with acrolein (Mello et al., 2007), human neuroblastoma SH-SY5Y cells treated with 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂) (Aldini et al., 2007c), and Alzheimer's disease brain (Butterfield et al., 2002).

Adduction of acrolein to β -actin may result in a disruption of the actin cytoskeleton in cells treated with the PC mixture. To test this hypothesis, we exposed cells to various concentrations of the PC mixture for 24 h and then examined the actin cytoskeleton organization by fluorescence microscopy using a rhodamine-phalloidin stain. Treatment of the cells with PC caused a disorganization of the ring-like cytoskeleton structure which was most evident in cells treated with 50 and 100 μ g/ml PC (Fig. 8). Disruption of the actin cytoskeleton structure has been observed in human gingival fibroblasts treated with acrolein (Poggi et al., 2002) and in HT-29 cells exposed to 1 mM formaldehyde for 1 h (Feick et al., 2006). Disorganization of the actin cytoskeleton was also seen in bovine lung microvascular endothelial cells treated with HNE (25 μ M) for 30 min (Usatyuk et al., 2006). Thus, alterations in the cytoskeleton as a result of increased carbonylation of β -actin by acrolein and HNE may be one possible mechanism of cell death induced by oxidative damage in HT-29 cells treated with hop PCs.

Acrolein has a propensity to undergo Michael addition with cysteine, histidine, and lysine and is considered the most reactive of the α,β -unsaturated aldehydes produced by lipid peroxidation (Aldini et al., 2007a; Aldini et al., 2007b; Esterbauer et al., 1991; Uchida, 1999). In *in vitro* studies employing G-actin, Cys-374 was identified as the site of carbonylation by acrolein (Aldini et al., 2007b; Aldini et al., 2007c; Dalle-Donne et al., 2007). 2-Alkenals are bifunctional electrophiles and Michael type-addition would allow for secondary reactions, such as Schiff's base formation, resulting in protein crosslinks. Noteworthy, the 2D gel electrophoretic maps indicated that in PC-treated HT-29 cells β -actin was found to be extensively modified as evident in the large number of spots visible in the 2D gel. Clearly, other modifications occurred beside the adduction to the two identified lipid peroxidation products, acrolein and HNE. Possible oxidative modifications may include hydroxyl radical-triggered side chain oxidations, direct oxidation of cysteine thiols, S-glutathionylation, S-alkylation involving polyphenolic semiquinones/quinones and adduction to other reactive lipid peroxidation products, e.g. 15d-PGJ₂, a reactive α,β -unsaturated cyclopentenone derived from arachidonic acid via the

cyclooxygenase pathway with particular relevance to colorectal adenoma carcinomas (Gayarre et al., 2006). Thus, further work is needed to evaluate to what extent the increased formation of β -actin-acrolein and β -actin-HNE adducts contribute towards the disruption of the actin cytoskeleton and the cytotoxic activity of hop PCs.

PDI-acrolein adduct formation in HT-29 cells treated with the PC mixture was evident in the Western blots probed with anti-acrolein antibody (Fig. 6B and Fig. 7B, right bottom panel). PDI is an endoplasmic reticulum chaperone which promotes correct disulfide formation, thereby participating in the maturation of newly synthesized proteins. An active site cysteine residue of PDI has been shown to be modified by both HNE and acrolein resulting in the inhibition of its enzyme activity (Carbone et al., 2005). The reduced form of PDI can be inactivated by acrolein ($IC_{50} = 10 \text{ mM}$) at pH 6.3, and to a lesser extent, at pH 7 (Liu and Sok, 2004). However, to which extent PDI inactivation contributes to possible mechanisms by which hop PCs exert their cytotoxic effects in HT-29 cells would need further investigation.

