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Assessment of the Anti-Genotoxic, Anti-Proliferative, and Anti-Metastatic Potential of Crude Watercress Extract in Human Colon Cancer Cells

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Abstract: *Although it is known to be a rich source of the putative anti-cancer chemicals isothiocyanates, watercress has not been extensively studied for its cancer preventing properties. The aim of this study was to investigate the potential chemoprotective effects of crude watercress extract toward three important stages in the carcinogenic process, namely initiation, proliferation, and metastasis (invasion) using established in vitro models. HT29 cells were used to investigate the protective effects of the extract on DNA damage and the cell cycle. The extract was not genotoxic but inhibited DNA damage induced by two of the three genotoxins used, namely hydrogen peroxide and fecal water, indicating the potential to inhibit initiation. It also caused an accumulation of cells in the S phase of the cell cycle indicating (possible) cell cycle delay at this stage. The extract was shown to significantly inhibit invasion of HT115 cells through matrigel. Component analysis was also carried out in an attempt to determine the major phytochemicals present in both watercress leaves and the crude extract. In conclusion, the watercress extract proved to be significantly protective against the three stages of the carcinogenesis process investigated.*

Introduction

Epidemiological studies link vegetable consumption with a reduced risk of colorectal cancer (CRC), with evidence being particularly strong for *Brassica* and related *Capparales* vegetables (1–2). Extensive investigations in animal models, human trials, and cell culture systems support these epidemiological findings and provide insights into the mechanisms involved. Potential phytochemical components of *Brassicaceae* (originally called the *Cruciferae*) that are responsible for the anti-cancer activity have also been studied. The main focus of these studies has been glucosinolates (GLS), an important group of sulfur-containing, water-soluble phytochemicals that are found in the *Brassicaceae* (including watercress and broccoli) and the related *Capparales* families. When the vegetables are cut, crushed, or masticated, myrosinases (thioglucosidases) come into contact with the vacuolar-stored glucosinolates and hydrolyzes them to an unstable intermediate, glucose and sulfate. These unstable intermediates can then be converted into a variety of products dependent on the R group, pH, metal ions, and epithiospecifier protein (ESP). Common hydrolysis products include isothiocyanates (ITCs), such as phenethyl isothiocyanate (PEITC), sulforaphane (SFN), and indoles such as indole 3-carbinol (I3C) that can react with endogenous ascorbic acid to form ascorbigen or dimerize to form 3,3′-di-indolylmethane (DIM) (3). Watercress is a rich source of PEITC, which is generated by the hydrolysis of the GLS gluconasturtiin (4). Watercress also contains lower levels of two long-chain GLS (7-methylsulfinylheptyl and 8-methylsulfinyl) that are hydrolyzed to ITCs with potential anti-cancer effects. Depending on the cultivar, trace amounts of the structurally-related 7-methylthioheptyl- and 8-methylthiooctyl GLS can also be present (5). The protective effect of PEITC against cancer has been attributed to its ability to modulate the activity of phase I and phase II enzymes that are responsible for the bio-activation and detoxification of carcinogens. In addition to this mechanism, PEITC, along with other ITCs, have been demonstrated to induce apoptosis and cell cycle arrest, and inhibit proliferation of cancer cells both in vitro and in vivo (6–9). Finally, both PEITC and 8-methylsulfinyloctyl have been demonstrated to result in a dose-dependent decrease in lipopolysaccharide-induced nitrate and prostaglandin E2 synthesis, overproduction of which has been associated with cancer development (10).

During this study in vitro model systems, biologically relevant to colorectal cancer, were chosen, together with appropriate human colon cell lines. The induction of DNA damage and mutations, brought about by a range of environmental (dietary) genotoxins, are key early stages in the initiation of colon carcinogenesis. To investigate the protective effects of

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watercress against initiation, the single cell gel electrophoresis assay (SCGE) or Comet assay was used. This assay has previously been used to evaluate anti-carcinogenic effects of a wide range of dietary components including probiotics and phytochemicals, both in vivo and in vitro (11–12). To test the effect of the extract against a range of environmental genotoxins, H_2O_2, 4-Hydroxy Nonenal (4-HNE) and fecal water were used in the assay. H_2O_2 is produced by numerous enzymes in the body and is a source of endogenous oxidative stress, resulting in oxidative damage to DNA in the form of strand breaks and oxidized bases (13). Therefore, this agent was used during this assay to initiate oxidative DNA damage. 4-HNE is an endogenously formed product of lipid peroxidation and has therefore been suggested to contribute significantly to the cytopathological effects observed in biological systems exposed to oxidizing agents capable of causing lipid peroxidation (14). It is considered to be a putative cancer-causing agent after its epoxidation (15) due to its ability to cause DNA damage. This effect has been observed in colonic cell lines, including HT29 (16–18) and was therefore used as a damaging agent during this study.

Fecal water was chosen as the third genotoxic agent as it is likely that the agents involved in the etiology of colon cancer are associated with the aqueous phase of the fecal stream in the gut. This aqueous phase, or fecal water, has been shown to contain biologically active substances that are cytotoxic to mammalian cells. As this may lead to mutations in critical genes and therefore the initiation and development of CRC, the use of fecal water in conjunction with the Comet assay and human colon cell lines proves a highly relevant in vitro model to investigate dietary components for potential anti-cancer activity in the colon (19). The human colon adenocarcinoma cell line HT29 has previously been demonstrated a useful cell line for the detection of genotoxic activities of different classes of genotoxic carcinogens (5,16,20–22); therefore, this cell line was chosen for use in this assay with the response to the aforementioned carcinogens observed.

