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Antiproliferative activity is predominantly associated with ellagitannins in raspberry extracts

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Abstract

Raspberry extracts enriched in polyphenols, but devoid of organic acids, sugars and vitamin C, were prepared by sorption to C18 solid phase extraction matrices and tested for their ability to inhibit the proliferation of human cervical cancer (HeLa) cells in vitro. The raspberry extract reduced proliferation in a dose-dependent manner whether this was judged by cell number or measurements of cell viability. However, measurements based on cell viability were more accurate and gave an EC_{50} value of 17.5 μg/ml gallic acid equivalents (GAE) at day 4 of culture.

Raspberry extracts were fractionated by sorption to Sephadex LH-20 into an unbound fraction, which was obviously enriched in anthocyanins, and a bound fraction. The unbound anthocyanin-enriched fraction was much less effective in reducing proliferation then the original extract and gave an EC_{50} value estimated at 67 μg/ml. The LH-20 bound fraction was more effective than the original raspberry extract ($EC_{50} = 13$ μg/ml) suggesting that the main anti-proliferative agents were retained in the bound fraction. Analysis of the original extract, the unbound and the LH20 bound fractions by LC–MS confirmed that the unbound fraction was enriched in anthocyanins and the bound fraction primarily contained ellagitannins. The ellagitannin-rich bound fraction had the highest antioxidant capacity as measured by the ferric reducing antioxidant potential (FRAP) assay. The mechanism by which the ellagitannins inhibit proliferation of cancer cells is discussed.

Keywords: Anthocyanins; Antioxidant; Antiproliferation; Cancer; Cell viability; Ellagic acid; Ellagitannins; Growth inhibition; Raspberry

1. Introduction

There is substantial epidemiological evidence that diets rich in fruit and vegetables (FAV) are associated with reduced risk of a number of chronic health disorders including cancers (Anon., 2002; Steinmetz and Potter, 1996; Hertog et al., 1996). There is a growing interest in identifying components of FAV responsible for anti-cancer effects (Kris-Etherton et al., 2002). FAV are rich sources of antioxidant components capable of scavenging oxygenated free radicals which can damage cellular components such as DNA, proteins or membrane lipids (Halliwell, 1996). Such oxidative damage may be involved in the onset of carcinogenesis (Mates and Sanchez-Jimenez, 2000).

Unregulated proliferation and suppression of apoptosis are key steps in initiation and progression of cancers (Evan and Vousden, 2001). Many studies have confirmed that berry extracts inhibit proliferation of cancer cells cultured in vitro (Yoshizawa et al., 2000; Liu et al., 2002; Meyers et al., 2003; Katsube et al., 2003; Olsson et al., 2004; Juranic et al., 2005; Han et al., 2005; Schmidt et al., 2005). Although such in vitro studies are essentially artificial, they can provide leads on potentially useful cytotoxic or antiproliferative compounds and guide further studies, such as the inhibition of transformation into cancerous cells in vitro (Xue et al., 2000) or the initiation and progression of experimentally induced tumours in laboratory animals (Han et al., 2005; Carlton et al., 2001), which may be more physiologically relevant. Berries provide a rich, complex and species-specific mixture of antioxidant components including vitamin C (ascorbic acid), carotenoids and

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xanthophylls, and polyphenols (Macheix et al., 1990; Kahkonen et al., 2001). Individual polyphenols such as anthocyanins or anthocyanidins (Cooke et al., 2005) and ellagic acid (Losso et al., 2004) have been shown to inhibit cancer proliferation in vitro. Furthermore, other studies have suggested considerable potential for synergetic effects between ascorbic acid, carotenoids and polyphenols (Olsson et al., 2004) and between polyphenol components (Seeram et al., 2004a, 2005).

This study examines the anti-proliferative effect of raspberry extracts enriched in polyphenols, but devoid of vitamin C and carotenoids, on human cervical carcinoma (HeLa) cells. This in vitro study attempts to rank the anti-proliferative effect of sub-fractions enriched in anthocyanins and ellagitannins using HeLa cells to identify compounds with enhanced anti-cancer properties.

2. Materials and methods

2.1. Plant material and extraction

Raspberries (*Rubus idaeus* L. variety Glen Ample) were purchased from local farmers. Polyphenol-rich fractions were obtained using an adaptation of a well-described method (Rommel and Wrolstad, 1993). Briefly, frozen raspberries (100 g) were thawed then homogenised in a Waring Blender using 100 ml of 0.5% (v/v) glacial acetic acid in water. The extract was filtered through a glass sinter and applied to C18 solid phase extraction units (Strata C18-E, GIGA units, 10 g capacity Phenomenex Ltd., Macclesfield, UK) pre-washed in 0.5% (v/v) glacial acetic acid in acetonitrile then pre-equilibrated in 0.5% (v/v) glacial acetic acid in water. The raspberry filtrate was applied and unbound material, which contained the free sugars, organic acids and vitamin C, was collected. The units were washed with three column volumes of 0.5% (v/v) aqueous acetic acid then the polyphenol-enriched bound extracts eluted with 0.5% (v/v) glacial acetic acid in acetonitrile. The C18-bound extracts were evaporated to dryness with repeated additions of methanol to drive off acetic acid.

Sorption to Sephadex LH-20 in aqueous ethanol and selective debinding with aqueous acetone is an established method for separating tannins from non-tannin phenolics (Strumeyer and Malin, 1975). The method was adapted from the Tannins Handbook (kindly made available from the Hagermann laboratory at www.users.muohio.edu/hagermae/tannin.pdf). Briefly, a column of Sephadex LH-20 was washed in 80% (v/v) ethanol/water then 50% (v/v) acetone/water before being equilibrated with three volumes of 80% ethanol. The raspberry C18 extract was dissolved in 80% ethanol, applied to the column and the run-through material plus a column volume of 80% ethanol collected as the unbound fraction. This material was bright red and obviously contained the bulk of the anthocyanins. The column was washed with three column volumes of 80% ethanol. The bound fraction was eluted with three volumes of 50% acetone. The unbound and bound fraction were evaporated to near dryness then stored frozen. Phenol content was measured using a modified Folin–Ciocalteu method (Singleton and Rossi, 1965) and anthocyanin content was measured by the differential colorimetric method (Ribereau-Gayon and Stonestreet, 1965).

2.2. Cell proliferation and measurements of cell viability

Human cervical cancer (HeLa) cells were grown as a monolayer in Dulbecco's modified eagle medium (DMEM) containing 1 g/l glucose and L-glutamine and pyruvate (Invitrogen product no. 31885, Paisley, UK) supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin (Gibco product no. 15070-063) and 25 μg/ml Gentamycin (Gibco product no. 15750). The cells were grown in 50 ml, 25 cm^2 flasks at 37 °C in an atmosphere of 5% CO_2/95% air and at constant humidity.

Prior to each experiment, cells from a number of flasks were harvested by treatment at 37 °C with a solution of Trypsin-EDTA (1×), washed thoroughly with phosphate-buffered saline (PBS) (1×) and re-suspended in the growth medium. They were then counted to determine the volume required to give a final density of approximately 250,000 cells in flasks containing 5 ml growth media inclusive of the different treatment solutions. Raspberry extracts were diluted in PBS then filter-sterilised. Phenol contents were checked to adjust for losses due to filtration. Cells were harvested daily over a 7-day period and cell number and viability were assessed. Cell viability was assayed using a kit (Dojindo CCK-8 kit, from NBS Biologicals, Cambridge, UK) which uses a tetrazolium salt derivative [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt] which is converted to a water-soluble formazon dye directly proportional to the number of living cells.