ROS generated from other polyphenolics such as epicatechin and EGCG have been shown to induce apoptosis and inhibit the growth of cancer cells (Azam et al., 2004; Chan et al., 2006) which may explain the anticancer activities of these compounds. In HT-29 cells, the antiproliferative and pro-apoptotic effects of EGCG have been attributed to increased oxidative stress in the treated cells (Chen et al., 2003). Other compounds, such as alpha-lipoic acid, have been shown to induce apoptosis in HT-29 cancer cells by a prooxidant mechanism (Wenzel et al., 2005a). There is a large body of evidence that a variety of cancer cells essentially operate under oxidative stress. Colorectal adenoma carcinoma cells express high levels of Nox1, a superoxide generation NADPH oxidase (Laurent et al., 2008), and cyclooxygenases (Antonakopoulos and Karamanolis, 2007). In rapidly growing cancer cells, metabolism is characterized by limited oxidative phosphorylation in order to provide protection from reactive oxygen species (ROS)-mediated cellular damage during phases of DNA replication and high biosynthetic activities (Wenzel et al., 2005b). In contrast in normal cells, the cellular redox status is tightly controlled and levels of ROS are counterbalanced by enzymatic and non-enzymatic antioxidant defense systems. Accordingly, the use of phytochemicals that increase ROS production and overburden the antioxidative capacity may drive cancer cells ultimately into apoptosis. The differential sensitivity of cancer cells to oxidative stress has been considered to be useful in alternative chemoprevention and chemotherapy strategies (McEligot et al., 2005). The low systemic bioavailability of PCs is an advantage when used in the prevention of intestinal/colon cancers in which the cancerous tissues could be exposed to high levels of PCs after ingestion. Unabsorbed PCs will go through the colon where the PCs may come in direct contact with the cancer cells and reach high micromolar concentrations at the cancer site.

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References

- Aldini G, Orioli M, Carini M. Alpha, beta-unsaturated aldehydes adducts to actin and albumin as potential biomarkers of carbonylation damage. *Redox Rep* 2007a;12:20–25. [PubMed: 17263903]