The effects of watercress on proliferation were investigated by examining its effects on the cell cycle as ITCs have been demonstrated to induce apoptosis and cell-cycle arrest of highly proliferating cancer cells (23–24). Recently it has been found that PEITC [in watercress and salad *Barbarea* species (*B. praecox* and *B. verna*)] and SFN (present in many *Brassica* species including broccoli and cabbage) can induce cell cycle arrest at the G2/M phase in HT29 cells (23,25). Therefore, the effect of the extract on the cell cycle of HT29 cells was also investigated during this study.

The final stage of the carcinogenesis pathway to be investigated was metastasis. Effects on invasion, a key stage in metastasis, were assessed by studying attachment and invasion of HT115 human colorectal adenocarcinoma cells through matrigel (26). In addition to this cell line, MRC5 human lung fetal fibroblasts were used as a chemoattractant for the invasion of HT115 cells to occur (11,27). These specific cell lines were chosen due to the ability of MRC5 cells to stimulate the invasion of HT115 cells, a well known, highly invasive cell line. This is due to MRC5 cells producing large quantities of bioactive Hepatocyte Growth Factor (HGF)/Scatter Factor (SF). HT115 cells have been demonstrated to express the receptor mRNA and protein for HGF/SF (26), and therefore, using these two cell lines together in this assay encourages tumor cell invasion to occur, an important property for matastatic spread. This assay has been used to assess anti-invasive effects of a number of phytochemicals including isoflavonoids (28). Although there are studies examining the effects of a range of vegetable extracts and specific ITCs, few have examined the preventive effects of watercress.

The watercress extract, in addition to the leaves from which the extract was prepared, were analysed by high-performance liquid chromatography electro-spray mass spectrometry (LC/MS) in both positive and negative ion modes to determine the types and amounts of GLS and phenolic components present.

The aim of this study, therefore, was to examine the effects of crude juice extracted from watercress in these in vitro model systems biologically relevant to colorectal cancer.

Materials and Methods

Chemicals

4-HNE was obtained from Alexis Biochemical's (Bingham, Nottingham, UK) and was prepared in ethanol. All glucosinolate standards had been previously purified and flavonoid standards were either obtained from Extrasynthese (Genay, France) or had been previously purified from broccoli [quercetin-3-*O*-sophoroside and various hydroxycinnamic acid (HCA) gentiobiose derivatives] and lettuce [quercetin-3-*O*-(6″-malonyl-glucoside)]. All other chemicals were obtained from Sigma-Aldrich (Poole, Dorset, UK), and all solvents were of high performance liquid chromatography (HPLC) grade.

Tissue Culture

HT29, HT115, and MRC5 cells were obtained from the European Collection of Animal Cell Cultures (ECACC; Salisbury, UK). Dulbecco's Modified Eagle's Medium (DMEM) and Minimal Essential Medium (MEM) were obtained from Sigma-Aldrich. HT29 and HT115 cells were cultured in Roux flasks as monolayers in DMEM containing 10% and 15% fetal bovine serum (FBS), respectively, 2 mM glutamine and 100 units/liter.

Penicillin/Streptomycin

MRC5 cells were cultured in Roux flasks as monolayers in MEM containing 10% FBS, 2 mM glutamine, 100 units/liter penicillin/streptomycin, and 1% nonessential amino acids (NEAA). Cells were cultured for 7 days (<75% confluence) at 37°C with 5% CO_2 and 95% filtered air. The medium was changed every 2 days. Cells were washed with phosphate buffered saline (PBS) for 2 min and resuspended by the addi-

tion of trypsin (0.25% trypsin-EDTA) at 37°C for 5 min. Cells were centrifuged at 258 *g* for 3 min and cells resuspended in the appropriate medium.

Preparation of Watercress Extract

This method was modified from that previously reported by Kassie et al. (29). Fresh watercress (5 × 85 g) was bought from local supermarkets, chopped into pieces, and juices homogenized in a Cookworks juice maker machine for 10 min at 4°C; subsequently the juices were centrifuged (9,000 *g*, 10 min, 4°C), the supernatant decanted, filter sterilized (45 μm and then 22 μm), and aliquoted into sterile 1.5 ml tubes (1 ml/tube). This one batch preparation was used for all experiments and stored at –70°C until required.

Component Analysis

Watercress leaves were processed using methods previously detailed by Mellon et al. (30) using LC/MS. Samples were freeze-dried and milled to a fine powder prior to extraction. All samples were analyzed in triplicate, using ion-pair LC with photo-diode array detection (200–600 nm overall) and also ion-pair LC/electrospray Ionization (ESI) MS in positive and negative ion modes (to further confirm identities). The LC gradient for glucosinolate and phenolic analysis is a multipurpose chromatographic method that simultaneously separates glucosinolates and phenolics. Triplicate 40 mg samples were extracted in 1 ml 70% methanol at 70°C for 20 min before being processed by the method previously detailed, using sinigrin and quercitrin (quercetin-3-O-rhamnoside; Extrasynthese, Genay, France) as the extraction standard (30). An injection volume of 20 μl was used. Glucosinolate and phenolic analyses were performed using the negative ion electrospray ramped cone voltage method and also in positive ion mode with a fixed cone voltage . Hydrolysis product analyses were performed using the positive ion LC/MS system. The watercress extract was analyzed using both positive and negative ion LC/MS for residual GLS. For analysis of homogenized watercress in both modes, a 0.1% Trifluoroacetic Acid (TFA)/water versus 0.1% TFA/Methanol (MeOH) was used. The replacing of Acetonitrile (MeCN) with MeOH for the homogenate analyses was necessary because MeCN causes ion suppression and nitriles cannot then be detected by MS in the presence of MeCN. In addition, pure standards of PEITC, 2-phenylpropionitrile, I3C, DIM, ascorbigen, and indole-3-acetonitrile were used for identification and retention time determinations and also for calibration curves for detection limit determinations. Data was collected at 227 nm (glucosinolates and most UV-absorbing compounds) and 270 nm (phenolic compounds). No anthocyanins were present in these samples.