2.3. Effect of incubation with HeLa cells on ellagitannins

HeLa cells were grown to confluence in DMEM media as above, harvested by treatment with trypsin and seeded at 50,000 cells/cm^3. After 3 days, the media was removed, the cells washed with PBS and 4.5 ml DMEM without Phenol Red (Gibco cat. no. 11880-036) supplemented to normal levels of glutamine (80 μM). Raspberry ellagitannins (500 μg GAE) were dissolved in 500 μl PBS, made up to 5 ml with media without Phenol Red and added to the cells. Controls contained no ellagitannins. Triplicate cultures were harvested after 2 h, 8 h, 24 h, 48 h and 72 h growth. To assess the breakdown of ellagitannins in media alone, the same procedure was repeated without cells.

Cells and debris were removed by centrifugation (10,000*g*, 10 min, 4 °C) and the media was stored frozen prior to analysis. The media was acidified with 500 μl of 5 N formic acid whilst thawing then 1 ml of 0.5% (v/v) aqueous formic acid was added. Any insoluble material was removed by centrifugation. The supernatants were

applied to SPE units (Phenomenex, Strata C18-E units, 50 mg capacity) that had been wetted with acetonitrile containing 0.5% (v/v) formic acid then pre-equilibrated in 0.5% (v/v) formic acid in water. The unbound material was collected along with a wash of 2 ml of 0.5% (v/v) aqueous formic acid. The bound material was then eluted with 1.5 ml of acetonitrile containing 0.5% (v/v) formic acid. The bound material was dried in a speed-vac (Thermo-Finnegan Ltd.). All samples were frozen prior to analysis. Control samples were prepared that contained the same amount of raspberry LH 20 bound material in fresh media without cells. These were immediately acidified and applied to SPE and were used to judge the recovery of ellagitannins after SPE.

2.4. Liquid chromatography–mass spectroscopy (LC–MS)

Raspberry samples containing 20 μg phenols (gallic acid equivalents) were analysed on a LCQ-Deca system, comprising Surveyor autosampler, pump and photo diode array detector (PDAD) and a ThermoFinnigan mass spectrometer iontrap. The PDAD scanned discrete channels at 280 nm, 365 nm and 520 nm. The samples were applied to a C-18 column (Synergi Hydro C18 with polar end capping, 4.6 mm × 150 mm, Phenomonex Ltd.) and eluted over a gradient of 5% acetonitrile (0.5% formic acid) to 30% acetonitrile (0.5% formic acid) over 60 min at a rate of 400 μl/min. The LCQ-Deca LC–MS was fitted with an ESI (electrospray ionisation) interface and analysed the samples in positive and negative ion mode. There were two scan events; full scan analysis followed by data dependant MS/MS of most intense ions using collision energies (source voltage) of 45%. The capillary temp was set at 250 °C, with sheath gas at 60 psi and auxiliary gas at 15 psi.

3. Results and discussion

The raspberry extract inhibited the proliferation of HeLa cells in a dose dependent manner whether the proliferation was assessed by cell number (Fig. 1a) or by measurement of cell viability (Fig. 1b). Up to day 4, the two graphs are similar but the continuing increase in cell number between day 4 and day 7 includes non-viable cells and/or dying cells not detected by the viability test. Therefore, the viability figures provided the more accurate estimation of antiproliferative effectiveness. The antiproliferative effect was estimated at day 4 because the cells were actively growing and had the largest proportion of viable cells. The raspberry extract gave a median effective dose (EC_{50}), where a low EC_{50} value represents greater anti-proliferative activity, of 21.0 μg/ml GAE which was extrapolated using comparison of % inhibition values at day 4 of culture (Fig. 1c).

The original raspberry extract was more effective in inhibiting proliferation than the raspberry LH-20 unbound fraction (compare Fig. 2a and b) which gave an EC_{50} value estimated at 67 μg/ml GAE. Also, treatment with this

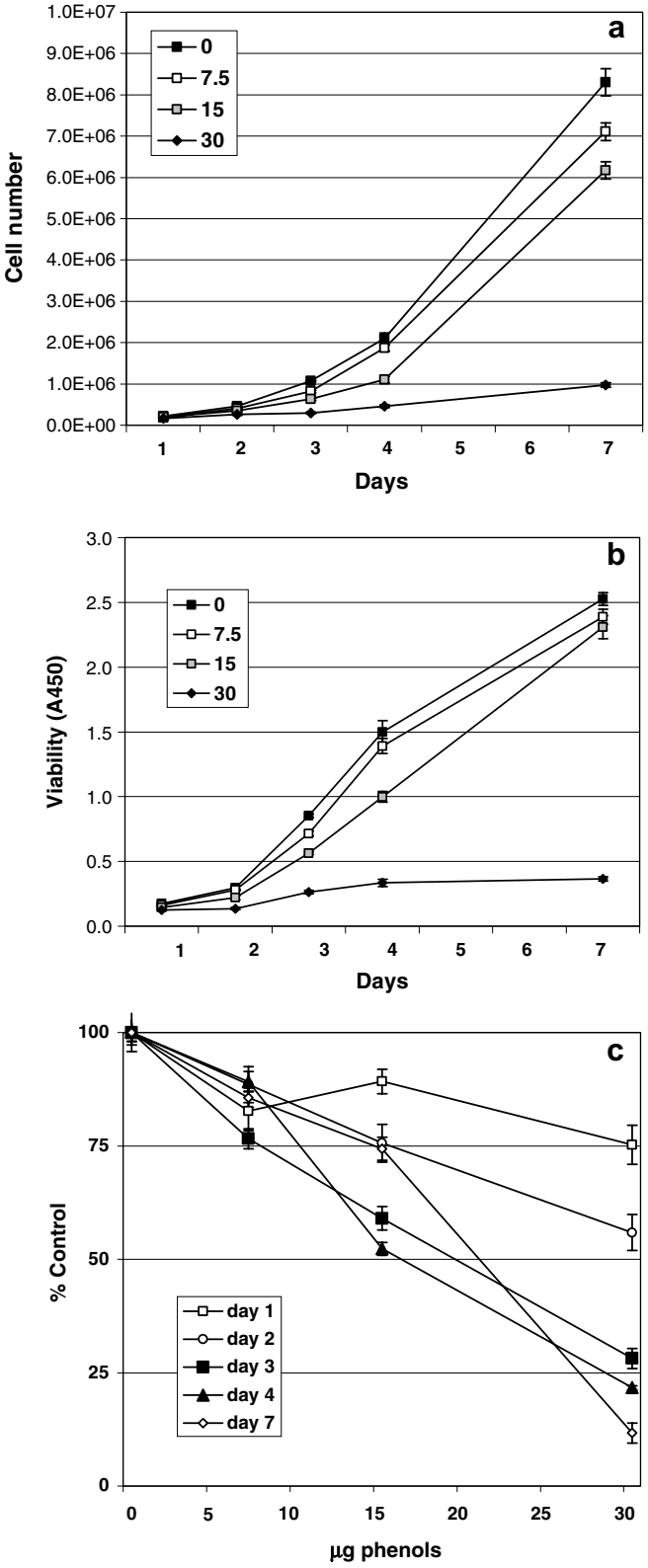

Fig. 1. Effect of raspberry extract on proliferation of HeLa cells. Proliferation is measured in terms of cell number (a) and cell viability (b). Each value is the mean of, at least, quadruplicate assays ± standard error. Each graph is representative of at least three repeat assays. Panel (c) shows the cell viability results re-plotted to calculate the EC_{50} value.

anthocyanin-enriched unbound extract caused an apparent increase in cell viability at day 7. The LH-20 bound fraction was more effective than the original raspberry extract and gave an EC_{50} value of 13 μg/ml GAE (Fig. 2c). In addition, the LH-20 bound fraction was notably more effective earlier (compare Fig. 2a and c) suggesting that it prevented rather than inhibited proliferation.

