POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES
www.pan.olsztyn.pl/journal/
e-mail: joan@pan.olsztyn.pl
Pol. J. Food Nutr. Sci.
2007, Vol. 57, No. 3, pp. 307–314

ASSESSMENT OF THE ANTIPROLIFERATIVE ACTIVITY OF CARROT AND APPLE EXTRACTS

Katarzyna Przybylska[1], Richard N. Bennett[2], Krystyna Kromer[1], Jennifer M. Gee[2]

[1]Plant Tissue Culture Laboratory, Botanical Garden, Wroclaw University, Wroclaw, Poland;
[2]Institute of Food Research, Norwich, United Kingdom

Key words: antiproliferative potential, apple, carrot, phenolic compounds, human colon cancer cell line

Phenolic extracts of apple and carrot juices and apple and carrot tissue, and selected pure compounds were examined for their ability to decrease the viability of human colon cancer cells (HT29 and LoVo) *in vitro*. In addition, apple and carrot juices were supplemented with phloretin and chlorogenic acid, respectively, to simulate genetically modified products with enhanced levels of these phytochemicals and screened as described above. Amongst the pure compounds tested, phloretin was shown to have the greatest effect on the viability of both cell lines. Here we report that phloretin has a marked effect on the survival of colon cancer cells at concentrations as low as 50 μmol/L, and therefore shows significant anticarcinogenic potential. With regard to the effect of the phenolic extracts of apple and carrot tissue, that from transgenic carrot decreased cell viability by as much as 20% at the highest concentration (200 μg/mL). Phenolic extracts of control carrot and control apple tissue had little effect. However similar extracts of apple juice supplemented with either 500 or 1000 μmol/L phloretin reduced the survival of LoVo cells to 10% and showed marked activity at 100 μmol/L. Apple juice alone did not reduce cell viability, probably because the concentration of phloretin was too low. This information supports the recommendation that consumers should be encouraged to eat more fruit and vegetables, especially those containing compounds such as phloretin. There is also potential for food producers and manufacturers to develop products with enhanced flavonoid content, either by conventional breeding, transgenically or by supplementation.

INTRODUCTION

Colon cancer is a very serious problem in most developed countries and is the third leading cause of cancer mortality throughout the world [Yu *et al*., 2004]. However, the mechanisms of action involved and their molecular targets remain unclear. Epidemiological studies of colorectal cancer incidence suggest that development of this disease can be modulated by dietary factors. Polyphenolic compounds are among micronutrients and non-nutrients showing significant potential for tumor prevention and, being plant secondary metabolites, are present in a wide range of fruits and vegetables. Data from population studies strongly suggest that the regular intake of fruits and vegetables is associated with a reduced risk of developing chronic diseases such as cardiovascular disease [Dauchet *et al*., 2004], lung dysfunction [Kelly *et al*., 2003], and various cancers [Johnson, 2004; Maynard *et al*., 2003; Talalay & Fahey, 2001; Vainio & Weiderpass, 2006]. Flavonoids, a subgroup of polyphenolic compounds, have been associated with a reduced risk of for example heart, cardiovascular and neurological diseases and other chronic ailments. Indeed several reports have shown that there is positive correlation between intake of plant polyphenols and reduced risk of colon cancer [Mahmoud *et al.,* 2000; Kuntz *et al.,* 1999; Caderni *et al.,* 2000].

Apples (*Malus domestica*) are among the most important sources of polyphenolic antioxidants, which are present in this fruit as flavonols (predominantly quercetin), flavanols and their oligomers and polymers. In addition dihydrochalcones (phloretin) and phenolic acids (such as chlorogenic acid) are also present in apples. Phloretin exists in the fruit in the glucosidic form, namely, phloridzin (phloretin 2'-O-glucoside) and is a powerful antioxidant. Both phloridzin and phloretin are present mainly in the peel of apples (72.3 and 40.2 μg/g fresh weight (f.w.), respectively) but are also found in the pulp (14.4 and 4.9 μg/g f.w., respectively) [Tsao *et al.,* 2003]. Processing of apples into juice causes considerable reduction in the amount of antioxidant polyphenolics. Commercial apple juice contains only 3–10% of the polyphenolics in the fruit from which it was produced [van der Sluis *et al.,* 2004].

Carrots (*Daucus carota*) are a major vegetable in diets worldwide mainly due to their pleasant flavour and perceived health benefits, which have been associated with their vitamin, mineral and dietary fiber content. Carrots are also a major single source of provitamin A, and carotenes (α-, β-, γ- and ζ-carotenes, lycopene and β-zeacarotene), terpenoids (terpenes, sesquiterpenes) and polyphenols. As regards polyphenolic content, carrot contains mainly hydroxycinnamic acid derivatives, namely neochlorogenic acid (3'-caf-

Author's address for correspondence: Katarzyna Przybylska, Plant Tissue Culture Laboratory, Botanical Garden, Wroclaw University, ul. Sienkiewicza 23, 50-355 Wrocław, Poland; e-mail: kasia_przybylska@poczta.onet.pl

feoylquinic acid), chlorogenic acid (5'-caffeoylquinic acid), 3'-p-coumaroylquinic acid, 3'-feruloquinic acid, 3'4'-dicaffeoylquinic acid, 5'-feruloylquinic acid, 5'-p-coumaroylquinic acid, 4'-feruloylquinic acid, 3'5'-dicaffeoylquinic acid, 3'4'-diferuloylquinic acid and 3'5'-diferuloyoquinic acid [Alasalvar *et al.,* 2001].

Over the past decade consumers in Western countries of Europe have been encouraged to increase their daily consumption of fruits and vegetables, as either the raw food or in processed products. There has consequently been a growing interest in obtaining further information on the benefits of fruit and vegetable intake and an identifying the components with protective properties. One hypothesis is that compounds in plants with antioxidant activity are able to scavenge reactive oxygen species, which have the potential to damage cell components such as DNA, proteins and lipids. Oxidative stress is thought to be involved in initiating events leading to the development of cancer, and free radicals may help to induce apoptosis, or cell suicide, thus deleting damaged cells. It is also now considered possible that low concentrations of phytochemicals are capable of affecting signalling cascades and gene expression.