Preparation of Fecal Water

A fecal sample from a healthy volunteer was kept at 4°C for up to 2 h prior to processing. Fecal water was prepared according to the method of Venturi et al. (19). The fecal sample was homogenized in a stomacher for 2 min before adding in ice cold PBS (1:1 ratio). The sample was then centrifuged at 38,000 *g* for 2 h. Samples were then filtered and frozen at –80°C until required.

Single Cell Gel Electrophoresis (Comet) Assay

This assay was carried out following the method of Gill et al. (11). For use in the Comet assay, the well-established HT29 cell model was used. The cells were incubated in watercress extract (0–50 μl/ml) for 24 h before being harvested for use in the Comet analysis. Fresh medium was added and cell viability measured using trypan blue to provide a final viable cell concentration of 1×10^5cell/ml. Suspended cells (500 μl) were then challenged with one of three genotoxic treatments (50 μl): 250 μM of 4HNE, 75 μM of H_2O_2, or fecal water. 4HNE suspensions were incubated for 30 min in a shaking water bath at 37°C, however, those containing H_2O_2 and fecal water were incubated for 5 min at 4°C. Solvent controls of watercress pretreated and nontreated HT29 cells were included to assess any genotoxic effects of the watercress extract alone. Positive (75 μM H_2O_2) and negative controls (PBS) were included in all experiments (HT29 cells that had not been exposed to the extract). Genotoxic agents were removed via centrifugation (258 *g* for 5 min). Cell pellets were mixed with 0.85% low melting point agarose (Sigma-Aldrich), and 450 μl of the cell suspensions were distributed onto 3 microscope slides precoated with 100 μl of 1% normal melting agarose. Slides were precoated by the method of Rieger et al. (31).

After the agarose solidified, slides were immersed in a lysis buffer (2.5 M NaCl, 0.1 M EDTA, 0.01 M Tris, 1% Triton X100) for at least 60 min. All slides were placed in an electrophoresis chamber containing alkaline solution (0.2 M EDTA, 10 M NaOH) for DNA unwinding. After 20 min the current was switched on and electrophoresis was carried out at 25 V, 300 mA for 20 min. The slides were removed from the alkali solution and washed three times for 5 min with neutralizing buffer (0.4 M Tris, pH 7.5). Slides were stained with ethidium bromide (Sigma-Aldrich; 20 μg/ml, 20 μl/slide).

Images were analyzed at ×400 magnifications using a Nikon eclipse 600 epi-fluorescence microscope. The % Tail DNA was recorded using Komet 3.0 image analysis software (Kinetic Imaging Ltd, Liverpool, UK). For each slide, 100 cells were scored. Positive (H_2O_2 = 75 μM) and negative (PBS) controls were included for all experiments. The mean was calculated from 100 cells/gel (each sample in triplicate), and the experiment repeated independently three times. The mean of each set of data was used in the statistical analysis. Differences between means were evaluated by analysis of variance (ANOVA), post-hoc LSD ($P < 0.05$).

Determination of Cell Cycle Progression

Cell cycle progression was measured using the method documented by Ortega (32). HT29 cells were grown until

70% confluency in 25 cm^2 Roux flasks at a density of 1 × 10^5cells/ml. Watercress extract (0–50 µl/ml) was added to the cells and incubated at 37°C and 5% CO_2 for 24 h prior to the assay. Monolayers were washed to remove watercress extract and were suspended by the addition of 1 ml 0.25% trypsin-EDTA and incubated at 37°C for 5 min. Cells were then transferred to polypropylene tubes (Becton Dickinson, Cowley, Oxford, UK) and centrifuged at 258 *g* for 3 min. The supernatant was removed and the pellet resuspended in 200 ml of ice cold PBS and 2 ml of 70% ethanol/30%PBS, incubated on ice for 30 min, and centrifuged at 258*g* for 3 min. The supernatant was discarded, and the cells were resuspended in 800 ml of ice cold PBS, 100 ml of RNase A (1 mg/ml), and 100 ml of propidium iodide (400 mg/ml). The cells were then incubated at 37°C for 30 min before the emission spectra was measured at 585 nm on a FACSCalibur flow cytometer (Becton Dickinson). Subsequently these emission spectra were analysed for DNA content using WinMDI software (J. Trotter, Scripps Inst.). Each treatment was run in duplicate and the complete data set is the mean of four independent experiments. The complete dataset was analysed using ANOVA performed in SPSS for Windows, version 12.0, using Games-Howell post-hoc tests ($P = 0.05$).

Determination of Effects on Invasive Potential

The assay was modified from the method of Gill et al. (11). MRC5 cells were used to enhance invasion rates of the HT115 cells through secretion of hepatocyte growth factor, which is a strong chemo-attractant and has been shown to increase cell scattering and motility in HT115 cells (33). MRC5 cells were seeded at a concentration of 2 × 10^5 cells/ml in DMEM, supplemented with 15% FBS, onto 6-well plates, as a chemo attractant for 2 h. HT115 cells were seeded onto inserts coated with matrigel; at a concentration of 1 × 10^5/ml per well of the assay plates before the watercress extract (0–50 µl/ml) was added. The plates were then incubated at 37°C for 24 h before carrying out the assay. The experiment was repeated three times and all data were statistically analysed using Mann-Whitney U test.