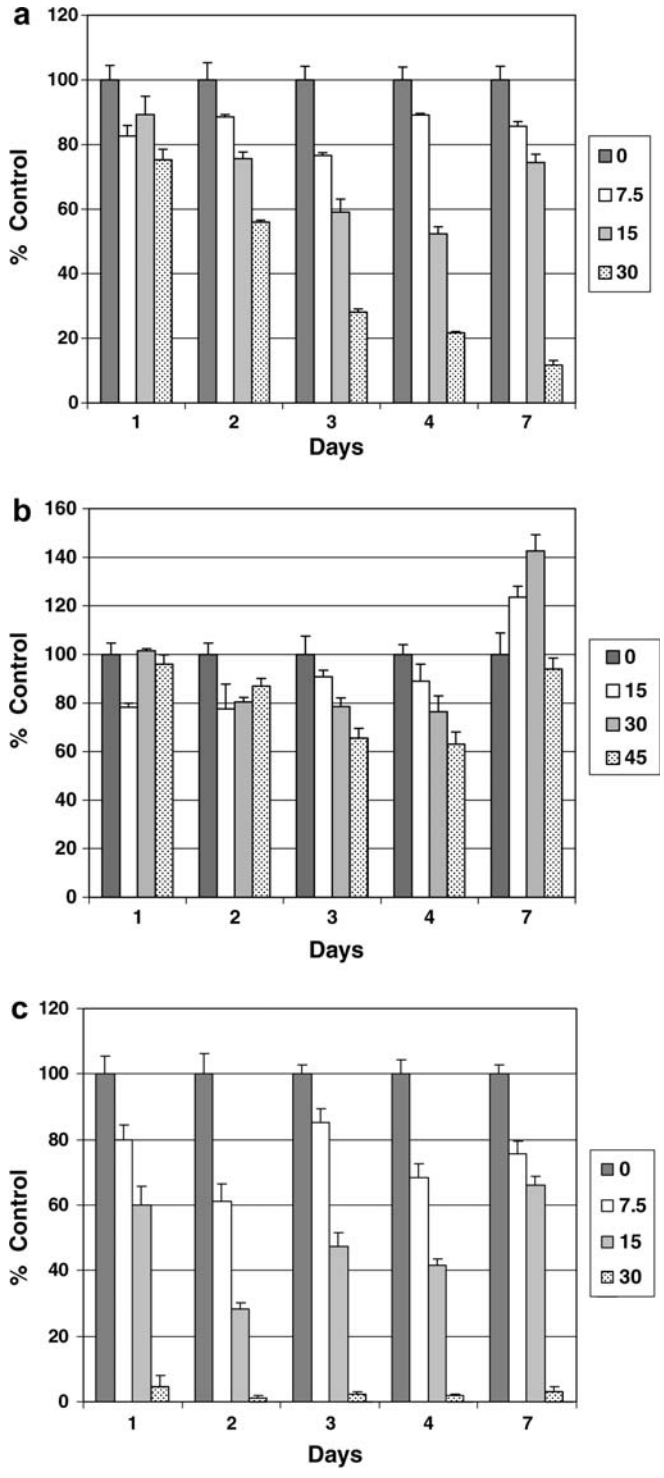

Fig. 2. Effect of raspberry fractions on proliferation of HeLa cells. Raspberry extract (a), the LH-20 unbound fraction (b) and the LH-20 bound fraction (c) were tested for anti-proliferation effects. The extent of inhibition of proliferation by each phenol concentration is expressed as % of the control figures at each time point. The control figures are given to show the extent of variation. Each value is the mean of quadruplicate assays ± standard error. Each graph is representative of a number of repeat assays.

The EC_{50} value for the anti-proliferative effect of the polyphenol-rich raspberry extracts in this work ($EC_{50} = 17.5$ μg GAE/ml) was lower but in the same general range as the figures calculated from data on previous work on raspberry (Liu et al., 2002) and strawberry (Meyers et al., 2003) extracts (EC_{50} values of 50 and 43 μg GAE/ml, respectively). However, these figures were obtained for extracts obtained with 80% (v/v) acetone in water and would contain vitamin C, sugars and other non-phenolic compounds that interfere with the Folin–Ciocalteu method (George et al., 2005). They are also not comparable because they refer to effects on HepG2 human liver cancer cells and substantial differences in sensitivity between different cancer cell lines have been documented (Losso et al., 2004).

The raspberry extract had a similar composition to previous reports (Kahkonen et al., 2001; Deighton et al., 2000) with an anthocyanin content of 15.9 μg/ml compared to 100 μg/ml for total phenols. The HPLC profile of the raspberry extract was similar to previous reports (Mullen et al., 2002; Beekwilder et al., 2005) and was mainly comprised of anthocyanins (peaks 1–4; Table 1) and ellagitannins (peaks 5–6; Table 1) with a number of other minor components (Fig. 3a). The anthocyanins were detected by their absorption at 520 nm and their structure confirmed by mass spectrometry (Table 1). The most abundant anthocyanin was cyanidin-3-*O*-sophoroside (peak 1), peak 2 was composed of cyanidin-3-*O*-(2^G)-glucosylrutinoside, peak 3 was composed of cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-sophoroside and peak 4 was composed of cyanidin-3-*O*-rutinoside and pelargonidin-3-*O*-(2^G)-glucosylrutinoside. Smaller amounts of pelargonidin-3-*O*-glucoside and pelargonidin-3-*O*-rutinoside eluted later in the gradient and were confirmed by searching the MS data at the relevant masses.

The LH-20 unbound sample (Fig. 3b) was greatly enriched in anthocyanins (peaks 1–4) and some other earlier eluting minor components. The total anthocyanin content was 3.4-fold higher then the original extract. This was reflected in the increase in abundance of individual peaks, i.e. peak 1 was enriched 3.33-fold and peak 2 by 3.97-fold over the original extract when the peak areas at 520 nm were compared. Peaks 5 and 6 were not detected in the unbound fraction.

The LH-20 bound fraction was predominantly composed of peaks 5 and 6 (Fig. 3c) with two new peaks 7 and 8 uncovered by the removal of anthocyanins. By comparing peak areas at 280 nm, peak 5 was enriched by 1.52-fold and peak 6 by 1.25-fold compared to the original extract. By the same token, peaks 1 and 2 were reduced to 0.98% and 0.70% of the original sample. The total anthocyanin content of this fraction as estimated by the colorimetric assay was 0.1 μg/ml.