- Aldini G, Dalle-Donne I, Facino RM, Milzani A, Carini M. Intervention strategies to inhibit protein carbonylation by lipoxidation-derived reactive carbonyls. *Med Res Rev* 2007b;27:817–868. [PubMed: 17044003]
- Aldini G, Carini M, Vistoli G, Shibata T, Kusano Y, Gamberoni L, Dalle-Donne I, Milzani A, Uchida K. Identification of actin as a 15-deoxy-Delta12,14-prostaglandin J2 target in neuroblastoma cells: mass spectrometric, computational, and functional approaches to investigate the effect on cytoskeletal derangement. *Biochemistry* 2007c;46:2707–2718. [PubMed: 17297918]
- Allingham JS, Klenchin VA, Rayment I. Actin-targeting natural products: structures, properties and mechanisms of action. *Cell Mol Life Sci* 2006;63:2119–2134. [PubMed: 16909206]
- Antonakopoulos N, Karamanolis DG. The role of NSAIDs in colon cancer prevention. *Hepatogastroenterology* 2007;54:1694–1700. [PubMed: 18019697]
- Antunes F, Cadenas E. Estimation of H₂O₂ gradients across biomembranes. *FEBS Lett* 2000;475:121–126. [PubMed: 10858501]
- Aron PM, Kennedy JA. Flavan-3-ols: nature, occurrence and biological activity. *Mol Nutr Food Res* 2008;52:79–104. [PubMed: 18081206]
- Azam S, Hadi N, Khan NU, Hadi SM. Prooxidant property of green tea polyphenols epicatechin and epigallocatechin-3-gallate: implications for anticancer properties. *Toxicol In Vitro* 2004;18:555–561. [PubMed: 15251172]
- Buss IH, Winterbourn CC. Protein carbonyl measurement by ELISA. *Methods Mol Biol* 2002;186:123–128. [PubMed: 12013759]
- Butterfield DA, Castegna A, Lauderback CM, Drake J. Evidence that amyloid beta-peptide-induced lipid peroxidation and its sequelae in Alzheimer's disease brain contribute to neuronal death. *Neurobiol Aging* 2002;23:655–664. [PubMed: 12392766]
- Carbone DL, Doorn JA, Kiebler Z, Petersen DR. Cysteine modification by lipid peroxidation products inhibits protein disulfide isomerase. *Chem Res Toxicol* 2005;18:1324–1331. [PubMed: 16097806]
- Chan MM, Soprano KJ, Weinstein K, Fong D. Epigallocatechin-3-gallate delivers hydrogen peroxide to induce death of ovarian cancer cells and enhances their cisplatin susceptibility. *J Cell Physiol* 2006;207:389–396. [PubMed: 16402374]
- Chavez J, Wu J, Han B, Chung WG, Maier CS. New role for an old probe: affinity labeling of oxylipid protein conjugates by N'-aminooxymethylcarbonylhydrazino d-biotin. *Anal Chem* 2006;78:6847–6854. [PubMed: 17007505]
- Chen C, Shen G, Hebbbar V, Hu R, Owuor ED, Kong AN. Epigallocatechin-3-gallate-induced stress signals in HT-29 human colon adenocarcinoma cells. *Carcinogenesis* 2003;24:1369–1378. [PubMed: 12819184]
- Chung WG, Miranda CL, Maier CS. Epigallocatechin gallate (EGCG) potentiates the cytotoxicity of rotenone in neuroblastoma SH-SY5Y cells. *Brain Res* 2007;1176:133–142. [PubMed: 17900545]
- Dalle-Donne I, Carini M, Vistoli G, Gamberoni L, Giustarini D, Colombo R, Maffei Facino R, Rossi R, Milzani A, Aldini G. Actin Cys374 as a nucleophilic target of alpha,beta-unsaturated aldehydes. *Free Radic Biol Med* 2007;42:583–598. [PubMed: 17291982]
- Dixon RA, Xie DY, Sharma SB. Proanthocyanidins-a final frontier in flavonoid research? *New Phytol* 2005;165:9–28. [PubMed: 15720617]
- Doroshov JH, Akman S, Chu FF, Esworthy S. Role of the glutathione-glutathione peroxidase cycle in the cytotoxicity of the anticancer quinones. *Pharmacol Ther* 1990;47:359–370. [PubMed: 2290853]
- Engelbrecht AM, Mattheyse M, Ellis B, Loos B, Thomas M, Smith R, Peters S, Smith C, Myburgh K. Proanthocyanidin from grape seeds inactivates the PI3-kinase/PKB pathway and induces apoptosis in a colon cancer cell line. *Cancer Lett* 2007;258:144–153. [PubMed: 17923279]
- Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med* 1991;11:81–128. [PubMed: 1937131]
- Feick P, Haas SR, Singer MV, Bocker U. Low-dose exposure of intestinal epithelial cells to formaldehyde results in MAP kinase activation and molecular alteration of the focal adhesion protein paxillin. *Toxicology* 2006;219:60–72. [PubMed: 16352387]
- Gali-Muhtasib HU, Younes IH, Karchesy JJ, el-Sabban ME. Plant tannins inhibit the induction of aberrant crypt foci and colonic tumors by 1,2-dimethylhydrazine in mice. *Nutr Cancer* 2001;39:108–116. [PubMed: 11588891]