The potential for apple and carrot polyphenols, as pure compounds, or in extracts of juices or extracts of normal and transgenic plant tissue, to protect the gut against disease was assessed using two human colon cancer cell lines (HT29 and LoVo cells) in an *in vitro* cell culture model. Root culture under laboratory conditions offered the possibility of generating transgenic tissue with elevated polyphenolic content. For the latter, the species of fruit (apple) and vegetable (carrot) were chosen from those known to contain phytochemicals associated with a reduced risk of colon cancer. The aim was to provide additional information on the modulation of human colon cancer cell proliferation and viability by these polyphenolic phytochemicals, using a simple screening procedure.

MATERIAL AND METHODS

Preparation of juices. Apple juice (Grove Fresh Ltd.) was obtained from a local health food store in Norwich, UK. It was opened under aseptic conditions, divided into 50 mL aliquots in sterile containers and stored at -20°C until further required. Carrots were purchased from a local supermarket (Sainsbury, UK), 'topped', 'tailed' and peeled and washed in tap water. Prepared vegetables were homogenised in a domestic food processor. The juice was separated by centrifugation, transferred to sterile Falcon tubes (50 mL) and stored at -20°C until required. Sub-samples of both apple and carrot juice were clarified by centrifugation at 8700 × g and 4°C for 5 and 10 min, respectively, on the day of the experiment.

Preparation of carrot root tissues. Hairy root cultures of tissue explants (combined phloem, cambium, and xylem) from roots of edible carrots were prepared as follows under aseptic conditions. Root explants were maintained in agar medium either without any plant hormone (transgenic roots) or supplemented with plant hormone, indole-3-butyric acid (control roots). Plant hormone was added to the agar medium to encourage the growth of control roots. To obtain transgenic tissue, explants were infected with *Agrobacterium rhizogenes* (strain LBA1334) plasmid to stimulate the production of hairy roots, resistant to both ryphampicilin and spectynomicin antibiotics. Cultivation was carried out in a controlled-environment chamber at a temperature of 21°C with a 16-h light/8-h dark cycle for one month. Infected roots were then dissected out and transferred into liquid medium supplemented with antibiotics for two weeks to eliminate bacteria. The control root line was transferred directly into liquid medium without any antibiotics. Then both control and infected (transgenic) carrot root cultures were sub-cultured into liquid medium without any plant hormone and incubated at 21°C under the same dark-light conditions mentioned above until required (approximately 1–2 months).

Preparation of apple root tissues. The apple rootstock culture was cultivated in agar medium supplemented with plant hormones (indole-3-butyric acid, gibberellic acid and 6-benzylaminopurine) in a controlled-environment chamber having a temperature 21°C, a 16-h light/8-h dark cycle for about two months. The plant hormones content in medium promoted root development. The apple roots were then cut off and transferred to liquid medium without any plant hormones and cultivated under the same conditions as above. Transgenic apple material was not successfully produced.

Both carrot and apple root cultures were propagated by transferring them into fresh liquid media every 3 weeks (carrot root cultures) and every 5 weeks (apple root cultures) and continuing cultivation under the conditions mentioned above for each species of plant.

Preparation of transgenic apple and carrot models. In order to prepare simulated transgenic material with higher polyphenol content, juices were supplemented with either chlorogenic acid or phloretin. Pure chemicals were purchased from the Sigma Chemical Company (Poole, UK). Variable amounts of chlorogenic acid (10–1000 μmol/L) were added to carrot roots and to domestically prepared carrot juice. Phloretin (10–1000 μmol/L) was added to apple juice to create a similar model of apple juice with enhanced level of this compound.

Preparation of polyphenolic extracts of plant material and juices for cell viability assay. Polyphenol extracts of apple and carrot root tissue were prepared. Roots were removed from the liquid medium, rinsed with distilled water and freeze-dried. The freeze-dried material was incubated with 70% methanol at 30°C for 30 min and subsequently centrifuged for 30 min at 4°C (7500 × g). The supernatants were used without or with added chlorogenic acid or phloretin respectively to assess their effects on mammalian cell viability. A polyphenol extract of both carrot and apple juices was obtained by adding 100% methanol (0.5 mL per 0.5 mL juice) and mixing well, followed by incubation and separation by centrifugation under the same conditions as stated above, except that apple juice was centrifuged for 5 min and carrot juice for 10 min. These polyphenol extracts of juice were prepared immediately prior to use and added to cell incubation media either undiluted or after ×5 or ×10 dilution, to yield final concentrations equivalent to 0.5, 0.1 and 0.05% (v/v) juice.

Preparation of apple and carrot root extracts for HPLC. The total polyphenol content of extracts of carrot and apple tissue was determined semi-quantitatively with HPLC and LC/MS. These data allowed estimation of the polyphenol level to which human colon cancer cells were exposed in subsequent experiments. Ethanol (1 mL; 70%) and 50 μL of an internal standard (0.1 mg/mL rhamnetin in 100% methanol) were added to 20 mg of freeze-dried plant tissue (apple and carrot roots, respectively) and vortexed thoroughly. The mixtures were incubated at 70°C for 30 min, with vortexing every 10 min and then centrifuged at 4°C at 7500 × g for 30 min. Supernatants were transferred to new vials and filtered through 0.2 μm PTFE filters, prior to HPLC and MS analyses.

Preparation of apple and carrot juice extracts for HPLC. In order to establish the polyphenol content of both apple and carrot juices, the HPLC analyses were made. The mixtures made with 500 μL of juice, 450 μL of 100% of methanol and 50 μL of an internal standard (0.1 mg of naringenin in 1 mL of methanol) were vortexed thoroughly and incubated at 70°C for 20 min. All samples were vortexed every 5 min during the incubation time. Following this all samples were centrifuged at 4°C at 7500 × g for 20 min. The supernatants were removed to new tubes and filtered through 0.2 μm PTFE filter prior to HPLC and LC/MS analyses.