Results

Analysis of watercress leaves collected from 8 bags was carried out to determine average levels of GLS and phenolic compounds present. The data showed that high levels of PEITC existed within the leaves. A number of other GLS were also present at lower levels (Table 1). From the data looking at the phenolic components present in watercress leaves (Table 1), it can be seen that there are various quercetin glycosides present including rutin, in addition to a large variety of HCA derivatives. Figure 1 shows example data of the major phytochemicals in leaves of watercress used in this study at 227 nm (Fig. 1A) and 270 nm (Fig. 1B).

Figure 2 shows example data at 227 nm (Fig. 2A) and 270 nm (Fig. 2B) produced on analysis of the crude extract of watercress. No GLS were detected in the homogenized leaf extract. A number of low molecular weight, unidentified phenolics were present, in addition to a complex mix of phenolics/flavonoids. 4-Methoxyascorbigen was also identified. LC-ES+MS analysis (Tables 2 and 3) demonstrated that neither PEITC nor 2-Phenylpropionitrile were detected in the extract. There were trace amounts of 7-methylsulfinylheptyl ITC and also the indole glucosinolate derived ascorbigen and 4-methoxy-ascorbigen (derived from the corresponding glucosinolates.

At 270 nm (Fig. 2B), a complex mixture of phenolic compounds was observed. Table 4 shows MS data and the provisional identities of the peaks observed. It can be seen that a number of phenolic derivatives were identified. Various quercetin glycosides, including rutin, were present in the watercress leaf extract, in addition to a number of caffeoyl, sinapoyl, feruloyl, and *p*-coumaroyl derivatives.

The effects of a range of watercress extract concentrations (0, 5, 10, and 50 µl/ml) on DNA (single strand breaks) damage in HT29 cells are shown in Fig. 3. The watercress extract itself was not genotoxic at any of the concentrations tested. When the cells were challenged with genotoxic agents a significant reduction in DNA damage was observed in cells pretreated with 50 µl/ml extract compared with controls, against both the H_2O_2 and fecal water challenge ($P = 0.011$ and $P = 0.039$, respectively). The reduction in 4HNE-in-

Table 1. Average Levels of Glucosinolates/Phenolics Present in Watercress Leaves Over 8 Wk Harvesting Period[a]

	Dry Weight (µmoles/g)	Fresh Weight (µmoles/g)
Glucosinolates (GLS)		
7-Methylsulfinylheptyl-GLS	1.067 ± 0.025	0.091 ± 0.002
8-Methylsulfinylheptyl-GLS	0.682 ± 0.148	0.058 ± 0.013
3-Indolylmethyl-GLS	0.430 ± 0.260	0.037 ± 0.074
2-Phenylethyl-GLS	17.977 ± 4.319	1.528 ± 1.410
4-Methoxy-3-Indolylmethyl-GLS	0.792 ± 0.051	0.065 ± 0.017
Flavonoids and Phenolics		
Quercetin-3-*O*-Sophoroside, 7-*O*-Rhamnside	1.006 ± 0.223	0.088 ± 0.021
Quercetin -3-*O*-Glc-Glc-Malonyl	1.430 ± 0.295	0.125 ± 0.029
Quercetin -3-*O*-Sophoroside	0.585 ± 0.051	0.050 ± 0.005
Quercetin -3-*O*-Rutinoside (Rutin)	0.602 ± 0.017	0.052 ± 0.004
Total Hydroxycinnamic Acid Derivatives	109.026 ± 4.223	9.402 ± 0.833

a: Figures shown are a mean of 3 independent experiments with ± SEM.

