Differential Effects of Falcarinol and Related Aliphatic C_{17}-Polyacetylenes on Intestinal Cell Proliferation

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Quantitative major polyacetylenes of carrots (falcarinol and falcarindiol) and American ginseng roots (falcarinol and panaxydol) were isolated and tested in human intestinal epithelial cells of normal (FHs 74 Int.) and cancer (Caco-2) origin. A hormesis effect was seen for all isolated polyacetylenes when added to Caco-2 cells in concentrations ranging from 1 ng/mL to 20 μg/mL. The relative inhibitory potency was falcarinol > panaxydol > falcarindiol. No hormesis effect was observed when adding the polyacetylenes to FHs 74 Int. cells. Instead, an inhibitory growth response was observed above 1 μg/mL. The relative inhibitory potency was panaxydol > falcarinol > falcarindiol. Maximal inhibition at 20 μg/mL corresponded to approximately 95% and 80% inhibition of cell proliferation in normal and cancer cells, respectively. Combinations of falcarinol and falcarindiol added to normal and cancer cells showed a synergistic response for the inhibition of cell growth. Furthermore, the oxidized form of falcarinol, falcarinon, showed a significantly less growth inhibitory effect in intestinal cells of both normal and cancer origin; hence, a hydroxyl group at C-3 may be important for activity of falcarinol-type polyacetylenes. Extracts of carrots, containing different amounts of falcarinol, falcarindiol, and falcarindiol 3-acetate had significant inhibitory effects on both normal and cancer cell proliferation. In cancer cells, the extract containing the highest concentration of falcarinol tended to have the highest growth inhibitory effect, in accordance with a higher potency of falcarinol than falcarindiol. The present study demonstrates that aliphatic C_{17}-polyacetylenes are potential anticancer principles of carrots and related vegetables and that synergistic interaction between bioactive polyacetylenes may be important for their bioactivity.

KEYWORDS: Daucus carota; Panax quinquefolium; falcarinol; falcarindiol; panaxydol; falcarinon; polyacetylenes; bioactivity; intestinal epithelial cells; proliferation; synergistic interactions

INTRODUCTION

Epidemiological studies have provided evidence that a diet high in fruit and vegetables is associated with a reduced risk for the development of certain types of cancer, cardiovascular diseases, diabetes, and other diseases (I–7). Compounds associated with the health promoting effects of vegetables are glucosinolates and other organosulfur compounds and their degradation products, carotenoids, phytoestrogens, polyphenols, vitamins, and dietary fibers (I, 7). The mechanisms for the protection of these classes of natural products are mostly unknown and may only in part explain the health effects of vegetables. Consequently, in recent years focus has been on other types of potential health promoting compounds. One of these groups of compounds is aliphatic C_{17}-polyacetylenes of the falcarinol-type (Figure 1) (7–13), which are common in carrots and related vegetables such as parsley, celery, parsnip, and fennel (10, 11, 14) as well as in medicinal plants such as ginseng (15, 16). Polyacetylenes of the falcarinol-type have shown many interesting bioactivities including anti-inflammato-

tory (11, 13, 16), antiplatelet-aggregatory (11, 16–18), cytotoxic (10, 11, 16, 19, 20), and antitumor activity (9, 22) as well as activity against bacteria and mycoplasma (21). Falcarinol appears to be the most bioactive among polyacetylenes of the falcarinol-type. The bioactivity of falcarinol is probably associated with its hydrophobicity and its ability to form an extremely stable carbocation with the loss of water, thereby acting as a very reactive alkylating agent toward various biomolecules (9, 11). This is supported by the fact that falcarinol is a strong contact allergen (23). A similar mode of action is expected for other falcarinol-type polyacetylenes with a hydroxyl group at C-3 (9, 11). The beneficial effects of polyacetylenes of the falcarinol-type occur at nontoxic concentrations and thus represent pharmaceutically useful properties indicating that polyacetylenes may be important nutraceuticals of vegetables. In the human diet, carrots are the major dietary source of falcarinol-type polyacetylenes, in particular falcarinol and falcarindiol (8, 13, 14). It has been demonstrated that falcarinol but not β-carotene could stimulate differentiation of primary mammalian cells of mammary origin in concentrations between 1 ng/mL and 10 ng/mL falcarinol. Toxic effects were found > 50 ng/mL falcarinol, while β-carotene had
In an attempt to answer these questions, the differential effect of common polyacetylenes of the falcarinol-type (falcarinol, falcarindiol, and panaxydol) as well as different combinations of falcarinol and falcarindiol and carrot extracts with different polyacetylene profiles on the proliferation of human intestinal epithelial cells of normal (FHs 74 Int.) and cancer (Caco-2) origin were investigated. The hypothesis that a hydroxyl group at C-3 is important for the bioactivity of falcarinol-type polyacetylenes was investigated by comparing the growth inhibitory effect of falcarinol and the oxidized form of falcarinol, falcarinon, on intestinal cell proliferation.