- Gayarre J, Sanchez D, Sanchez-Gomez FJ, Terron MC, Llorca O, Perez-Sala D. Addition of electrophilic lipids to actin alters filament structure. *Biochem Biophys Res Commun* 2006;349:1387–1393. [PubMed: 16979589]
- Hadi SM, Bhat SH, Azmi AS, Hanif S, Shamim U, Ullah MF. Oxidative breakage of cellular DNA by plant polyphenols: a putative mechanism for anticancer properties. *Semin Cancer Biol* 2007;17:370–376. [PubMed: 17572102]
- Halliwell B. Oxidative stress in cell culture: an under-appreciated problem? *FEBS Lett* 2003;540:3–6. [PubMed: 12681474]
- Halliwell B, Clement MV, Ramalingam J, Long LH. Hydrogen peroxide. Ubiquitous in cell culture and in vivo? *IUBMB Life* 2000;50:251–257. [PubMed: 11327318]
- Hostanska K, Jurgenliemk G, Abel G, Nahrstedt A, Saller R. Willow bark extract (BNO1455) and its fractions suppress growth and induce apoptosis in human colon and lung cancer cells. *Cancer Detect Prev* 2007;31:129–139. [PubMed: 17418981]
- Kaur M, Singh RP, Gu M, Agarwal R, Agarwal C. Grape seed extract inhibits in vitro and in vivo growth of human colorectal carcinoma cells. *Clin Cancer Res* 2006;12:6194–6202. [PubMed: 17062697]
- Klenow S, Gleis M, Haber B, Owen R, Pool-Zobel BL. Carob fibre compounds modulate parameters of cell growth differently in human HT29 colon adenocarcinoma cells than in LT97 colon adenoma cells. *Food Chem Toxicol* 2008;46:1389–1397. [PubMed: 17950517]
- Lambert JD, Sang S, Yang CS. Possible controversy over dietary polyphenols: benefits vs risks. *Chem Res Toxicol* 2007;20:583–585. [PubMed: 17362033]
- Laurent E, McCoy JW 3rd, Macina RA, Liu W, Cheng G, Robine S, Papkoff J, Lambeth JD. Nox1 is over-expressed in human colon cancers and correlates with activating mutations in K-Ras. *Int J Cancer* 2008;123:100–107. [PubMed: 18398843]
- Li HJ, Deinzer ML. Structural identification and distribution of proanthocyanidins in 13 different hops. *J Agric Food Chem* 2006;54:4048–4056. [PubMed: 16719533]
- Liu XW, Sok DE. Inactivation of protein disulfide isomerase by alkylators including alpha,beta-unsaturated aldehydes at low physiological pHs. *Biol Chem* 2004;385:633–637. [PubMed: 15318812]
- Lizarraga D, Lozano C, Briede JJ, van Delft JH, Tourino S, Centelles JJ, Torres JL, Cascante M. The importance of polymerization and galloylation for the antiproliferative properties of procyanidin-rich natural extracts. *FEBS J* 2007;274:4802–4811. [PubMed: 17824958]
- Long LH, Clement MV, 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. [PubMed: 10873562]
- McEligot AJ, Yang S, Meyskens FL Jr. Redox regulation by intrinsic species and extrinsic nutrients in normal and cancer cells. *Annu Rev Nutr* 2005;25:261–295. [PubMed: 16011468]
- Mello CF, Sultana R, Piroddi M, Cai J, Pierce WM, Klein JB, Butterfield DA. Acrolein induces selective protein carbonylation in synaptosomes. *Neuroscience* 2007;147:674–679. [PubMed: 17570602]
- Oikawa S, Furukawa A, Asada H, Hirakawa K, Kawanishi S. Catechins induce oxidative damage to cellular and isolated DNA through the generation of reactive oxygen species. *Free Radic Res* 2003;37:881–890. [PubMed: 14567448]
- Poggi P, Rota MT, Boratto R. The volatile fraction of cigarette smoke induces alterations in the human gingival fibroblast cytoskeleton. *J Periodontal Res* 2002;37:230–235. [PubMed: 12113559]
- Prior RL, Gu L. Occurrence and biological significance of proanthocyanidins in the American diet. *Phytochemistry* 2005;66:2264–2280. [PubMed: 15904940]
- Rasmussen SE, Frederiksen H, Struntze Krogholm K, Poulsen L. Dietary proanthocyanidins: occurrence, dietary intake, bioavailability, and protection against cardiovascular disease. *Mol Nutr Food Res* 2005;49:159–174. [PubMed: 15635686]
- Requena JR, Chao CC, Levine RL, Stadtman ER. Glutamic and aminoadipic semialdehydes are the main carbonyl products of metal-catalyzed oxidation of proteins. *Proc Natl Acad Sci USA* 2001;98:69–74. [PubMed: 11120890]