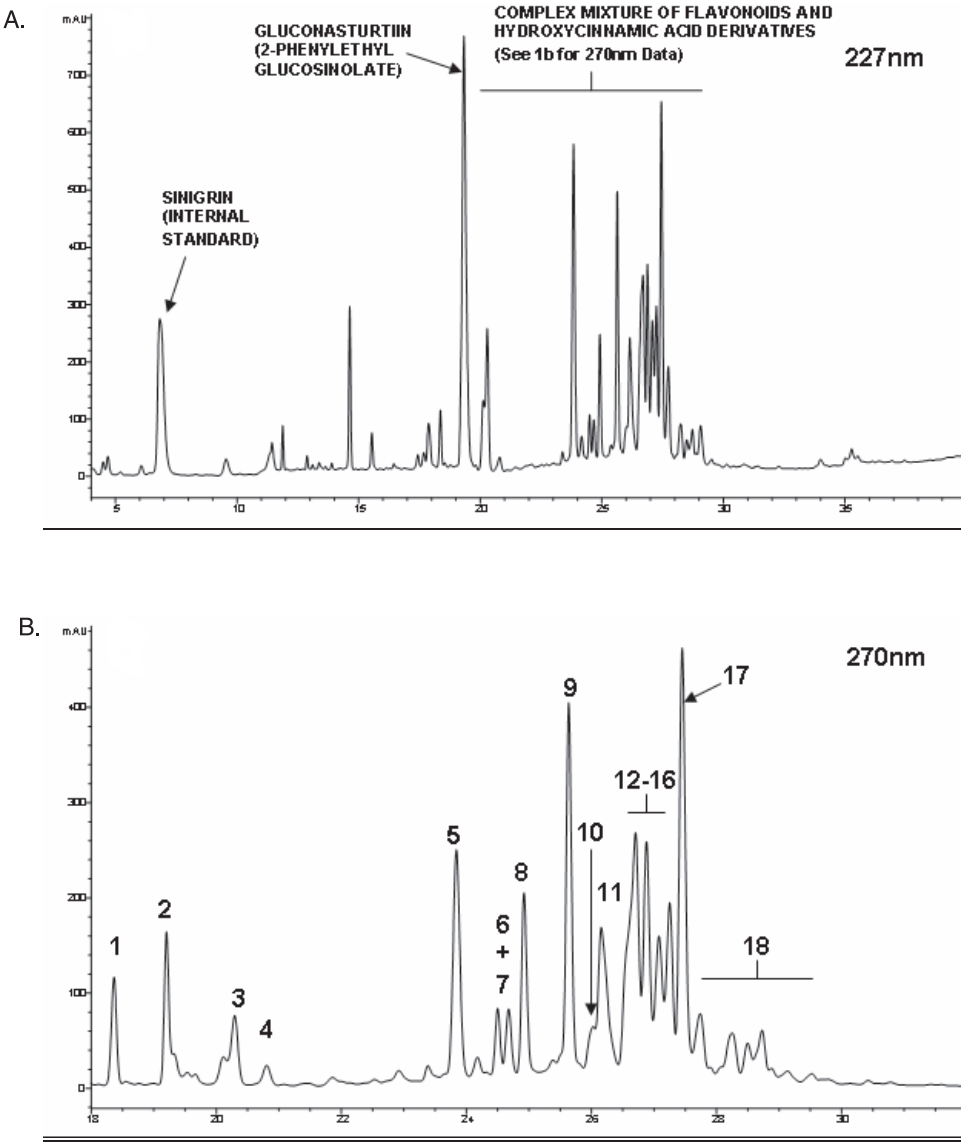

Figure 1. Liquid chromatography electro-spray mass spectrometry data for watercress extract (20 µl) measured at A: 227 nm and B: detail of the phenolic region at 270nm [0.1% trifluoroacetic acid (TFA)/water vs. 0.1% TFA/MeCN gradient was used]. Peak ID: 1 = Q-3-O-Soph, 7-0-Rha; 2 = Q-3-O-Glc-(6″-Malonyl-Glc); 3 = unidentified phenolic, 4 = 4-MeO-3-Indolylmethyl-GLS; 5 = Caffeoyl-derivative; 6 = Q-3-O-Soph; 7 = Q-3-O-Rutinoside; 8 = hydroxycinnamic acid (HCA)-derivative; 9 = HCA-derivative; 10 and 11 = Sinapoyl-derivative isomer 1; 12, 13, 14, and 15 = HCA-derivatives; 16 and 17 = Feruloyl-derivatives; and 18 = unidentified minor phenolics.

duced damage, however, was not statistically significant. The extract proved most protective against the oxidative damaging agent, with the 50 µl/ml concentration providing a 28% reduction in DNA damage. This concentration also caused a 19% reduction in DNA damage against the fecal water challenge.

The data shown in Fig. 4 demonstrate that 20 µl/ml and 50 µl/ml watercress extract significantly altered the progression of the normal cell cycle of HT29 cells ($P = 0.013$ and $P = 0.002$, respectively). It shows that an accumulation of the cells occurred in the S phase and may therefore suggest that cell cycle delay was occurring at this phase of the cell cycle.

The effect of a range of watercress extract concentrations on HT115 cell invasion through matrigel is shown in Fig. 5. At all concentrations used, the addition of the watercress extract significantly inhibited HT115 invasion levels as compared to control values ($P = 0.002$, 0.001, <0.001, and <0.001, respectively). An estimate of cell numbers showed no significant decrease in total cell count (attached cells) for any of the concentrations used.

Discussion

In this study we have shown that a crude extract of watercress had effects on a range of in vitro bioassays designed to model different stages in colon carcinogenesis, namely 1) initiation, the events leading to DNA damage; 2) proliferation, the continued uncontrolled growth of transformed cells; 3) metastasis, the invasion of the cells into surrounding tis-