Table 1
Putative identification of main components of raspberry extracts

Peak	T^R	M^+ (m/z)	MS^2	Tentative identification
1	28.05	**611.1**	**287.2**	Cy-3-sophoroside
2	28.72	**757.2**	**287.2**, 611.1	Cy-3-(2^G-glucosylrutinoside)
3	30.51	**449.1**	**287.2**	Cy-3-glucoside
3	–	**595.1**	**271.2**	Pg-3-sophoroside
4	31.52	**595.1**	**287.2**, 449.1	Cy-3-rutinoside
4	–	**741.2**	**271.2**, 595.1	Pg-(2^G-glucosylrutinoside)
		M^- (m/z)		
5	38.35	1869.0, 1567.1, **1401.3**, 1235.0, **933.1**, **633.2**	Many	Lambertianin C, Sanguiin H6, Sanguiin H10 isomer?
6	39.71	**1869**, 1567.1, 1235.0, 933.1, 633.2	Many	Sanguiin H6, Sanguiin H10 isomer?
7	26.30	**1567.1**	Many	Sanguiin H10 isomer
8	33.70	**1567.1**	Many	Sanguiin H10 isomer

Peaks refer to Fig. 3. Anthocyanins are detected in positive mode and ellagitannins by negative mode. The most abundant ions are in bold. Identifications are supported by previous work (Mullen et al., 2003; McDougall et al., 2005).

Peak 6 yielded one predominant mass ion at m/z 1869 in negative mode (Table 1) with smaller peaks at 1567, 1235, 933, and 633 which may arise from in-source fragmentation of the m/z 1869 ion. Indeed, the m/z 1869 ion gave MS^2 ions at m/z values of 1567, 1235, 1103, 933, 897 and 633. The mass ion and the fragment ions were essentially identical to the pattern obtained for Sanguiin H6 (Mullen et al., 2003) (Fig. 4), previously identified in essentially identical extracts of raspberry (McDougall et al., 2005). Peaks 7 and 8 both yielded a dominant ion at m/z 1567 in negative mode (Table 1) which suggests that they contain different isomers of Sanguiin H10 (Fig. 4), which has a similar structure to Sanguiin H6 but lacking one hexahydroxydiphenoyl (HHDP) unit. Peak 5 gave an MS spectrum similar to that of peak 6 except it also contained an appreciable signal at m/z 1401 (Table 1) which has been assigned to Lambertianin C (Mullen et al., 2003). Therefore, peak 5 was composed of a mixture of Lambertianin C and Sanguiin H6 similar to that described in previous work (McDougall et al., 2005). It was not possible to discern whether the m/z 1567 signal observed in peaks 5 and 6 was due to in-source fragmentation of Sanguiin H6 (m/z 1869) or isomers of Sanguiin H10.

Although past studies have suggested potent effects of anthocyanin-rich berry extracts (Zhao et al., 2004), pure anthocyanins (Shih et al., 2005) and anthocyanidins (Cooke et al., 2005) on cancer cell proliferation, our findings suggest that anthocyanins were less effective than the ellagitannins present in raspberry. No significant correlation was established between anthocyanin content and the anti-proliferative activity of raspberry varieties (Liu et al., 2002) even though one of the varieties effectively lacked anthocyanins. The authors concluded that "phytochemicals other than anthocyanins in the raspberries were responsible for inhibition of tumour cells". There was also no correlation between anthocyanin content and anti-proliferative effectiveness in a range of berry extracts (Olsson et al., 2004). However, in these studies using whole berry extracts, ascorbic acid would be present and may act synergistically with carotenoid and polyphenols in determining anti-proliferative effects. In this study, ascorbic acid and carotenoids were removed by solid phase extraction so that the anti-proliferative effects of the polyphenols in the raspberry extracts could be directly compared. Other studies have indicated that water soluble extracts of raspberry seeds had appreciable anti-proliferative activity (Juranic et al., 2005) and it is known that the seeds are ellagitannin-rich (Daniel et al., 1989). In a study of the anti-mutagenic activity of berry extracts (Smith et al., 2004), the ellagitannin-containing fraction from strawberries was most effective.

In contrast, the anthocyanin-rich fractions from blueberry gave the highest antiproliferative effect on human colon cancer cell lines whereas the tannin and flavonol enriched fractions were less effective (Yi et al., 2005). However, the tannin fraction produced from blueberry would mainly be composed of proanthocyanidins not ellagitannins (Macheix et al., 1990; Oszmianski et al., 1988). Also, the difference in effectiveness between anthocyanin and tannin fractions was much less pronounced using Caco-2 cells than observed using HT-29 colon cancer cell lines. It would be interesting to compare the anti-proliferative effect of anthocyanidins, proanthocyanidins and ellagitannins on an equal basis.

The ellagitannin-rich raspberry fraction had considerably higher antioxidant capacity than the original raspberry extract or the anthocyanin-rich fraction (Fig. 5). Deighton et al. (2000) found that although the antioxidant capacity of *Rubus* extracts was closely correlated with total phenol content, it was not correlated with anthocyanin content. Mullen et al. (2002) noted that the antioxidant capacity of raspberry extracts was dominated by the contribution made by ellagitannins. This was confirmed in another study (Beekwilder et al., 2005) where over 50% of the total antioxidant capacity of raspberry was contributed by Sanguiin H6 and Lambertianin C. It has been proposed that cancer cells over-produce hydrogen peroxide (H_2O_2) and other reactive oxygen species which constantly stimulate redox-sensitive cell-signalling pathways to maintain proliferation (Loo, 2003). In this model, therefore,