- Sakano K, Mizutani M, Murata M, Oikawa S, Hiraku Y, Kawanishi S. Procyanidin B2 has anti- and pro-oxidant effects on metal-mediated DNA damage. *Free Radic Biol Med* 2005;39:1041–1049. [PubMed: 16198231]
- Scalbert A, Williamson G. Dietary intake and bioavailability of polyphenols. *J Nutri* 2000;130:2073S–2085S.
- Seeram NP, Adams LS, Zhang Y, Lee R, Sand D, Scheuller HS, Heber D. Blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts inhibit growth and stimulate apoptosis of human cancer cells in vitro. *J Agric Food Chem* 2006;54:9329–9339. [PubMed: 17147415]
- Singletary KW, Meline B. Effect of grape seed proanthocyanidins on colon aberrant crypts and breast tumors in a rat dual-organ tumor model. *Nutr Cancer* 2001;39:252–258. [PubMed: 11759289]
- Stevens JF, Miranda CL, Wolthers KR, Schimerlik M, Deinzer ML, Buhler DR. Identification and in vitro biological activities of hop proanthocyanidins: inhibition of nNOS activity and scavenging of reactive nitrogen species. *J Agric Food Chem* 2002;50:3435–3443. [PubMed: 12033808]
- Taylor AW, Barofsky E, Kennedy JA, Deinzer ML. Hop (*Humulus lupulus* L.) proanthocyanidins characterized by mass spectrometry, acid catalysis, and gel permeation chromatography. *J Agric Food Chem* 2003;51:4101–4110. [PubMed: 12822954]
- Uchida K. Current status of acrolein as a lipid peroxidation product. *Trends Cardiovasc Med* 1999;9:109–113. [PubMed: 10639724]
- Usatyuk PV, Parinandi NL, Natarajan V. Redox regulation of 4-hydroxy-2-nonenal-mediated endothelial barrier dysfunction by focal adhesion, adherens, and tight junction proteins. *J Biol Chem* 2006;281:35554–35566. [PubMed: 16982627]
- Watson AJ, Askew JN, Sandle GI. Characterisation of oxidative injury to an intestinal cell line (HT-29) by hydrogen peroxide. *Gut* 1994;35:1575–1581. [PubMed: 7828976]
- Wenzel U, Nickel A, Daniel H. alpha-Lipoic acid induces apoptosis in human colon cancer cells by increasing mitochondrial respiration with a concomitant O²-generation. *Apoptosis* 2005a;10:359–368. [PubMed: 15843897]
- Wenzel U, Nickel A, Daniel H. Increased carnitine-dependent fatty acid uptake into mitochondria of human colon cancer cells induces apoptosis. *J Nutr* 2005b;135:1510–1514. [PubMed: 15930461]
- Yamamoto T, Lewis J, Wataha J, Dickinson D, Singh B, Bollag WB, Ueta E, Osaki T, Athar M, Schuster G, Hsu S. Roles of catalase and hydrogen peroxide in green tea polyphenol-induced chemopreventive effects. *J Pharmacol Exp Ther* 2004;308:317–323. [PubMed: 14569057]

Abbreviations

Ac-DEVD-AMC	N-acetyl-Asp-Glu-Val-Asp-AMC (7-amino-4-methylcoumarin)
CM-H ₂ DCFDA	5-(and -6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate
DCF	dichlorofluorescein
DNPH	2,4-dinitrophenylhydrazine
DTT	dithiothreitol
EGCG	epigallocatechin gallate
GSH	glutathione
HNE	4-hydroxy-2-nonenal
HRP	horseradish peroxidases
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	phosphate buffered saline
PI3-kinase	phosphatidylinositol 3-kinase
PCs	proanthocyanidins
PDI	protein disulfide isomerase

PKB	protein kinase B
ROS	reactive oxygen species
TMB	3,3'-5,5' tetramethylbenzidine

Fig. 1. Percent viability of HT-29 cells treated with increasing concentrations of hop PCs in the presence and absence of catalase (100 units/ml) in the culture medium
Values are mean $\pm$ SE, $n = 4$. * $P < 0.05$ vs. control group.