HPLC methodology. The total polyphenol content of apple and carrot juice and polyphenol extracts of carrot and apple tissue was determined with HPLC and LC-MS according to Mandalari *et al* [2006]. The HPLC analyses were carried out using an Agilent HP1100 HPLC System equipped with a binary pump, cooled auto-sampler, thermostatically controlled column oven and a photo-diode array (PDA) detector. Flavonoids and phenolic acids were identified by a combination of retention time and compared with standards, matching UV-visible spectral characteristics with a library database and LC-MS analysis. The relative concentration of the individual compounds was calculated from the response at 270 nm on HPLC chromatograms.

Colon cancer cell viability assay. Two human colon cancer cell lines (HT29 and LoVo) were used to assess the effect of plant flavonoids on their viability *in vitro*. The HT29 cell line carries a p53 mutation, whereas the LoVo cell line carries an MMR mutation. Cultures of both cell lines were maintained in media supplemented with 1% penicillin-streptomycin mixture (100 unit/mL and 0.1 mg/mL final concentration respectively), 1% L-glutamine (2 mmol/L final concentration) and Fetal Bovine Serum (5% for HT29 and 10% for LoVo cells, respectively). The cells were seeded at a low density (7.5×10^4 cells per mL) into basal media (Dulbecco's Modified Eagles Medium for HT29 cells and McCoy's 5A medium for LoVo cells) and cultured under the same incubation conditions (37°C, 5% of CO_2 in air and 90% humidity). All chemicals and media were obtained from Sigma (Poole, UK).

Pure phenolic compounds, apple and carrot juice and the phenolic extracts of apple and carrot roots were tested for an antiproliferative activity using 96-well plate methodology, to detect changes in viability of human colon cancer cells by a neutral red uptake. Pure compounds and root extracts were tested over a concentration range between 10 and 1000 μmol/L in the cell culture medium. Both apple and carrot juice extracts were tested at a dilution factor 1, 5 and 10× which corresponds to approximately 100, 500 and 1000 μmol/L of phenolic extract. For the assay cells were grown in 96-well plates for 48 h in the basal media, prior to exposure to test materials, to allow the cells adhere to the wells. Media were then removed and fresh media (200 μL) without (control) or supplemented with test compounds, or extracts of juices or plant tissue were added to the wells. The effect of the following pure compounds was assessed: phloretin, phloridzin, quercetin, quercetin-3-O-glucoside, chlorogenic acid, 4-hydroxybenzoic acid, caffeic acid and ferulic acid (final concentration range 10–1000 μmol/L). Cells were also exposed to extracts of commercial apple juice (whole and clarified by centrifugation) and home-made carrot juice (whole and clarified by centrifugation) at a range of dilutions equivalent to 0.5, 0.1 and 0.05% (v/v) juice. In addition, extracts of control apple roots, control and transgenic carrot roots (20–200 μg/mL), and models of both transgenic apple and carrot juices (phloretin and chlorogenic acid – 10–1000 μmol/L – added to apple and carrot juice, respectively) were tested. Control incubation media contained the equivalent volume of extraction solvent to the corresponding extracts under test.

Both cell lines were incubated for 5 and 24 hs using 3 plates with 6 complete rows per plate (avoiding edges), giving 18 replicates for each incubation time and each test mixture and account for any inter- and intra-plate variations. These incubation times were selected on the basis of previous studies and are representative of short and longer exposure within the colon [Daskiewicz *et al.*, 2005]. At the end of the incubation period the supernatant was removed and neutral red dye in basal medium (50 μg/mL) was added. After a further 2-h incubation period the medium was again removed, the cells fixed by addition of a solution containing formaldehyde: $CaCl_2$ (0,5% : 0,1%) for 30 sec and the plates were washed twice for 30 sec in phosphate buffered saline (PBS). After air-drying the plates, 200 μL of a solution of ethanol: water: acetic acid (50:49:1%) was added to each well to lyse the cells and release the intracellular dye. The intensity of the colour was then measured using a plate reader (Spectra-Count™, Packard BioScienceCompany) at 550 nm to assess cell viability.

The concentration of a compound that decreased cell viability by 50% (IC_{50}) was determined for those showing potential in reducing cell numbers (phloretin, quercetin and apple juice supplemented with phloretin),

Statistical analysis. The data, comparing a response to test compound, juice or apple or carrot extract against the appropriate untreated control, were analysed for significance using the general linear model in ANOVA, with a Tukey's pair-wise comparison, using Minitab statistical software.

RESULTS

Polyphenolic content of both apple and carrot juices and roots

HPLC and LC-MS analysis of apple and carrot roots and juices revealed a number of flavonoids and pheno-

TABLE 1. All individual flavonoids and phenolic acids in apple and carrot juices and roots.

Source of phenolics		Compound	Content (μg/g d.w.)
Apple roots		phloridzin	3025
		phloridzin xyloside	507
		Total	3532
Carrot roots	control	chlorogenic acid	373
		4-hydroxybenzoic acid	270
		Total	643
	transgenic	chlorogenic acid	712
		4-hydroxybenzoic acid	1241
		unknown phenolic	1019
		Total	2972
Source of phenolics		Compound	Content (μg/mL of juice)
Apple juice		chlorogenic acid	94.3
		procyanidin B1	5.6
		procyanidin B2	22.2
		epicatechin	18.4
		coumaroylquinic acid (isomer 1)	25.04
		coumaroylquinic acid (isomer 2)	8.4
		phloretin-2-O-xyloglucoside	12.5
		phloretin-2-O-xyloglucoside (isomer)	3.02
		phloridzin	18.7
		Total	208.57
Carrot juice		chlorogenic acid	194
		caffeic acid	235
		phloridzin	54.3
		phloretin	219
		quercetin	17.3
		Total	648