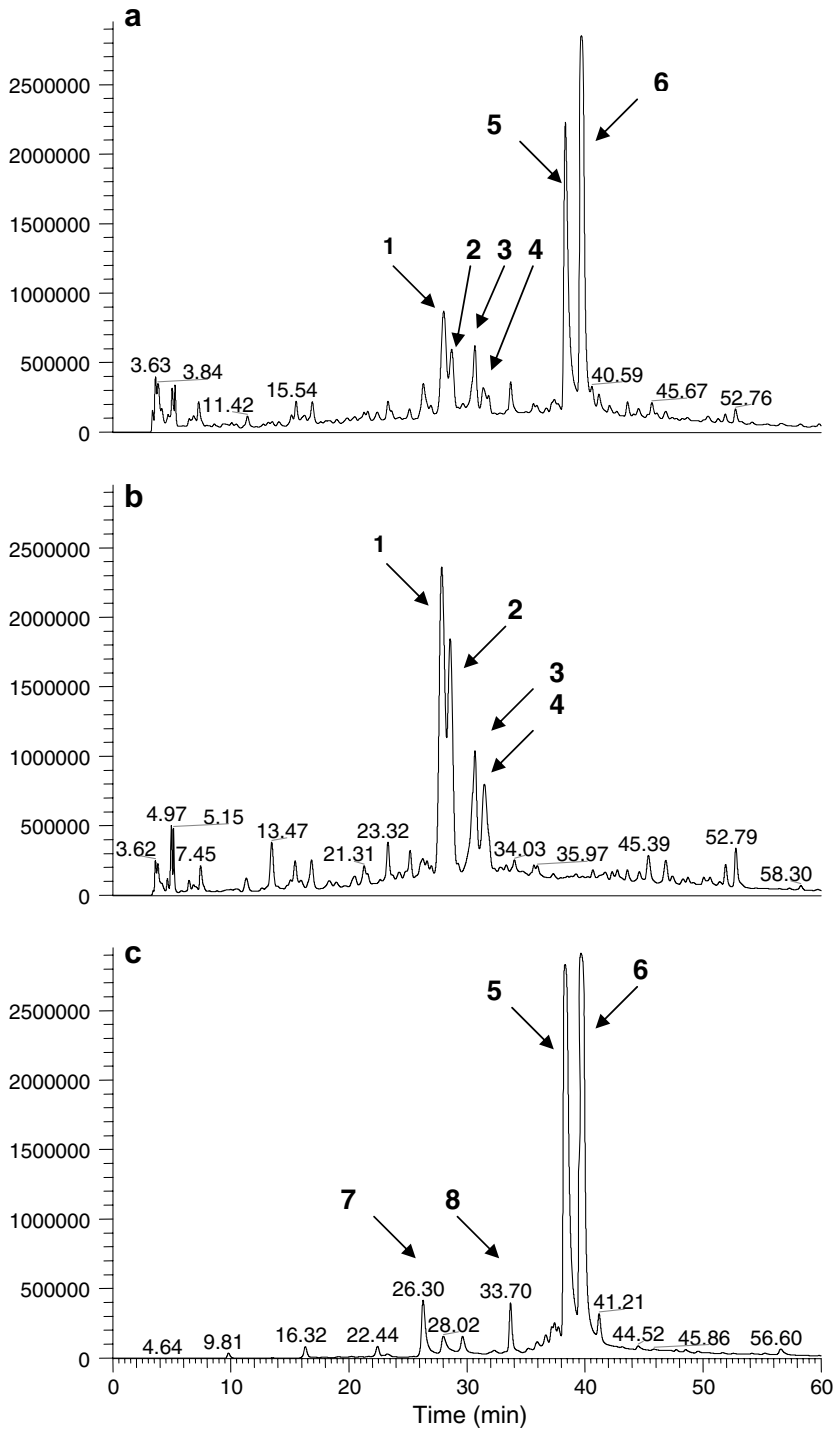

Fig. 3. HPLC traces of raspberry fractions. Equal amounts (20 μg GAE) of raspberry extract (a), the LH-20 unbound fraction (b) and the LH-20 bound fraction (c) were separated on HPLC and detected by absorbance at 280 nm. Peaks are described in the text and putative identifications are given in Table 1.

those phytochemicals with the highest antioxidant capacity would be the most effective anti-proliferative agents.

The survival of the ellagitannins in the raspberry LH-20 bound fraction was monitored upon incubation with HeLa cells (Fig. 6). At 2 h, a major A280 absorbing peak eluting at 41.26 min which gave MS and MS^2 spectra consistent with Sanguiin H6 could be detected (Fig. 6b, peak 1). A second peak at 47.73 min could be detected in the media after 2 h and was identified as ellagic acid by its UV and MS spectra (Fig. 6b, peak 2). However, the amount of Sanguiin H6 that could be recovered after 2 h was less than 1% of the amount added whereas the levels of ellagic acid increased over 2–48 h incubation (Fig. 6b–f). The recovery of Sanguiin H6 after 2 h in media without cells was equally as low and ellagic acid levels rose in a similar fashion (compare Fig. 7a and b). The low recovery of Sanguiin H6 was

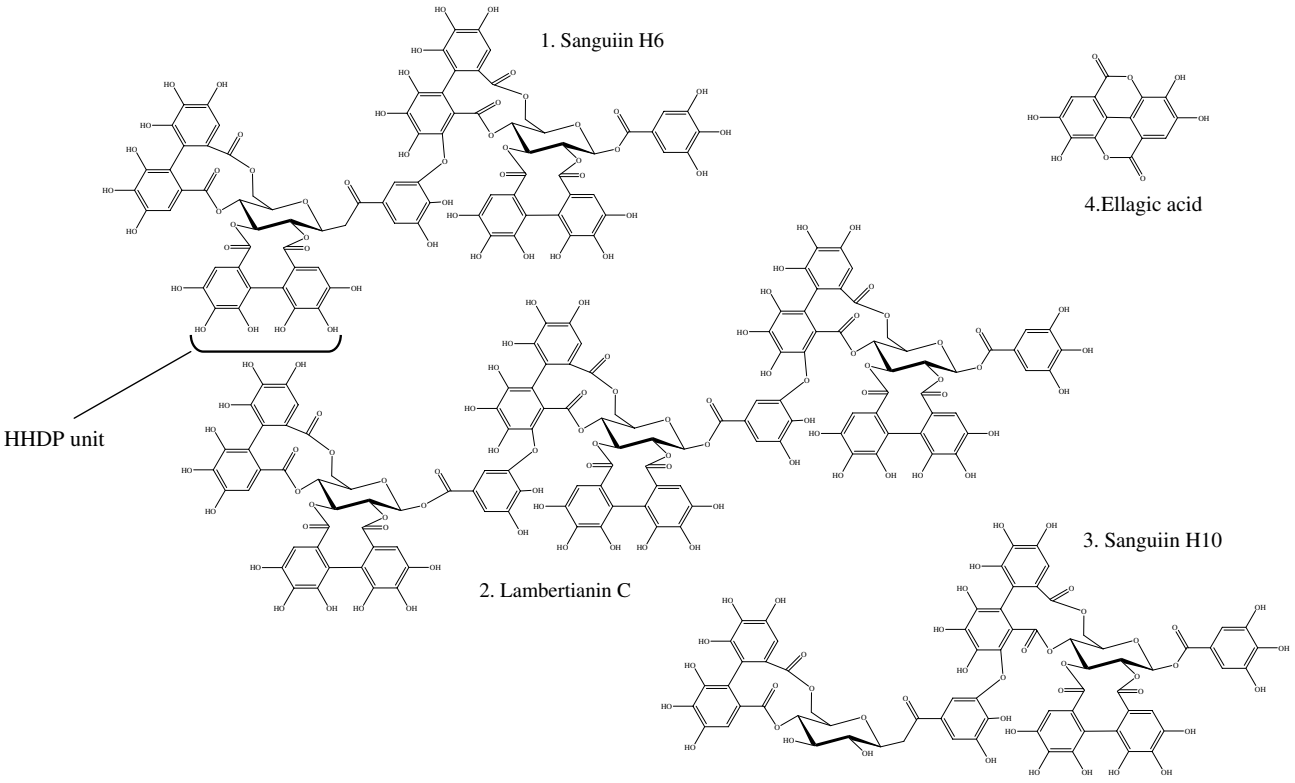

Fig. 4. Ellagitannin structures. The structure of an HHDP unit is annotated.

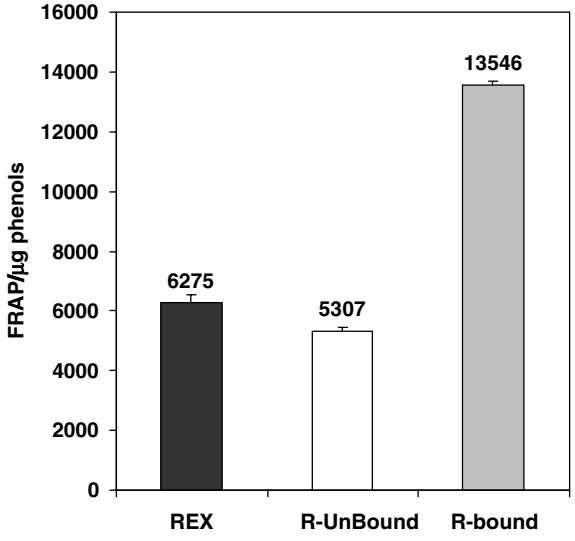

Fig. 5. Antioxidant potential of raspberry fractions. Each assay carried out with 1 µg phenol GAE. Each value is the mean of triplicates ± standard error.

due to binding to serum proteins in the media as Sanguiin H6 (and some of the other ellagitannins identified in the LH-20 bound fraction) were detected in the unbound fraction after solid phase extraction but only after drying and re-extraction with methanol. Even then, only up to 25% of the total was recovered (results not shown).