Fig. 2. (A) H₂O₂ production in fresh RPMI 1640 media (no cells, not incubated) and in media with or without cells incubated with 0 (control), 10, 50 or 100 μg/ml PCs for 24 h at 37 °C. Values are mean ± SE, n = 4. **P* < 0.05 vs. the respective control group. #*P* < 0.05 vs. no cells. (B) H₂O₂ content of RPMI 1640 media with cells incubated with various concentrations of H₂O₂ for 24 h at 37 °C. Values are mean ± SE, n = 4.

Fig. 3. Effect of hop PCs on caspase-3 activity of HT-29 cells. The cells were exposed to PC for 24 h before measuring caspase-3 activity of the cytosol fraction of HT-29 cells using Ac-DEVD-AMC as a substrate

Values are mean $\pm$ SE, $n = 4$. * $P < 0.05$ vs. control group.

Fig. 4. Effect of treatment of HT-29 cells with hop PCs on intracellular ROS levels as measured by fluorescence microscopy after staining the cells with 5-(and -6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate

Fig. 5.

(A) Protein carbonyl content of HT-29 cells incubated with 10, 50 or 100 $\mu\text{g/ml}$ PCs for 24 h at 37 °C. Values are mean $\pm$ SE, n = 4. * P < 0.05 vs. control group. **(B)** Protein carbonyl content of HT-29 cells incubated with increasing concentrations of H₂O₂ for 24 h at 37 °C. Values are mean $\pm$ SE, n = 4. * P < 0.05 vs. control group.

Fig. 6. SDS-PAGE (A) and Western blot using anti-acrolein antibody (B), anti-HNE antibody (C), anti- β -actin antibody + anti-PDI antibody (D) of cell lysates from HT-29 cells treated with 10, 50, and 100 $\mu\text{g/ml}$ PC for 24 h, and of cell lysates from HT-29 cells treated with 50 and 100 μM H_2O_2 for 24 h probed with anti-acrolein IgG (E)

In A, the proteins are identified as follows: 1: Myosin-9, 2: α -Actin 1, 3: Heat shock protein 90- α , β , 4: Glucose regulatory protein, 5: Heat shock protein 71, 6: Protein disulfide isomerase A3, 7: α -Enolase, 8: Actin cytoplasmic 1 (β -actin), 9: Keratin type I, 10: Keratin type II, 11: Glyceraldehyde 3-phosphate dehydrogenase, 12: 14-3-3 protein gamma, 13: Triosephosphate isomerase, 14: Glutathione S-transferase.

Fig. 7. Coomassie Blue G250-stained gels (A, control HT-29 cells and B, HT-29 cells treated with 100 µg/mL PCs and the respective Western blots (right top and right bottom panels) probed with anti-acrolein IgG

The identities of the spots (center panels) are as follows: 1, β -Actin; 2, Keratin type I cytoskeletal 18; 3, Keratin type II cytoskeletal 8; and 4, protein disulfide isomerase A3.

Fig. 8. Effect of hop PCs on actin cytoskeleton organization in HT-29 cells. Control cells and cells treated with 10, 50 or 100 µg/ml PCs for 24 h were stained with rhodamine phalloidin and examined by fluorescence microscopy (with 40× power objective)

A ring-like cytoskeletal organization is observed at the cell periphery in control cells and after PC treatment the actin cytoskeleton became disorganized and actin appeared as clusters or aggregates in treated cells.

Table 1

Mass spectrometry data of proteins identified in the gel regions 1 to 14 (Figure 6) and 1 to 4 (Figure 7).