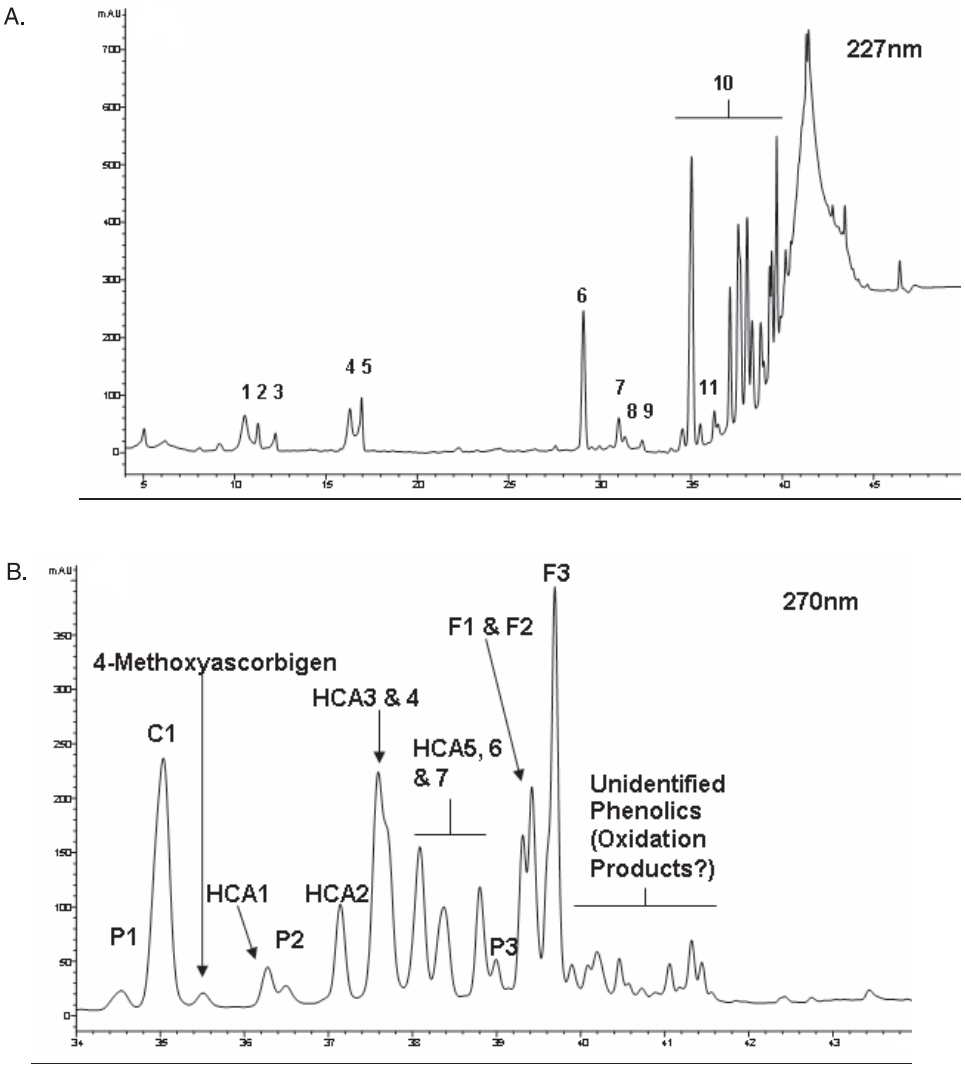

Figure 2. Example liquid chromatography data for watercress extract (60 µl) measured at A: 227nm and B: phenolic region at 270nm [0.1% trifluoroacetic acid (TFA)/water vs. 0.1% TFA/MeOH gradient was used]. Peak ID: P1 = unknown; C1 = Caffeoyl-derivative; HCA1 and P2 = *p*-Coumaroyl-derivatives; HCA2 = ferulic acid, HCA3 and HCA4 = *p*-Coumaroyl-derivatives; HCA5 = Feruloyl/Sinapoyl-derivative; HCA6=Feruloyl-derivative; HCA7 = 1,2-Di-Sinapoyl-Glc-Glc; P3 = unknown; F1 = Quercetin 3-O-Rutinoside (Rutin); F2 = Quercetin 3-O-Glc (Isoquercitrin); F3 = Quercetin 3-O-(6′-Malonyl-Glc).

sues. Using these in vitro models we have determined that the crude extract of watercress had potential beneficial effects on carcinogenesis.

In this study, a significant linear trend was evident for anti-genotoxicity with increasing watercress concentrations against a H_2O_2 and fecal water challenge in HT29 human colon cells. Because the watercress extract was removed from the cells before exposure to the genotoxic challenge, the reduction (approx 27% and 18% respectively) in induced DNA damage indicates an increased cellular capacity to protect against damage (Fig. 3). As such our findings appear to be consistent with the effects reported by other authors who have shown anti-genotoxic activity for cruciferous vegetables and various ITCs found within them in a variety of in vitro cell models (4,34). For example, an extract of cooked Brussels sprouts (10 µg/ml) inhibited H_2O_2-induced DNA strand breaks in human lymphocytes (35). A previous study of the effect of watercress extract on benzo (a) pyrene (B (a) P)-induced DNA damage in murine hepa1c1c7 cells, reported an inhibition of 20–38% after 24 h of preincubation, again at the 10 µg/ml concentration (36). However, although a protective effect was observed with the watercress extract, in response to B (a) P in the study by Kassie et al. (4), PEITC itself was found to be genotoxic. These results suggest that the anti-genotoxic effect demonstrated during the study were not due to the presence of PEITC. In agreement with these findings, the anti-genotoxic effects observed during the present study could not be attributed to PEITC content, since PEITC was not detected in the extract of the watercress (Table 3).

Previous studies investigating cruciferous vegetable constituents have identified a wide range of hydrolysis products, (4,5,37–40). From the data in Table 1, in addition to the findings from these previous studies, it was thought that a number of potential components would be observed following analysis of the watercress extract. However, a large variation

Table 2. LC-ES+MS Analyses of Watercress Extract (270 nm), 18–32 mins[a]

Phenolic	$[M+K]^+$	$[M+Na]^+$	$[M+H]^+$	Key Diagnostic Fragment Ions	Peak ID
1	811	795	773	627, 611, 465, 449, 303	Quercetin-3-*O*-Sophoroside, 7-O-Rhamnside
2	751	735	713	303	Quercetin-3-*O*-Glc- (6″-Malonyl-Glc)
3	—	—	—	MS data unclear	Unidentified phenolic
4	—	—	—	—	4-MeO-3-Indolylmethyl-GLS (ES data)
5	—	—	—	MS data unclear	Caffeoyl-derivative (from UV visible spectra)
6	ND	649	627	303	Quercetin-3-*O*-Sophoroside
7	ND	633	611	465, 303	Quercetin-3-*O*-Rutinoside
8	ND	811	789	671	HCA-derivative (from UV visible spectra)
9	761	745	723	MS Data Unclear	HCA-derivative (From UV visible spectra)
10	775	759	737	369	Sinapoyl-derivative Isomer 1
11	775	759	737	369	Sinapoyl-derivative Isomer 2
12	ND	ND	803	MS data unclear	HCA-derivative (from UV visible spectra)
13	ND	ND	803	MS data unclear	HCA-derivative (from UV visible spectra)
14	893	877	855	MS data unclear	HCA-derivative
15	957	941	919	MS data unclear	HCA-derivative
16	907	891	869	177	Feruloyl-derivative
17	ND	911	889	339, 177	Feruloyl-derivative
18	—	—	—	—	Unidentified minor phenolics

a: Abbreviations are as follows: LC, liquid chromatography; ES, electrospray; MS, mass spectrophotometry; GLS, glucosinolates;—, no molecular ion or fragment ions could be identified from MS data; UV, ultraviolet; ND, not detected.