lic acids which are listed in Table 1. Nine phenolic compounds were identified in the commercially available apple juice namely chlorogenic acid, procyanidin B1, procyanidin B2, epicatechin, two isomers of coumaroylquinic acid, two isomers phloretin-2-O-xyloglucoside and phloridzin. The total polyphenolic content, as determined by HPLC and LC-MS, amounted to 208.57 μg/g dry weight (d.w.) of apple juice. Chlorogenic acid was the main component (94.3 μg/g d.w.). Two compounds contributed around 10% (procyanidin B2, 10.6%; 22.2 μg/g d.w. and isomer 1 of coumaroylquinic acid, 12.0%; 25.04 μg/g d.w.). All the remaining compounds contributed less than 10% to the polyphenolic fraction. The range of phenolic compounds in apple roots was more restricted, consisting primarily of dihydrochalcones. (Table 1). Phloridzin was the major component at a concentration of 3025 μg/g d. w., while the concentration of phloridzin xyloside was 507 μg/g d.w.

By comparison, domestically prepared carrot juice contained a different spectrum of compounds. Although chlorogenic acid (194 μg/g d.w.) and two dihydrochalcones (phloridzin and phloretin) were present (Table 1) caffeic acid was the predominant phenolic acid at a concentration of 235 μg/g d.w.. The total concentration of all these compounds was 648 μg/g d.w.. With respect to the dihydrochalcones, phloretin was the major component at a concentration of 219 μg/g d.w., while phloridzin was found in the carrot juice at a much lower concentration (54.3 μg/g d.w.) than phloretin. The flavonol, quercetin, comprised a small portion of the total polyphenolic profile of the carrot juice, being only 2.6% of the total phytochemical content of the juice.

In the case of carrot roots, the transgenic material was richer in phytochemicals compared to the control roots (Table 1). Two phenolic acids, chlorogenic acid and 4-hydroxybenzoic acid, were detected in both types of roots, and a third unidentified compound in transgenic roots. Concentrations of chlorogenic acid were two-fold higher in transgenic tissue (373 μg/g d.w. in the control carrot roots and of 712 μg/g d.w. in the transgenic roots), while 4-hydroxybenzoic acid, present at 270 μg/g d.w. in control roots, increased to 1241 μg/g d.w. in transgenic roots. The concentration of the unidentified phenolic in transgenic tissue was calculated to be 1019 μg/g d.w. equivalent to 34% of total phenolics present.

Viability of human colon cancer cells exposed to pure phenolics

Cell survival data after a 24-h incubation period are listed in Table 2. Generally speaking, effects after 5 h were comparable (data not shown except for phloretin where effects were time-dependent). Amongst all the pure compounds tested, phloretin and quercetin were the most effective in decreasing the viability of human colon cancer cell lines (HT29 and LoVo cells). In the case of HT29 cells, the IC_{50} for phloretin was determined as 400 μmol/L and 260 μmol/L after 5-h and 24-h incubation respectively; whereas for LoVo cells the IC_{50} was determined as 380 μmol/L and 170 μmol/L after 5-h and 24-h incubation, respectively. Thus phloretin significantly decreased the viability of both HT29 and LoVo cells to 6.48% ($p<0.05$) and 4.73% ($p<0.05$) respectively at the highest concentration (1000 μmol/L) after 24 h of incubation. A concentration of 500 μmol/L was also very effective in decreasing the viability of both cell lines to 11.7% ($p<0.05$; HT29 cells) and 5.54% ($p<0.05$; LoVo cells), respectively, after 24 h, perhaps indicating that the LoVo cells are slightly more susceptible to this compound. A concentration of 100 μmol/L was also effective but less so, decreasing the viability of HT29 and LoVo cells to 78.75% ($p<0.05$) and 68.85% ($p<0.05$) respectively, after 24 h. A small but significant reduction in cell numbers was also seen after exposure of both cell lines to 50 μmol/L phloretin for 24 h (86.77% survival for HT29 cells; 82.26% survival for LoVo cells; $p<0.05$). There was no significant effect after exposure for 5 h. When exposure was reduced to 5 h, the highest concentration (1000 μmol/L) decreased viability of HT29 and LoVo cells to 19.09% ($p<0.05$) and 10.94% ($p<0.05$), respectively. A concentration of 500 μmol/L was also significantly effective, reducing the survival to 41.03% ($p<0.05$) and 30.03% ($p<0.05$), respectively.

By comparison, quercetin was generally less potent than phloretin to both cell lines, although once again the LoVo cells appeared to be more susceptible at lower concentrations (IC_{50} 100 μmol/L for LoVo cells; IC_{50} 380 μmol/L for

TABLE 2. Effect of apple and carrot root extracts and pure compounds on the viability of HT29 and LoVo cells *in vitro* after 5 (phloretin only) and 24-h exposure.

Colon cells		HT29			LoVo		
(μmol/L)		*100*	*500*	*1000*	*100*	*500*	*1000*
Phloretin	5 h	77.76	41.03*	19.09*	92.92	30.03*	10.94*
	24 h	78.75*	11.72*	6.48*	68.85*	5.54*	4.73*
Phloridzin		102.21	100.0	96.3	not tested		
Quercetin		100.38	32.95*	23.21*	51.69*	42.79*	124.02
Chlorogeic acid		101.39	90.93	73.42*	96.13	89.38	72.23*
Caffeic acid		120.36	62.06*	25.07*	98.51	93.54	68.27*
Amount of roots phenolic extract (mg/mL)		*20*	*100*	*200*	*20*	*100*	*200*
Control apple roots		105.62	96.6	76.28*	96.84	94.14	96.84
Control carrot roots		100.46	99.04	98.2	92.79	97.81	95.59
Transgenic carrot roots		91.42	87.03	79.62*	91.03	88.54	82.58*

* denoted value is significantly different than control ($p<0.05$)

HT29 cells after 24-h incubation). A clear effect was seen only at the two highest concentrations (500, 1000 μmol/L) for HT29 cells but at 100 μmol/L and 500 μmol/L for LoVo cells (Table 2), however, for some reason that is not clear, the highest concentration (1000 μmol/L) was consistently ineffective. Following a 5-h incubation (data not shown) trends were similar but the magnitude was decreased, compared to 24-h exposure. The glucosides of both phloretin and quercetin, phloridzin and quercetin-3-glucoside, respectively, had no effect on HT29 cells (data not shown). LoVo cells were not tested.