More EA was released over the first 8 h in the presence of cells than in the media-only experiment (see Fig. 7a and b). This suggests that the living cells facilitate ellagitannin breakdown. The lower levels of free extracellular ellagic acid at 24 h could be explained by uptake of ellagic acid into the cells. Although EA could be detected in methanol extracts of the cells (results not shown), we could not discriminate between EA uptake and binding to cell surfaces. The growth of the HeLa cells in these experiments was very slow as they were treated with 100 µg/ml of the raspberry LH-20 bound fraction, almost 8-fold higher then the estimated EC_{50}, which must have influenced their metabolic processes.

Ellagitannins are unstable at neutral to alkaline pH and break down to yield ellagic acid (Daniel et al., 1991; Clifford and Scalbert, 2000). Raspberry ellagitannins produced ellagic acid in cell media in the presence or the absence of HeLa cells, presumably by loss and lactonisation of HHDP groups, in a similar fashion to the pomegranate ellagitannin, punicalagin, in Caco-2 cells (Larrosa et al., 2005). Ellagic acid has potent antiproliferative effects (Castonguay et al., 1997; Losso et al., 2004) and gave an EC_{50} value of ~5 µg/ml in this study (results not shown). Although long-term exposure to pure ellagic acid has been shown to decrease immune responses in mice (Allen et al., 2003), the levels of free ellagic acid used were considerably higher than those achievable from food sources where free ellagic acid is rare (Clifford and Scalbert, 2000) and is obtained via degradation of ellagitannins.

The in vitro HeLa system is a useful model system to rank the effectiveness of different compounds but it is, by

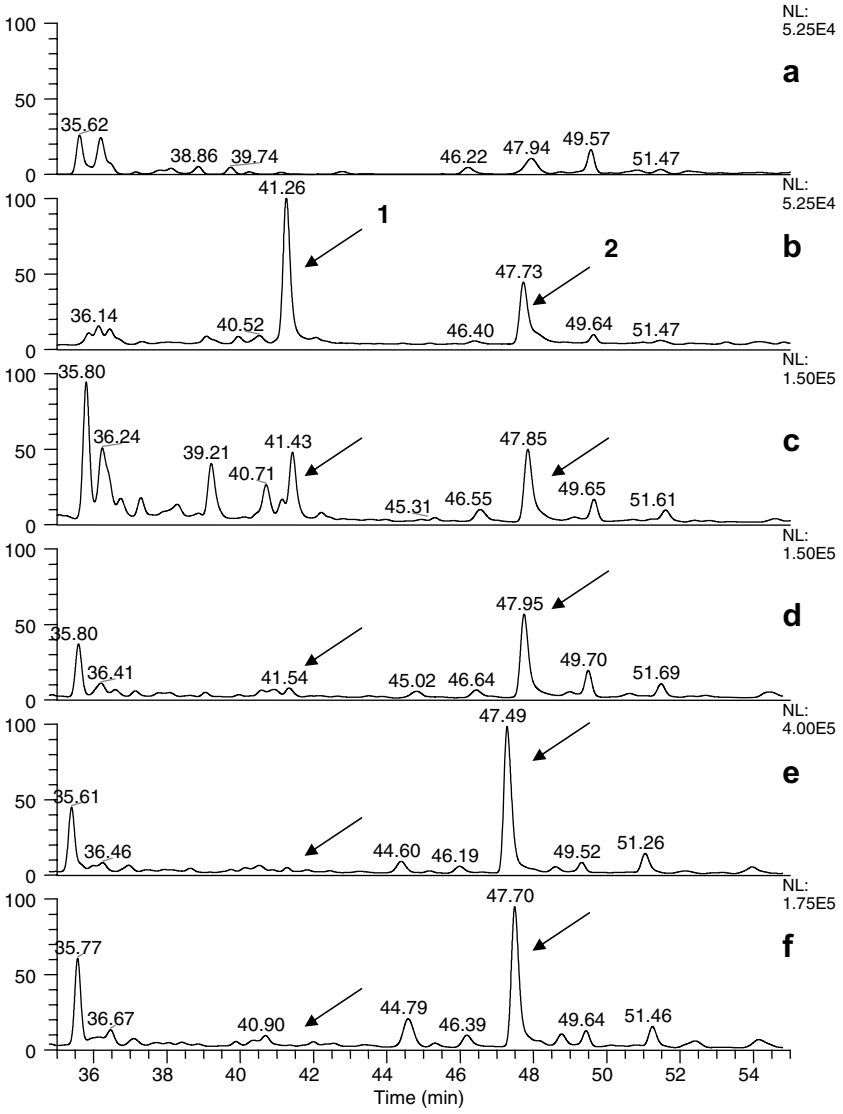

Fig. 6. HPLC traces of culture media from HeLa cells. (a) Control culture media at 2 h, (b) culture media at 2 h + 100 μg/ml raspberry LH20 unbound fraction, (c) culture media at 8 h + 100 μg/ml raspberry LH20 unbound fraction, (d) culture media at 24 h + 100 μg/ml raspberry LH20 unbound fraction, (e) culture media at 48 h + 100 μg/ml raspberry LH20 unbound fraction, (f) culture media at 72 h + 100 μg/ml raspberry LH20 unbound fraction. Each trace shows maximum detector response (FSD) at 280 nm. Arrows denote main Sanguiin H6 peak and ellagic acid, respectively. Traces are from one of three replicate experiments.

its nature, artificial and cannot be related to the normal physiological state. Animal studies suggest that only a very limited portion of ellagic acid is orally bioavailable (Teel and Martin, 1988) and human plasma concentrations of ellagic acid after consumption of pomegranate juice (Seeram et al., 2004b) or black raspberries (Stoner et al., 2005) were detectable but low. Therefore, ellagitannins or ellagic acid could not reach cervical cancer cells in the concentrations used in this study. However, urinary excretion of urolithin-type microbial degradation products of ellagic acid was judged to be ~14% of ellagitannin intake from raspberries (Cerda et al., 2005) which suggests that ellagitannins (and therefore ellagic acid) are retained in the gastrointestinal tract (GIT) and can be biotransformed by the colonic microflora. The retention of ellagitannins and ellagic acid within the GIT, in addition to the continuing release of bound ellagitannins during digestion of foods, may influence cancer cell proliferation in the epithelial cells of the GIT, from oesophagus to colon. Ellagic acid has been shown to accumulate in cultured Caco-2 colon cancer cells (Whitley et al., 2003) which may contribute to the reported protective effects of ellagitannin-rich berries on cancers of the aerodigestive tract (Han et al., 2005).

Many mechanisms whereby ellagic acid could influence cancer cell proliferation (Losso et al., 2004; Dorai and Aggarwal, 2004) have been proposed. However, from recent work, it seems most likely that ellagic acid released from dietary ellagitannins induces apoptosis through the intrinsic mitochondrial pathway (Larrosa et al., 2005).