MATERIALS AND METHODS

Chemicals and Materials. Ethyl acetate (EtOAc), ethanol (EtOH, 96%), and n-hexane of high-performance liquid chromatography (HPLC) grade (99.9% HPLC grade) and diethyl ether (Et2O, 99.9% p.a. for GC) were obtained from Sigma-Aldrich (Steinheim, Germany). Activated manganese(IV) oxide (MnO2; ReagentPlus ≥ 99%) and anhydrous sodium sulfate (Na2SO4) were obtained from Sigma-Aldrich. Preparative TLC plates (20 × 20 cm, silica gel 60 F254, 2.5 mm thickness) were obtained from Merck (Darmstadt, Germany).

Polyacetylene Standards. Authentic standards of the polyacetylenes (3R)-falcarinol and (3R, 8S)-falcarindiol (Figure 1) were isolated from 2.5 kg of carrot roots (Daucus carota, cv. Bolero) and (3R)-panaxydol and (3R)-falcarinol (Figure 1) from 2 kg of American ginseng (Panax quinquefolium) roots according to the procedure described by Kidmose et al. (14) and Christensen et al. (15), respectively. Falcarinol, falcarindiol, and panaxydol were obtained in a purity >98%, which was demonstrated by analyzing the isolated polyacetylenes by reverse phase (RP) HPLC—photodiode array detection (PAD) according to the method described by Christensen and Kreutzmann (34). Polyacetylenes were identified by UV, mass spectrometry (MS) [gas chromatography (GC)—MS (EI, 70 eV)], NMR (1H and 13C NMR) and 1H–1H correlation spectroscopy recorded in CDCl3 with TMS as internal standard), and optical rotation, and the complete spectral data set corresponded fully with literature values for falcarinol (9, 22, 26–28), falcarindiol (22, 27–29), and panaxydol (19, 30–32), respectively.

Falcarinol (Figure 1) was produced by the oxidation of falcarinol by activated MnO2 (33). Activated MnO2 (100 mg) was added to a solution of falcarinol (25 mg) in Et2O (50 mL) and the mixture stirred at room temperature for 2 h under dark conditions. The reaction mixture was filtered and the residue washed with Et2O. The filtrate was concentrated in vacuo (30 °C) in dim light and the residue purified by preparative TLC (n-hexane-Et2O, 3:1) to give 18 mg of falcarinol as a pale yellow oil in a purity >98% as shown by RP-HPLC-PAD (34). UV λmax (EtO): 228, 262, 276, 292 nm. GC—MS (EI, 70 eV); m/z: 242 [M]+ (9), 185 (5), 171 (11), 157 (57), 145 (36), 143 (20), 131 (63), 129 (80), 128 (45), 117 (100), 115 (83), 103 (87), 91 (59), 77 (76), 55 (94). 1H NMR (300 MHz, CDCl3, TMS as internal standard): δ 0.88 (3H, t, 7.1 Hz, H-17), 1.28 (8H, m, H-13–H-16), 1.37 (2H, m, H-12), 2.04 (2H, dt, 7.0, 7.5 Hz, H-11), 3.12 (2H, d, 6.9 Hz, H-8), 5.38 (1H, m, H-9), 5.57 (1H, m, dt, 1.8, 7.5, 10.5 Hz, H-10), 6.19 (1H, dd, 0.9, 10.1 Hz, H-1), 6.40 (1H, dd, 10.1, 17.4 Hz, H-4), 6.55 (1H, dd, 0.9, 17.4 Hz, H-2). 13C NMR (75 MHz, CDCl3, TMS as internal standard): δ 141.0 (CH, C-16), 137.6 (2H, C-15), 127.3 (CH2, C-11), 29.1 (CH, C-14), 29.2 (CH2, C-13), 29.2 (CH2, C-12), 31.8 (CH2, C-15), 63.6 (C, C-6), 70.6 (C, C-5), 77.1 (C, C-7), 88.0 (C, C-4), 120.7 (CH, C-9), 133.9 (CH, C-10), 134.1 (CH2, C-15), 137.8 (CH, C-2), 177.8 (C, C-3).