Region No.	Protein (Swiss-Prot entry) & matching peptides	Mass	pI	Sequence Coverage	Start-End	Peptide No.	Mass	Mascot Scores	Protein Peptide
1	Myosin-9 (MYH9_HUMAN) MQNQIQEELEEEESAR IAQLEEEEEEQGNTELINDR	226,392	5.50	3%	941 - 959 1731 - 1751	2	2332.96 2472.08	73 48 25	
2	Alpha-actinin-1 (ACTN1_HUMAN) LASDLLEWIR GYEEWLLNEIR	103,058	5.25	2%	282 - 291 377 - 387	2	1215.73 1421.77	75 50 25	
3	Heat shock protein 90- α (HS90A_HUMAN) HNDDEQYAWESSAGGSFTVR HNDDEQYAWESSAGGSFTVR Oxidation (HIW9) HFSVEGQLEFR ALLFVPR RAPFDLFENR APFDLFENR	84,607	4.94	8%	154 - 173 154 - 173 328 - 338 339 - 345 346 - 355 347 - 355	6	2255.91 2287.89 1348.66 815.56 1264.69 108.55	280 117 35 49 25 26 28	
4	78 kDa glucose regulatory protein (GRP78_HUMAN) IEIESFYEGEDFSETLTR DNHLLGTFDLTGIPPAPR	72,288	5.07	6%	307 - 324 475 - 492	2	2165.00 1934.06	149 103 46	
5	Heat shock protein 71 (HSP7C_HUMAN) QTQTFTTYSQNPQGVLIQVYEGER	70,854	5.37	4%	424 - 447	1	2774.35	38 38	
6	Protein disulfide-isomerase (PDIA3_HUMAN) DASIVGFDDDSFSEAHSEFLK 2 FAHTNVESLVNEYDDNGEGIIIFR 2 DLLIAYYDVIDYEK 2 TFSHELSDFGLESTAGEIPVVAIR 2 FVMQEEFSR 1,2 FLQDYFDGNLK 2 MDATANDVPSPYEVR 1 GFPTIYFSPANK 1	56,747	5.98	28%	153 - 173 184 - 207 259 - 271 306 - 329 336 - 344 352 - 362 434 - 448 449 - 460	9	2347.95 2752.16 1619.66 2575.07 1172.53 1359.56 1664.73 1341.66	577 107 149 38 111 29 36 32 43	

Region No.	Protein (Swiss-Prot entry) & matching peptides	Mass	pI	Sequence Coverage	Start-End	Peptide No. Mass	Mascot Scores	Protein Peptide
7	ELSDFIISYLQR ^{1,2}			472 - 482		1370.67		32
	α-Enolase (ENOA HUMAN)	47,139	7.01	16%		6		365
	AAVPSGASTGIYEALER			33 - 50		1804.86		54
	LAMQEFMILPVGAANFR Oxidation (M7)			163 - 179		1924.03		26
	LAMQEFMILPVGAANFR 2 Oxidation (M)			163 - 179		1940.04		26
	VVIGMDVAASEFFR			240 - 253		1540.72		69
	VVIGMDVAASEFFR Oxidation (M)			240 - 253		1556.71		71
	DYPVVSIEDPFDQDDWGAWQK			286 - 306		2510.01		119
	Actin cytoplasmic 1 (β-actin) (ACTB HUMAN)	41,710	5.29	38%		12		644
	AGFAGDDAPR ^{1,2}			19 - 28		976.47		28
8	AVFPSIVGR ^{1,2}			29 - 37		945.61		56
	AVFPSIVGRPR ¹			29 - 39		1198.67		25
	IWHHTFYNELR ^{1,2}			85 - 95		1515.77		37
	IWHHTFYNELR Oxidation (W2) ¹			85 - 95		1531.73		57
	VAPPEHPVLLTEAPLNPK ²			96 - 113		1953.99		30
	TTGIVMDSGDGVTHTVPIYEGYALPHAILR ¹			148 - 177		3183.47		62
	GYSFTTTAER ^{1,2}			197 - 206		1132.58		40
	SYELPDGQVITIGNER ^{1,2}			239 - 254		1790.94		115
	DLYANTVLSGGTTMYPGIADR ^{1,2}			92 - 312		2215.12		72
	DLYANTVLSGGTTMYPGIADR Oxidation (M) ²			292 - 312		2231.13		51
9	QEYDESGPSIVHR ^{1,2}			360 - 372		1516.65		71
	Keratin type I cytoskeletal 18 (K1C18_HUMAN)	48,029 ³	5.34	35%		11		748
	AQIFANTVDNAR ^{1,2}			138 - 149		1319.64		55
	IVLQIDNAR ^{1,2}			150 - 158		1041.59		36
	VKYETELAMR ¹			166 - 175		1239.62		55
	LQLETEIEALKEELLFMK Oxidation (M) ²			197 - 214		2193.09		109
	AQYDELAR ^{1,2}			254 - 261		965.45		34
	YWSQIEESTTVVTQTQSAEVGAAETTLTELAR ²			270 - 300		3428.68		117