The addition of chlorogenic acid (present in both carrot juice and roots) and caffeic acid (present in carrot juice) to the cell culture medium reduced the viability of both cell lines in a significant manner (Table 2). Caffeic acid was significantly more effective, decreasing survival of HT29 cells to 62.06% ($p<0.05$) and 25.07% ($p<0.05$) at 500 μmol/L and 1000 μmol/L, respectively. LoVo cells were less sensitive to caffeic acid, the highest concentration (1000 μmol/L) decreasing viability to 68.27% ($p<0.05$) after 24 h. Chlorogenic acid had less effect on the viability of both cell lines. In this respect the viability of HT29 and LoVo cells was only decreased by about 30% ($p<0.05$) at the highest concentration (1000 μmol/L) after 24-h incubation. A 5-h exposure to either of these compounds was much less effective. Interestingly, 4-hydroxybenzoic acid, one of the most abundant phenolics in transgenic carrot root tissue, did not have any significant effect on the viability of either of the cell lines after 24 h of incubation (data not shown).

Viability of human colon cancer cells exposed to polyphenol extracts of apple and carrot roots

Polyphenol extracts of plant tissue showed contrasting effects. Compared to control carrot root tissue, a polyphenol extract of control apple roots decreased the viability of HT29 cells to 76.28% ($p<0.05$, Table 2). However, LoVo cells appeared to be more resistant, the highest concentration of the apple extract (200 μg/mL) having little effect on cell survival after 24 h (91.08% n.s.). Further dilutions of the extracts had no effect on either HT29 or LoVo cells.

In contrast, the polyphenol extract of transgenic carrot roots had a small but clear effect on the viability of both colon cancer cell lines (HT29 and LoVo) after 24-h incubation. The viability of both cell lines was decreased to about 80% ($p<0.05$) at the highest level (200 μg/mL), whereas 100 μg/mL only decreased viability to about 88% after 24-h incubation. On this occasion, a 5-h incubation had similar effects, decreasing the viability of both cell lines to 75% and 86%, respectively, at the same level (200 μg/mL) (data not shown).

Viability of human colon cancer cells exposed to extracts of apple and carrot juices

The least diluted extract of whole carrot juice (0.5% (v/v) juice) decreased the viability of LoVo cells to 67.52% ($p<0.05$). However dilutions of the juice had no significant effect. By comparison, whole and clarified apple juice and clarified carrot juice had no effect on the viability of both colon cancer cell lines (HT29 and LoVo) when unsupplemented. Although phenolic compounds were detected in these juices (Table 1), concentrations were below the threshold for activity.

There was a significant decrease in the viability of cells treated with apple and carrot juices, supplemented with enhanced levels of phloretin and chlorogenic acid, respectively (Table 3). Both juices were supplemented with phloretin and chlorogenic acid in order to simulate genetically modified apple and carrot juices, respectively, having enhanced levels of these phenolics. Apple juice supplemented with phloretin had a much greater effect on cell viability than carrot juice with chlorogenic acid. IC_{50} values for phloretin supplementation were 100 μmol/L and 90 μmol/L for HT29 and LoVo cells respectively, significantly decreasing the viability of HT29 and LoVo cells to 35% ($p<0.05$) and 38.61% ($p<0.05$) respectively, at a 100 μmol/L concentration of phloretin. In contrast, at the same concentration, clarified carrot juice supplemented with chlorogenic acid did not significantly reduce cell viability in either HT29 or LoVo cell lines. At higher levels of supplementation (500 and 1000 μmol/L), the apple juice with phloretin reduced cell viability to approximately 10% ($p<0.05$), with the exception of HT29 cells exposed to 1000 μmol/L phloretin concentration, where survival then increased to 31.76% ($p<0.05$). Cancer cell viability was also decreased to a smaller extent by carrot

TABLE 3. Effect of apple and carrot juices, alone and supplemented, on the viability of HT29 and LoVo cells *in vitro* after 24-h exposure.

Cell survival after 24 h (% of unexposed control)							
Colon cells		HT29			LoVo		
Dilution factor (% v/v juice)		0.5%	0.1%	0.05%	0.5%	0.1%	0.05%
Apple juice	whole	100.2	98.73	98.97	106.24	104.79	102.31
	clarified	95.95	99.14	102.29	102.78	103.4	103.3
Carrot juice	whole	103.68	99.64	103.85	67.52*	89.97	102.17
	clarified	102.36	97.3	97.52	102.14	104.76	104.42
Concentration of added compounds to juices (μmol/L)		*100*	*500*	*1000*	*100*	*500*	*1000*
Carrot juice +chlorogenic acid		98.56	94.75	69.9*	103.96	86.7	74.19
Apple juice+phloretin		35*	3.26*	31.76*	38.61*	9.95*	11.25*

* denoted value is significantly different than control ($p<0.05$)

juice supplemented with chlorogenic acid. This combination significantly reduced the viability of both cell lines to about 70% ($p<0.05$) at the highest supplementation (1000 μmol/L) after 24-h incubation. The lower concentration of chlorogenic acid (500 μmol/L) had no significant effect (Table 3).