Table 3. LC-ES+MS Analyses of Watercress Extract (227 nm)[a]

Hydrolysis Products and Endogenous Conjugates	[M+H]+	Detected in Extract
PEITC	164	ND
PEITC-Glutathione	471	ND
2-Phenylpropionitrile	132	ND
7-Methylsulfinylheptyl ITC	220	Trace (41.7 min)
7-Methylsulfinylheptyl ITC-Glutathione	527	ND
8-Methylsulfinyloctyl ITC	234	ND
8-Methylsulfinyloctyl ITC-Glutathione	541	ND
3,3′-Di-indolylmethane (DIM)	247	ND
Ascorbigen (Indole-3-carbinol-ascorbic acid adduct)	306	Trace (31.4 min)
4-Methoxy-indole-3-carbinol	178	ND
4,4′-Mono-methoxy-(3,3′-Di-indolylmethane)	277	ND
4,4′-Di-methoxy-(3,3′-Di-indolylmethane)	307	ND
4-Methoxyascorbigen	336	Trace (35.5 min)
Indole-3-acetonitrile	157	ND
4-Methoxy-indole-3-acetonitrile	187	ND

a: Abbreviations are as follows: LC, liquid chromatography; ES, electrospray; MS, mass spectrophotometry; ND, not detected by LC/MS; Trace = detected by LC/MS but not quantifiable.

existed between the levels of the specific phytochemicals observed in the crude extract of watercress and the leaves, with regard to the GLS profile, as determined also by LC-ES+MS (Tables 1 and 3). These results are consistent with those observed by Rose et al. (5) who identified only the nonvolatile ITCs, derived from 7-methylsulfinylheptyl and 8-methylsulfinyloctyl GLS, in watercress extract. This loss of the highly volatile PEITC during the production of the extracts could be prevented or minimized via dichloromethane extraction followed by gas chromatography (GC)-MS as a better method for determining ITC formation from fresh extracts with analysis occurring immediately upon production of the extract.

A range of phenolic components, such as quercetin glycosides, were detected in the extract (Tables 2 and 4, Fig. 1 and Fig. 2). Quercetin is the main flavonoid found in the average Western diet and is present in many plant-derived foods. It has also been demonstrated to modulate directly H_2O_2-induced DNA damage in human lymphocytes in vitro (41). In addition to the quercetin glycosides, a number of HCA derivatives were also detected in the watercress extract (Table 4). In a recent study carried out to investigate whether the HCAs that occur in plant cell walls act as antioxidants, protect against DNA breakage in mammalian cells, and/or modulate the activity of enzymes relevant to the carcinogenic process, it was observed that ferulic acid and *p*-coumaric acid were shown to have high activity as free radical scavengers. Protection against DNA and chromosome breakage as well as modulation of enzyme activity associated with carcinogenesis was also observed for both compounds (42).

Table 4. LC-ES+MS Analyses of Watercress Extract (270 nm), 3444 mins[a]

Peak Number	Major Fragment Ions	Potential ID
P1	ND	Unknown
C1	163	Caffeoyl-Derivative (MS and spectra)
HCA1	ND	*p*-Coumaroyl-Derivative (spectra)
P2	165	*p*-Coumaroyl-Derivative (MS and spectra)
HCA2	177	Free Ferulic Acid (MS, spectra, and standard)
HCA3	ND	*p*-Coumaroyl-Derivative (spectra)
HCA4	ND	*p*-Coumaroyl-Derivative (spectra)
HCA5	207, 177	Feruloyl/Sinapoyl-Derivative (MS and spectra)
HCA6	177	Feruloyl-Derivative (MS and UV)
HCA7	777, 755, 369	1,2-Di-Sinapoyl-Gentiobioside (MS and spectra)
P3	ND	Unknown
F1	633, 611, 465, 303	Quercetin 3-*O*-Rutinoside Rhamnoglucoside (Rutin) (MS, spectra, and standard)
F2	487, 465, 303	Quercetin 3-*O*-Glucoside (Isoquercitrin) (MS, spectra, and standard)
F3	551, 303	Quercetin 3-*O*-(6"-Malonyl-Glc) (MS, spectra, and standard)

a: Abbreviations are as follows: LC, liquid chromatography; ES, electrospray; MS, mass spectrophotometry; HCA, hydroxycinnamic acid; UV, ultraviolet; ND: No characteristic ions detected either due to weak or too complex MS spectra.

In this study, the crude watercress extract caused an accumulation of HT29 cells during the S phase of the cell cycle, suggesting that cell cycle delay is occurring during this stage (Fig. 4). Most of the studies carried out in this area examine the effect of individual ITCs found in cruciferous vegetables on the progression of the cell cycle and apoptosis of transformed cells. SFN has previously been demonstrated to significantly reduce cell proliferation of the HT29 colon cancer cell line at concentrations of 0.015 mmol (23) and 0.02 mmol or above (43). The activation of cyclin D1, which acts at the mid portion of G1/S phase and is observed in most cancer types, results in phosphorylation of Rb protein and releases E2F transcription factors, which facilitates the transition and onset of DNA synthesis in the S phase (32). Therefore, a suggestion for the effects observed in response to those ITCs found in cruciferous vegetables on the cell cycle of the HT29 cells may be due to the transcriptional inhibition of cyclin D1 expression. This may indeed be the case in this study; however, this assay was not carried out in great enough detail to determine the specific mechanism of action that occurred in response to the watercress extract. Suggestions for further analysis would be to examine the apoptotic fraction observed in response to the same concentration range of watercress.

The presence of flavonoids, identified in the watercress extract during this study, would suggest that these compounds may be in part responsible for the effects of watercress extract on the proliferation rates of HT29 cells. Previously quercetin has been demonstrated to reduce significantly proliferation rates in this cell line in vitro (44–46). The large variety of phytochemicals identified in the crude extract in this study allows for the suggestion that the components present in watercress could work synergistically with one another. This would also account for the effects observed, even in the absence of PEITC in the extract.