Region No.	Protein (Swiss-Prot entry) & matching peptides	Mass	pI	Sequence Coverage	Start-End	Peptide No.	Mass	Mascot Scores	Protein Peptide
10	TVQSLIEDLDSMR ¹			302 - 314		1506.71		72	
	ASLENSLR ^{1,2}			318 - 325		889.45		39	
	YALQMEQLNGILLHLESELAQTR Deamidation(N) ^{1,2}			331 - 353		2671.25		114	
	QAQYEYALLNIK ^{1,2}			359 - 370		1419.77		83	
	LEAEIATYR ²			373 - 381		1065.53		34	
	Keratin type II cytoskeletal 8 (K2C8_HUMAN)	53,671 ³	5.52	30%		16		791	
	FLEQQNK ²			111,- 117		- 906.49		47	
	WSLLQQQK ¹			123 - 130		1030.55		36	
	SNMDNMFESYINNLR ^{1,2}			134 - 148		1847.82		68	
	SNMDNMFESYINNLR Oxidation (M3) ^{1,2}			134 - 148		1863.74		72	
	YEDEINKR ²			179 - 186		1066.54		39	
	TEMENEFVLIK ²			187 - 197		1352.70		35	
	TEMENEFVLIK Oxidation (M) ²			187 - 197		1368.70		44	
	DVDEAYMNKVELESR ¹			199 - 213		1797.77		31	
	LEGLTDEINFLR ^{1,2}			214 - 225		1419.77		79	
	QLYEEER ^{1,2}			226 - 233		1079.54		29	
	AQYEDIANR ¹			265 - 273		1079.54		34	
	LQAEIEGLKGQR ¹			317 - 328		1341.71		50	
	ASLEAAIADAEQR ^{1,2}			329 - 341		1344.65		61	
	ASLEAAIADAEQRGELAIK ¹			329 - 347		1955.96		37	
11	LSELEAALQR ^{1,2}			353 - 362		1129.60		59	
	LALDIEIATYR ²			382 - 392		1277.74		70	
	Glyceraldehyde-3-phosphate dehydrogenase (G3P_HUMAN)	36,030	8.57	8%		3		165	
12	LVINGNPITIFQER			67 - 80		1613.95		47	
	LISWYDNEFGYSNR			310 - 323		1763.85		51	
	LISWYDNEFGYSNR Oxidation (W)			310 - 323		1779.85		67	
	14-3-3 protein gamma (1433G_HUMAN)	28,065	4.76	4%		1		64	
	EMQPTHPIR			161 - 169		1108.54		64	

Region No.	Protein (Swiss-Prot entry) & matching peptides	Mass	pI	Sequence Coverage	Start-End	Peptide No.	Mass	Mascot Scores	Protein Peptide
13	Triphosphate isomerase (TPIS_HUMAN) VPADTEVVCAPPTAYIDFAR Propionamide (C)	26,653	6.45	8%	34 - 53	1	2206.01	42	42
14	Glutathione S-transferase P (GSTP1_HUMAN) PPYTVVYFPVR FQDGDLTLYQSNITLR ALPGQLKPFETLLSQNQGGK	23,341	5.43	22%	2 - 12	3	1337.69	163	50
				56 - 71			1883.88	64	64
				122 - 141			2126.08	49	49

¹ indicate peptides identified in the 1D and/or 2D gel regions, respectively (Figure 6A and 7),

² indicate peptides identified in the 1D and/or 2D gel regions, respectively (Figure 6A and 7),

³ The molecular weights observed for keratin I and II in the SDS PAGE (Figure 6) did not match the theoretical values given in the Swiss Prot protein database which may be due to proteolysis.