In conclusion, of all the apple and carrot phenolic compounds screened, phloretin appears to be the most effective in the decreasing the viability of colon cancer cells *in vitro*. Either alone or when added as a supplement to apple juice, phloretin had a significant effect after 24-h exposure. None of the single compounds tested nor the plant phenolic extracts nor the supplemented juices demonstrated such a significant effect as phloretin alone in this respect. However other phenolic compounds, notably quercetin and caffeic acid, significantly decreased cell viability ($p<0.05$ for both compounds) causing the death of about 75% of HT29 cells, but only at the highest concentration (1000 μmol/L) after 24 h of incubation (Table 2).

DISCUSSION

In the past few years, food-borne phenolics have attracted increased attention because of their potential to afford protection against a variety of disorders, including different types of cancer [Dauchet *et al.,* 2004; Johnson, 2004; Maynard *et al.,* 2003; Talalay & Fahey, 2001; Vainio & Weiderpass, 2006] and cardiovascular diseases [Kuntz *et al.,* 1999; Caderni *et al.,* 2000; Daskiewicz *et al.,* 2005]. Fruits and vegetables provide the most abundant food source of phytochemicals [Daskiewicz *et al.,* 2005]. Apples are an important fruit source in the Western diet, as they contain phloretin, quercetin and chlorogenic acid [Lotito & Frei, 2004]. As regards vegetables, carrots are being increasingly consumed, mainly due to their pleasant flavor and their perceived health benefits related to their phytochemicals (such as chlorogenic acid), vitamins and fiber [Alasalvar *et al.,* 2001]. A limited number of *in vitro* studies of phenolic extracts of plants have suggested that more attention should be given to their effects on human colon cancer cells. Furthermore, to date, screening studies have been mainly restricted to pure compounds. It is therefore of considerable interest to ascertain whether polyphenol extracts of plants possess the same antiproliferative activity as single components.

In this study, using two human colon cancer cell lines (HT29 and LoVo cells), we have investigated the effect of both pure phenolic compounds and phenolic extracts of apples and carrots *in vitro* on cancer cell proliferation in a concentration-dependent manner. We have found that, amongst all the pure phenolic compounds tested, phloretin showed the greatest potential for decreasing the viability of both cell lines. Phloridzin, quercetin, chlorogenic acid and caffeic acid also showed antiproliferative activity in the *in vitro* tests, but the effect of these compounds was far weaker compared to phloretin and varied depending on the compound, its concentration and the cell type. Phloretin was the only compound which, after 24 h of incubation, was effective in a dose-dependent manner, at concentrations ranging from 50 μmol/L to 1000 μmol/L. The mechanisms involved were not further investigated in this case. However, Kobori *et al* [1999] demonstrated that phloretin induced apoptosis at 200 μmol/L in B16 mouse melanoma cells and HL60 human leukemia cells, after 24 h of exposure. This was attributed to inhibition of protein kinase C activity in HL60 cells and fragmentation of nuclei in both B16 and HL60 cells. Phloretin has also been shown to induce apoptosis in B16 cells through the inhibition of glucose transmembrane transport causing inhibition the growth of the cancer cells [Kobori *et al.,* 1999]. Moreover dietary phenolic compounds have been shown to arrest cancer cell cycle *in vitro.* Flow-cytometric analysis revealed that flavone can arrest HT29 cells in a post-G_1--phase before apoptosis occurs or differentiation is initiated in apoptosis-resistant cells [Wenzel *et al*., 2000].

A significant body of experimental and epidemiological data indicates that dietary phenolics may play an important role in the prevention of colon cancer by blocking hyperproliferation of the gastrointestinal epithelium and by promoting apoptosis [Kuntz *et al.,* 1999; Daskiewicz *et al.,* 2005; Chen *et al.,* 1998]. This hypothesis is primarily based on data from the screening of pure phenolics *in vitro* [Yu *et al.,* 2004] or from *in vivo* studies with animal models [Mahmoud *et al.,* 2000]. In our work we also investigated the antiproliferative activity of phenolic extracts of apple and carrot juice and extracts of root tissue on the viability of HT29 and LoVo cells *in vitro*. Polyphenolic extracts of both apple and carrot roots possessed less antiproliferative activity than the pure compounds. However the transgenic carrot roots significantly reduced viability of both HT29 and LoVo cells more effectively than the control carrot roots, possibly due to a higher

level of phenolic compounds. In the case of the apple root extract only HT29 cells were susceptible. The apple roots were shown to possess phloridzin and phloridzin xyloside, whereas the carrot roots possess phenolic acids, *i.e.* chlorogenic acid and 4-hydroxybenzoic acid. It should be pointed out that the transgenic carrot roots contained twice as much chlorogenic acid and four fold the amount of 4-hydroxybenzoic acid, compared to the control carrot roots. In addition, a third compound, a simple phenolic, not seen in the control roots was also present in the transgenic tissue.

Although apple roots also contained phloridzin and phloridzin xyloside, the glucosidic forms of phloretin, the aglycone was most effective in decreasing the viability of HT29 and LoVo cells in the present study. In the human gastrointestinal tract, phloridzin is hydrolysed on the luminal side of the small intestinal brush border to phloretin and glucose, by the lactase domain of lactase-phloridzin hydrolase (LPH), prior to absorption and metabolism [Day *et al.,* 2000]. Crespy *et al*. [2002] showed that rats fed phloridzin and phloretin had glucuronidated or sulfated phloretin but no phloridzin in blood plasma. In studies by Liu *et al*. [2005], rats were gavaged with whole apple extract (equivalent in amount to a human consuming a few fruit daily) for 2 weeks prior to treatment with the carcinogen 7,12-dimethylbenz[*a*]anthrace ne (DMBA). A reduction of tumor burden was observed. In the present study the most abundant phenolic compound in apple roots, phloridzin (85% of all phenolics), seemed not to be hydrolysed by LPH and consequently phloretin was not released. This is probably due to the fact that many intestinal cell lines poorly express LPH activity, and could explain the weak activity of the apple root polyphenol extract reported here.