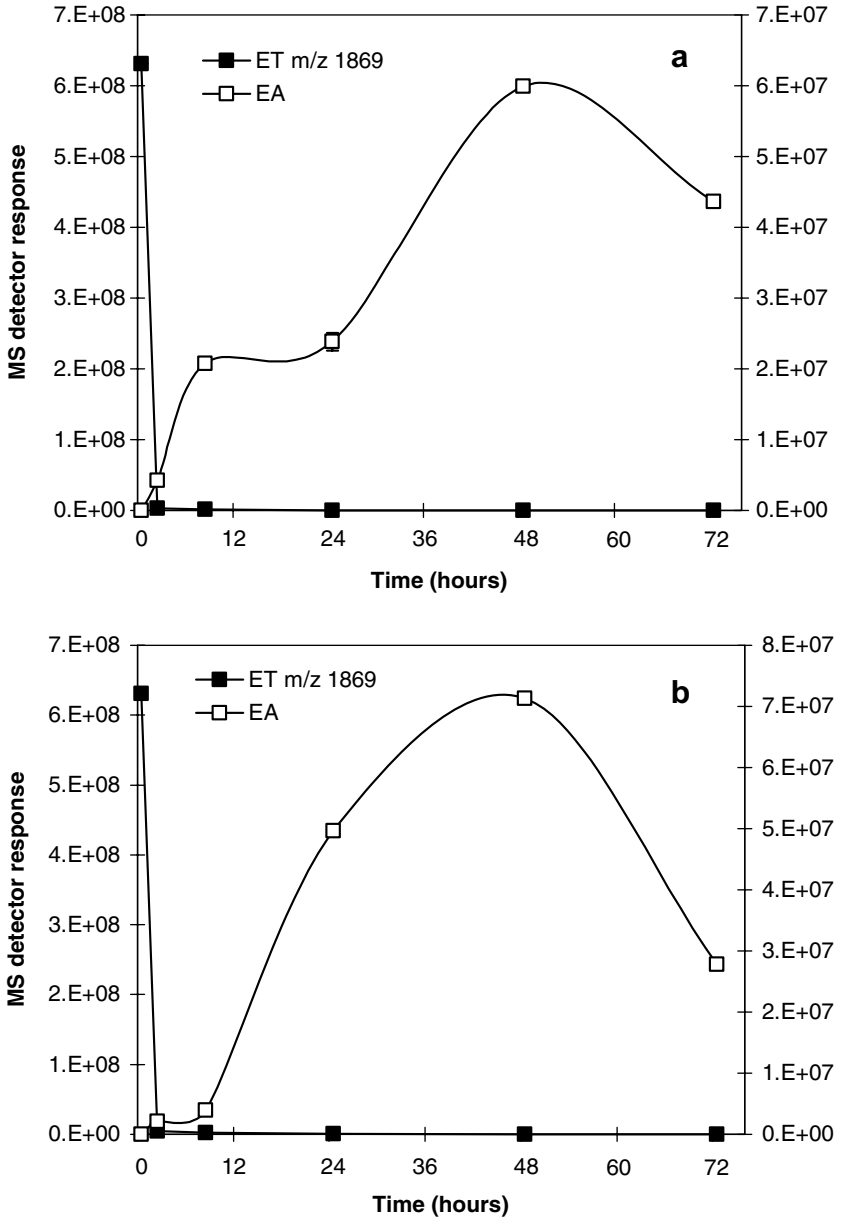

Fig. 7. Time courses of ellagic acid production and Sanguiin H6 stability in media plus HeLa cells (a) and without cells (b). Values are means of triplicate experiments ± standard error.

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References

Allen, C.T., Peden-Adams, M.M., EuDaly, J., Keil, D.E., 2003. Subchronic exposure to ellagic acid impairs cytotoxic T-cell function and suppresses humoral immunity in mice. Immunopharmacol. Immunotoxicol. 25, 409–422.

Anon., 2002. Joint WHO/FAO expert consultation on diet, nutrition and the prevention of chronic diseases, WHO Technical Report Series, No. 916, Geneva, Switzerland.

Beekwilder, J., Jonker, H., Meesters, P., Hall, R.D., van der Meer, I.M., de Vos, C.H.R., 2005. Antioxidants in raspberry: on-line analysis links antioxidant activity to a diversity of individual metabolites. J. Agric. Food Chem. 53, 3313–3320.

Carlton, P.S., Kresty, L.A., Siglin, J.C., Morse, M.A., Lu, J., Morgan, C., Stoner, G.D., 2001. Inhibition of *N*-nitrosomethylbenzylamine-induced tumorigenesis in the rat oesophagus by dietary freeze-dried strawberries. Carcinogenesis 22, 441–446.

Castonguay, A., Gali, H.U., Perchellet, E.M., Gao, X.M., Boukharta, M., Jalbert, G., Okuda, T., Yoshida, T., Hatano, T., Perchellet, J.P., 1997. Antitumorigenic and anti-promoting activities of ellagic acid, ellagitannins and oligomeric anthocyanin and procyanidin. Int. J. Oncol. 10, 367–373.

Cerda, B., Tomas-Barberan, F.A., Espin, J.C., 2005. Metabolism of antioxidant and chemopreventive ellagitannins from strawberries,

raspberries, walnuts, and oak-aged wine in humans: identification of biomarkers and individual variability. J. Agric. Food Chem. 53, 227–235.

Clifford, M.N., Scalbert, A., 2000. Ellagitannins – nature, occurrence and dietary burden. J. Sci. Food Agric. 80, 1118–1125.

Cooke, D., Steward, W.P., Gescher, A.J., Marczylo, T., 2005. Anthocyans from fruits and vegetables – does bright colour signal cancer chemopreventive activity? Eur. J. Cancer 41, 1931–1940.

Daniel, E.M., Krupnick, A.S., Heur, Y.-H., Blinzler, J.A., Nims, R.W., Stoner, G.D., 1989. Extraction, stability, and quantitation of ellagic acid in various fruits and nuts. J. Food Compos. Anal. 2, 338–349.

Daniel, E.M., Ratanayake, S., Kinstle, T., Stoner, G.D., 1991. The effects of pH and rat intestinal contents on the liberation of ellagic acid from purified and crude ellagitannins. J. Nat. Prod. 54, 946–952.

Deighton, N., Brennan, R., Finn, C., Davies, H.V., 2000. Antioxidant properties of domesticated and wild *Rubus* species. J. Sci. Food Agric. 80, 1307–1313.

Dorai, T., Aggarwal, B.B., 2004. Role of chemopreventive agents in cancer therapy. Cancer Lett. 215, 129–140.

Evan, G.I., Vousden, K.H., 2001. Proliferation, cell cycle and apoptosis in cancer. Nature 411, 342–348.

George, S., Brat, P., Alter, P., Amiot, M.J., 2005. Rapid determination of polyphenols and vitamin C in plant-derived products. J. Agric. Food Chem. 53, 1370–1373.

Halliwell, B., 1996. Antioxidants in human health and disease. Annu. Rev. Nutr. 16, 33–50.

Han, C.H., Ding, H.M., Casto, B., Stoner, G.D., D'Ambrosio, S.M., 2005. Inhibition of the growth of premalignant and malignant human oral cell lines by extracts and components of black raspberries. Nut. Cancer-Int. J. 51, 207–217.

Hertog, M.G.L., Bas Bueno-de-Mesquita, H., Fehily, A.M., Sweetnam, P.M., Elwood, P.C., Kromhout, D., 1996. Fruit and vegetable consumption and cancer mortality in the Caerphilly study. Cancer Epidemiol. Biomark. Prevent. 5, 673–677.

Juranic, Z., Zizak, Z., Tasic, S., Petrovic, S., Nidzovic, S., Leposavic, A., Stanojkovic, T., 2005. Antiproliferative action of water extracts of seeds or pulp of five different raspberry cultivars. Food Chem. 93, 39–45.

Kahkonen, M.P., Hopia, A.I., Heinonen, M., 2001. Berry phenolics and their antioxidant activity. J. Sci. Food Agric. 49, 4076–4082.