This, to the best of our knowledge, is the first study to demonstrate anti-invasive effects of watercress extract in vitro. It was observed that the watercress extract (6.25–50 µg/ml) significantly decreased the invasiveness of HT115 colon cancer cells through matrigel in the absence of changes in total cell numbers (Fig. 5). This indicates a specific effect

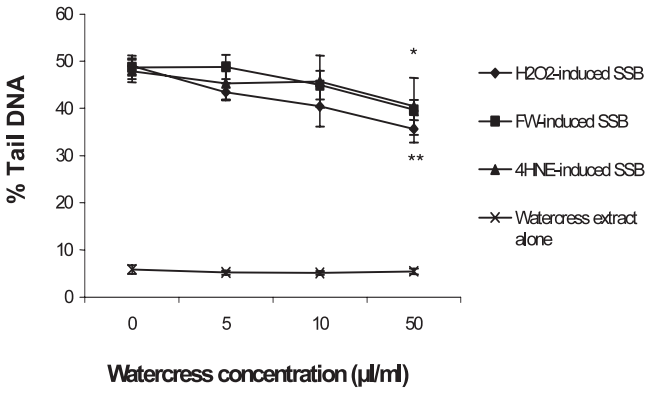

Figure 3. Effect of 24 h preincubation of HT29 cells with watercress extract on single strand DNA breaks. ◆, induced by H_2O_2 (75 µM); ■, fecal water (FW); ▲, 4HNE (250 µM); ×, no genotoxic treatment. *, $P < 0.05$; **, $P < 0.012$.

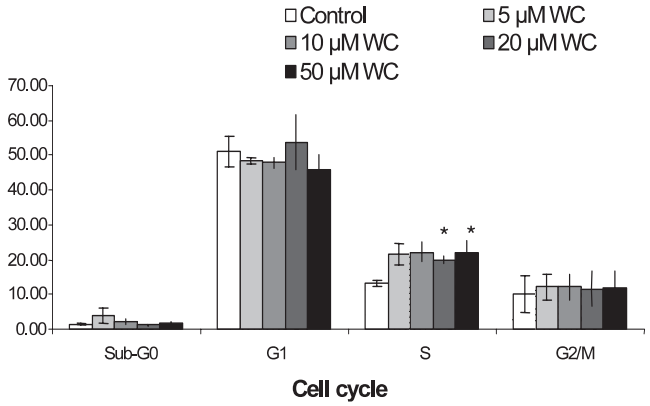

Figure 4. Cell cycle analysis of HT29 cells exposed to a range of watercress extracts (0–50 µl/ml) for 24 h before undergoing analysis. The results of this assay demonstrated a significant accumulation of cells in the S phase the 20 and 50 µl/ml concentrations. *, $P < 0.05$.

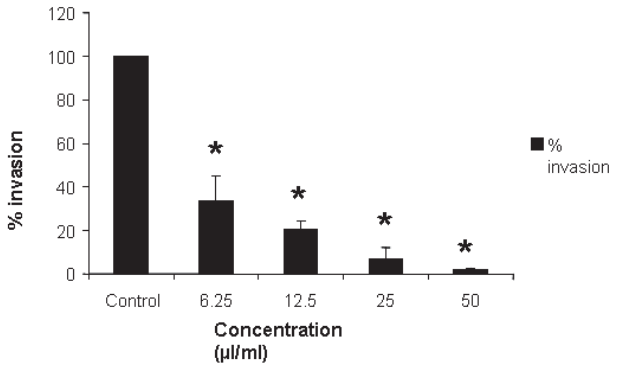

Figure 5. Effect of watercress extract on HT115 tumor cell invasion. HT115 cells were incubated with watercress extract (0–50 µl/ml) for 24 h and the percentage invasion through matrigel measured. Values marked with an asterisk were significantly different from the control. *, $P < 0.05$.

on the invasion process rather than a general effect on cytotoxicity.

It was demonstrated that the crude watercress extract is capable of inhibiting, at least in vitro, key stages in the colon carcinogenesis pathway including initiation, proliferation, and metastasis. From these observations it can be suggested that the protective effects observed in response to watercress extract may in fact be due to a complex mixture of compounds present in the extract, including the nonvolatile hydrolysis products of the GLS identified and a range of phenolic compounds.

The range of anti-carcinogenic activities of specific plant phenolics and flavonoids observed in previous studies suggests that the presence of the same compounds in the watercress used during this study may also play a part in the anti-carcinogenic effects observed. Quercetin and rutin, in the form of herbal supplements, have been demonstrated to cause a 4.2- and 1.2-fold reduction in aberrant crypt foci (ACF) in F334 rats. Histological examination of the colon mucosa also demonstrated an increase in apoptosis (47). Caffeic acid derivatives were also detected in the watercress leaves during this study. Caffeic acid esters, such as caffeic acid phenethyl ester (CAPE) have also been demonstrated to reduce the formation of ACF and tumors induced by azoxymethane (AOM) in the rat colon (48). With regard to human health, >95% of these cell wall HCAs are likely to be released in the human colon, and, therefore, it is possible that these components could reach biologically significant levels in parts of the colon, exerting their protective effects in both human and animal populations (42).

By using the crude extract during this study, information has been obtained on the overall effect of mixtures of potential anti-carcinogenic agents (known and unknown) in watercress and allowed for the possibility of synergistic activity not achievable through study of single compounds or even limited combinations. Thus the current studies support the view that watercress may be an effective agent for reducing cancer risk in humans although clearly further studies are required.

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