A significant decrease in the viability of HT29 and LoVo cells was observed in the case of both apple and carrot juices supplemented with either phloretin or chlorogenic acid to simulate products with enhanced levels of these particular phenolics. Much stronger antiproliferative potential was observed compared to the unsupplemented juices, which had virtually no activity. This can probably be explained by the fact that the phloretin and chlorogenic acid concentrations in the juices were below the thresholds for an effect on viability. Nevertheless, antiproliferative activity of fruit extracts has been reported in other cancer cell lines *in vitro*. Boyer and Liu showed that when Caco-2 colon cancer cells were treated with apple extracts, cell proliferation was inhibited in a dose-dependent manner reaching a maximum inhibition of 43% at a dose of 50 mg/mL [Boyer & Liu., 2004]. The same trend was seen in Hep G2 liver cancer cells with maximal inhibition reaching 57% at a dose of 50 mg/mL. It should be emphasized we observed an inhibition in viability of 24% for HT29 cells exposed to 0.2 mg/mL of apple root extract, a much lower concentration compared to the 50 mg/mL reported by Boyer & Liu [2004] with apple fruit extracts.

CONCLUSIONS

Our results support the hypothesis that some dietary polyphenols may have a significant role to play in protection against colon cancer in humans. From this point of view, it is important to know if orally administered polyphenolics reach the colon intact. The optimal dose of phenolics has yet to be ascertained, bearing in mind the fact that during transit through the esophagus, stomach and small intestine they may be degraded or metabolised, before reaching the colon. However, most dietary phenolics, such as quercetin and chlorogenic acid resist degradation in the upper gut and reach the colon, where they may undergo microbial transformations [Olthof *et al*., 2003] possibly yielding additional photoprotectants. In these studies we have shown that the threshold for phloretin to have an effect on colon cancer cell viability appears to be around 50 μmol/L. Given that phloretin glycosides can be hydrolysed in the upper intestinal tract, but that the aglycone is poorly absorbed, it can be calculated that eating at least half a kilogram of apples at one sitting could deliver effective concentrations to the large bowel. In support of this, Tsao *et al.* [2003] determined the mean concentration of phloretin glucosides in unpeeled certain varieties of fresh apples as 51.4 μg/kg F.W. potentially yielding concentrations of almost 100 μmol/L of phloretin. This is identical to the IC_{50} for apple juice supplemented with phloretin in our tests. Our results therefore indicate that the development of fruit or products with enhanced levels of phloretin is a promising strategy. In contrast, where the effective concentration of a particular phytochemical is much higher, as in the case of chlorogenic acid (1 mmol/L), it is virtually impossible to deliver sufficiently high concentrations to the colon, solely by eating fresh products. In this case consumers could still benefit from the use of supplements, or from genetically manipulated products containing enhanced levels of these polyphenolic compounds, although safety aspects would need to be evaluated. In view of the evidence that a diet rich in fruits and vegetables could protect any part of the gastrointestinal tract, consumers should be encouraged to eat more fruits and vegetables, in fresh form in particular.

REFERENCES

1. Alasalvar C., Grigor J.M., Zhang D., Quantick P.C., Shahidi F., Comparison of volatiles, phenolics, sugars, antioxidant vitamin, and sensory quality of different colored carrot varieties. J. Agric. Food Chem., 2001, 49, 1410–1416.
2. Boyer J., Liu R.H., Apple phytochemicals and their health benefits. Nutr. J., 2004, 3, 1–15.
3. Caderni G., De Filippo C., Luceri C., Salvadori M., Giannini A., Biggeri A., Remy S., Cheynier V., Dolara P., Effects of black tea, green tea and wine extracts on intestinal carcinogenesis induced by azoxymethane in F344 rats. Carcinogenesis, 2000, 21, 1965–1969.
4. Chen Z.P., Schell J.B., Ho Ch.T., Chen K.Y., Green tea epigallocatechin gallate shows a pronounced growth inhibitory effect on cancerous cells but not on their normal counterparts. Cancer Lett., 1998, 129, 173–179.
5. Crespy V., Aprikian O., Morand Ch., Besson C., Manach C., Demigne., Remesy Ch., Bioavailability of phloretin and phloridzin in rats. J. Nutr., 2002, 132, 3227–3230.
6. Daskiewicz J.B., Depeint F., Viornery L., Bayet Ch., Comte-Sarrazin G., Comte G., Gee J.M., Johnson I.T., Ndjoko K., Hostettmann K., Barrow D., Effects of flavonoids on cell proliferation and caspase activation in a human colonic cell line HT29: An SAR study. J. Med. Chem., 2005, 48, 2790–2804.
7. Dauchet L., Ferrieres J., Arveiler D., Yarnell J.W., Gey F., Ducimetriere P., Ruidavets J.-B., Haas B., Evans A., Bingham A., Amouyel A., Dallongeville D., Frequency of fruit and vegetable consumption and coronary heart disease in France and Northern Ireland: the PRIME study. Br. J. Nutr., 2004, 92, 963–972.