Katsube, N., Iwashita, K., Tsushida, T., Yamaki, K., Kobori, M., 2003. Induction of apoptosis in cancer cells by bilberry (*Vaccinium myrtillus*) and the anthocyanins. J. Agric. Food Chem. 51, 68–75.

Kris-Etherton, P.M., Hecker, K.D., Bonanome, A., Coval, S.M., Binkoski, A.E., Hilpert, K.F., Griel, A.E., Etherton, T.D., 2002. Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. Am. J. Med. 113, 71S–88S.

Larrosa, M., Tomas-Barberan, F.A., Espin, J.C., 2005. The dietary hydrolysable tannin punicalagin releases ellagic acid that induces apoptosis in human colon adenocarcinoma Caco-2 cells by using the mitochondrial pathway. J. Nutr. Biochem. 17, 611–625.

Liu, M., Li, X.Q., Weber, C., Lee, C.Y., Brown, J., Liu, R.H., 2002. Antioxidant and anti-proliferative activities of raspberries. J. Agric. Food Chem. 50, 2926–2930.

Loo, G., 2003. Redox-sensitive mechanisms of phytochemical-mediated inhibition of cancer cell proliferation. J. Nutr. Biochem. 14, 64–73.

Losso, J.N., Bansode, R.R., Trappey, A., Bawadi, H.A., Truax, R., 2004. In vitro anti-proliferative activities of ellagic acid. J. Nutr. Biochem. 15, 672–678.

Macheix, J.-J., Fleuriet, A., Billot, J., 1990. Fruit Phenolics. CRC Press, Boca Raton, FL.

Mates, J.M., Sanchez-Jimenez, F.M., 2000. Role of oxygen species in apoptosis: implications for cancer therapy. Int. J. Biochem. Cell Biol. 32, 157–170.

McDougall, G.J., Shpiro, F., Dobson, P., Smith, P., Blake, A., Stewart, D., 2005. Different polyphenolic components of soft fruits inhibit alpha-amylase and alpha-glucosidase. J. Agric. Food Chem. 53, 2760–2766.

Meyers, K.J., Watkins, C.B., Pritts, M.P., Liu, R.H., 2003. Antioxidant and antiproliferative activities of strawberries. J. Agric. Food Chem. 51, 6887–6892.

Mullen, W., McGinn, J., Lean, M.J., MacLean, M.R., Gardiner, P., Duthie, G.G., Yokota, T., Crozier, A., 2002. Ellagitannins, flavanoids, and other phenolics in red raspberries and their contribution to antioxidant capacity and vasorelaxation properties. J. Agric. Food Chem. 50, 5191–5196.

Mullen, W., Yokota, T., Lean, M.E., Crozier, A., 2003. Analysis of ellagitannins and conjugates of ellagic acid and quercetin in raspberry fruits by LC–MS^n. Phytochemistry 64, 617–624.

Olsson, M.E., Gustavsson, K.E., Andersson, S., Nilsson, A., Duan, R.D., 2004. Inhibition of cancer cell proliferation in vitro by fruit and berry extracts and correlations with antioxidant levels. J. Agric. Food Chem. 52 (24), 7264–7271.

Oszmianski, J., Ramos, T., Bourieix, M., 1988. Fractionation of phenolic compounds in red wine. Am. J. Enol. Vitic. 39, 259–262.

Ribereau-Gayon, P., Stonestreet, E., 1965. Determination of anthocyanins in red wine. Bull. Soc. Chim. Fr. 1965, 2649–2652.

Rommel, A., Wrolstad, R.E., 1993. Composition of flavanols in red raspberry juice as influenced by cultivar, processing and environmental factors. J. Agric. Food. Chem. 41, 1941–1950.

Schmidt, B.M., Erdman, J.W., Lila, M.A., 2005. Differential effects of blueberry proanthocyanidins on androgen sensitive and insensitive human prostate cancer cell lines. Cancer Lett. 231, 240–246.

Seeram, N.P., Adams, L.S., Hardy, M.L., Heber, D., 2004a. Total cranberry extract versus its phytochemical constituents: antiproliferative and synergistic effects against human tumour cell lines. J. Agric. Food Chem. 52, 2512–2517.

Seeram, N.P., Lee, R., Heber, D., 2004b. Bioavailability of ellagic acid in human plasma after consumption of ellagitannins from pomegranate (*Punica granatum* L.) juice. Clin. Chim. Acta 348, 63–68.

Seeram, N.P., Adams, L.S., Henning, S.M., Niu, Y.T., Zhang, Y.J., Nair, M.G., Heber, D., 2005. In vitro antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. J. Nutr. Biochem. 16, 360–367.

Shih, P.H., Yeh, C.T., Yen, G.C., 2005. Effects of anthocyanidins on the inhibition of proliferation and induction of apoptosis in human gastric adenocarcinoma cells. Food Chem. Toxicol. 43, 1557–1566.

Singleton, V.L., Rossi Jr., J.A., 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Viticult. 16, 144–158.

Smith, S.H., Tate, P.L., Huang, G., Magee, J.B., Meepagala, K.M., Wedge, D.E., Larcom, L.L., 2004. Antimutagenic activity of berry extracts. J. Med. Food 7, 450–455.

Steinmetz, K.A., Potter, J.D., 1996. Vegetables, fruit and cancer prevention: a review. J. Am. Diet. Assoc. 96, 1027–1039.

Stoner, G.D., Sardo, C., Apseloff, G., Mullet, D., Wargo, W., Pound, V., Singh, A., Sanders, J., Aziz, R., Casto, B., Sun, X.L., 2005. Pharmacokinetics of anthocyanins and ellagic acid in healthy volunteers fed freeze-dried black raspberries daily for 7 days. J. Clin. Pharmacol. 45, 1153–1164.

Strumeyer, D.H., Malin, M., 1975. Condensed tannins in grain sorghum: isolation, fractionation, and characterization. J. Agric. Food Chem. 23, 909–914.

Teel, R.W., Martin, R.M., 1988. Disposition of the plant phenol ellagic acid in the mouse following oral-administration by gavage. Xenobiotica 18, 397–405.

Whitley, A.C., Stoner, G.D., Darby, M.V., Walle, T., 2003. Intestinal epithelial cell accumulation of the cancer preventive polyphenol ellagic acid – extensive binding to protein and DNA. Biochem. Pharmacol. 66, 907–915.

Xue, H.W., Aziz, R.M., Sun, N.J., Cassidy, J.M., Kamendulis, L.M., Xu, Y., Stoner, G.D., Klaunig, J.E., 2000. Inhibition of cellular transformation by berry extracts. Carcinogenesis 13, 121–125.

Yi, W., Fischer, J., Krewer, G., Casimir, C., Akoh, Y., 2005. Phenolic compounds from blueberries can inhibit colon cancer cell proliferation and induce apoptosis. J. Agric. Food Chem. 53, 7320–7329.

Yoshizawa, Y., Kawaii, S., Urashima, M., Fukase, T., Sato, T., Tanaka, R., Murofushi, N., Nishimura, H., 2000. Antiproliferative effects of small fruit juices on several cancer cell lines. Anticancer Res. 20, 4285–4289.

Zhao, C., Giusti, M.M., Malik, M., Moyer, M.P., Magnuson, B.A., 2004. Effects of commercial anthocyanin-rich extracts on colonic cancer and non-tumorigenic colonic cell growth. J. Agric. Food Chem. 52, 6122–6128.