8. Day A.J., Canada F.J., Diaz J.C., Kroon P.A., Mclauchlan R., Faulds C.B., Plumb G.W., Morgan M.R.A., Williamson G., Dietary flavonoid and isoflavone glycosides are hydrolyzed by the lactase site of lactase phloridzin hydrolaze. FEBS Letters, 2000, 468, 166–170.
9. Johnson I.T., Micronutrients and cancer. Proc. Nutr. Soc., 2004, 63, 587–595.
10. Kelly Y., Sacker A., Marmot M., Nutrition and respiratory health in adults: findings from the Health Survey for Scotland. Eur. Respir. J., 2003, 21, 664–671.
11. Kobori M., Iwashita K., Shinmoto H., Tsushida T., Phloretin-induced apoptosis in B16 melanoma 4A5 cells and HL60 human leukemia cells. Biosci. Biotechnol. Biochem., 1999, 63, 719–725.
12. Kuntz S., Wenzel U., Daniel H., Comparative analysis of the effects of flavonoids on proliferation, cytotoxicity, and apoptosis in human colon cancer cells. Eur. J. Nutr., 1999, 38, 133–142.
13. Lotito S.B., Frei B., Relevance of apple polyphenols as antioxidants in human plasma: contrasting in vitro and *in vivo* effects. Free Radic. Biol. Med., 2004, 36, 201–211.
14. Liu R., Liu J., Chen B., Apples prevent mammary tumors in rats. J. Agric. Food Chem., 2005, 53, 2341–2343.
15. Mahmoud N.N., Carothers A.M., Grunberger D., Bilinski R.T., Churchill M.R., Martucci Ch., Newmark H.L., Bertagnolli M.M., Plant phenolics decrease intestinal tumors in an animal model of familial adenomatous polyposis. Carcinogenesis, 2000, 21, 921–927.
16. Mandalari G., Bennett R.N., Bisignano G., Saija A., Dugo G., Curto R. B., Faulds C.B., Waldron K.W., Characterization of flavanoids and pectins from bergamot (*Citrus bergamia* Risso) peel, a major byproduct of essential oil extraction. J. Agric. Food Chem., 2006, 54, 197–203.
17. Maynard M., Gunnell D., Emmett P., Frankel S., Smith G.D., Fruit, vegetables, and antioxidants in childhood and risk of adult cancer: the Boyd Orr cohort. J. Epidemiol. Community Health, 2003, 57, 218–225.
18. Olthof M.R., Hollman P.C., Buijsman M.N., van Amelsvoort J.M., Katan M.B., Chlorogenic acid, quercetin-3-rutinoside and black tea phenols are extensively metabolized in humans. J. Nutr., 2003, 133, 1806–1814.
19. van der Sluis A.A., Dekker M., Skrede G., Jongen W.M., Activity and concentration of polyphenolic antioxidant in apple juice. 2. Effect of novel production methods J. Agric. Food Chem., 2004, 52, 2840–2848.
20. Talalay P., Fahey J.W., Phytochemicals from cruciferous plants protect against cancer by modulating carcinogen metabolism. J. Nutr., 2001, 131, 3027S–3033S.
21. Tsao R., Yang R., Young Ch., Zhu H., Polyphenolic profiles in eight apple cultivars using high-performance liquid chromatography (HPLC). J. Agric. Food Chem., 2003, 51, 6347–6353.
22. Vainio H., Weiderpass E., Fruit and vegetables in cancer prevention. Nutr. Cancer, 2006, 54, 111–142.
23. Wenzel U., Kuntz S., Brendel M.D., Daniel H., Dietary flavone is a potent apoptosis inducer in human colon carcinoma cells. Cancer Res., 2000, 60, 3823–3831.
24. Yu Z. Li W., Liu F., Inhibition of proliferation and induction of apoptosis by genistein in colon cancer HT-29 cells. Cancer Lett., 2004, 215, 159–166.

Received October 2006. Revision received January and accepted March 2007.

OCENA ANTYPROLIFERUJĄCYCH WŁAŚCIWOŚCI EKSTRAKTÓW FENOLOWYCH Z MARCHWI I JABŁEK

Katarzyna Przybylska[1], Richard N. Bennett[2], Krystyna Kromer[1], Jennifer M. Gee[2]

[1]Pracownia Kultur Tkankowych, Ogród Botaniczny Uniwersytetu Wrocławskiego, Uniwersytet Wrocławski, Wrocław, Polska;
[2]Institute of Food Research, Norwich, United Kingdom

W pracy badano wpływ ekstraktów fenolowych z surowców roślinnych (sok z jabłek i marchwi, hodowle tkankowe korzeni jabłek i marchwi) oraz wybranych czystych chemicznie związków fenolowych na przeżywalność dwóch linii komórek nowotworowych jelita grubego (HT29 i LoVo) w warunkach *in vitro*. Ponadto wzbogacono naturalny sok z jabłek i marchwi, odpowiednio, floretyną i kwasem chlorogenowym w celu symulacji produktów genetycznie modyfikowanych posiadających podwyższony poziom związków fenolowych i zbadano ich wpływ na przeżywalność komórek nowotworowych. Wśród testowanych czystych chemicznie związków fenolowych, floretyna wykazała największą skuteczność w obniżaniu przeżywalności badanych linii komórek nowotworowych (tab. 2), już w stężeniu 50 μmol/L znacznie obniżyła przeżywalność badanych komórek. Spośród surowców roślinnych, ekstrakty fenolowe z transgenicznych korzeni marchwi najskuteczniej obniżyły przeżywalność komórek nowotworowych (tab. 2), ekstrakt fenolowy w stężeniu 200 μg/mL obniżył przeżywalność komórek o 20%. Natomiast ekstrakty fenolowe z kontrolnych korzeni marchwi i jabłoni wykazały znacznie mniejszy efekt na przeżywalność badanych komórek (tab. 2). Warto zaznaczyć, że wzbogacenie soku z jabłek floretyną doprowadziło do obniżenia przeżywalności badanych komórek już przy stężeniu floretyny wynoszącym 100 μmol/L a stężenia powyżej 500 μmol/L obniżyły przeżywalność komórek LoVo aż o 90% (tab. 3). Sam sok z jabłek nie był skutecznym czynnikiem w obniżaniu żywotności komórek w warunkach *in vitro* (tab. 3), prawdopodobnie z powodu zbyt niskiego naturalnego stężenia floretyny. Zamieszczone informacje powinny skłonić konsumentów do regularnego spożywania większych ilości warzyw i owoców, szczególnie tych produktów, które zawierają duże ilości floretyny (jabłka). Ponadto doniesienia te powinny nakłonić przedsiębiorców do uzyskiwania produktów o podwyższonym poziomie związków fenolowych poprzez tradycyjne techniki rolnicze lub poprzez dodatki do żywności.