Falcarinol extract from testa. Frozen carrot leaves (20 g) from the cultivars cv. Bolero (carrot extract 1) and cv. Purple Haze (carrot extract 2) were homogenized to a puree using an Ultra-Turrax T25 for 1 min followed by extraction with 60 mL of EtOAc overnight under stirring at room temperature. Before stirring, the samples were exposed to ultrasound for 10 min. After the first extraction, the samples were extracted with an additional 60 mL of EtOAc for 3 h under stirring at room temperature. The EtOAc extracts were combined and the water removed from the combined extract by adding anhydrous Na2SO4 (15 g) followed by filtration through a Munktell filter paper, grade 3W. The dried extract was evaporated in vacuo at 25 °C in dim light, and the residue obtained was dissolved in EtOH (5 mL) and filtered through a 0.45 μm
Cell Culture. Caco-2 cells (a kind gift from Dr. Jan Trige Rasmussen, Protein Chemistry Laboratory, University of Aarhus, Denmark) were cultured in 96-well plates at a density of 4000 cells/well in Dulbecco’s minimal essential medium (DMEM) (Gibco 11960–044), supplemented with fetal calf serum (FCS, 10%; Cambrex Bio Science, Copenhagen, Denmark), N-(2-hydroxyethyl)piperazine-N’-ethanesulfonic acid (HEPES) (10 mM; Invitrogen A/S), glutamax (2 mM; Invitrogen A/S), and antibiotic solution (P-3539; Sigma-Aldrich A/S, Vallensbæk Strand, Denmark) containing penicillin and streptomycin for 24 h (pretreatment period). FHs 74 Int. cells (ATCC; purchased from LGC Nordic AB, Boras, Sweden) were cultured in 96-well plates at a density of 1500 cells/well in DMEM (Gibco 42430–125), supplemented with fetal calf serum (FCS, 10%; Cambrex Bio Science, Copenhagen, Denmark), HEPES (10 mM; Invitrogen A/S, Tästrup, Denmark), glutamax (2 mM; Invitrogen A/S), human recombinant EGF (30 μg/mL; Austral Biologicals, San Ramon CA, USA), NCTC-135 with glutamin (8%; Invitrogen A/S), nonessential amino acids (1%; Invitrogen A/S), sodium puruvate (0.4 mM; Invitrogen A/S), oxaloacetate (0.1 g/L; Sigma-Aldrich A/S), glutamax (2 mM; Invitrogen A/S), and antibiotic solution (P-0781; Sigma-Aldrich A/S) containing penicillin and streptomycin for 24 h (pretreatment period). At 60 to 80% confluence, cells were washed carefully with phosphate buffered saline (PBS) (Invitrogen A/S), and treatment medium (200 μL per well in 96-well plates) was added to individual wells for a total of 72 h, with medium changed after 48 h. For each cell line, growth curves were obtained with different numbers of cells and different concentrations of FCS for 8–10 successive days of culture. In this way, we ensured that cells were in the sigmoid phase of the growth curve when components to be tested were added to the culture medium.

Figure 2. Effect of increasing concentrations of falcarinol, falcarindiol, and panaxydol on cell proliferation in (A, B) Caco-2 and (C, D) FHs 74 Int. cells cultured for 72 h in either (A, C) 0.625% FCS or (B, D) 10% FCS. Values are least-squares means obtained from cultures in quadruplicate samples from two experiments and presented as relative to proliferation obtained in medium without polyacetylenes (basal medium). Standard error of the mean (SEM) values obtained in the ANOVA analysis were 0.15, 0.08, 0.06, and 0.04 (relative values) for A, B, C, and D, respectively. Values significantly different from proliferation obtained in basal medium are indicated: c, P < 0.05; b, P < 0.01; a, P < 0.001.

Treatment medium contained falcarinol, falcarindiol, or panaxydol in different concentrations (0.001, 0.01, 0.1, 1, 2.5, 5, 10, and 20 μg/mL), falcarinol or falcarinon in different concentrations (1, 2.5, 5, 10, and 20 μg/mL), or carrot extracts (0.01, 0.05, 0.1, 0.5, and 1% in culture medium). These treatment media were prepared from stock concentrations of polyacetylenes in 96% EtOH or carrot extracts resolubilized in 96% EtOH. Concentrations of EtOH in culture media never exceeded 1%. Experiments also included studies of potential synergistic and/or antagonistic effects of quantitative major and most bioactive polyacetylenes in carrots. For these studies, falcarinol and falcarindiol were added to culture medium in combinations. Keeping one of the polyacetylenes constant at 10 ng/mL or 1 μg/mL, the two compounds were tested for effects in the ratios 1:1, 1:5, 1:10, 5:1, and 10:1. Treatment medium furthermore contained FCS (Cambrex Bio Science, Copenhagen, Denmark) in concentrations of either 0.625% or 10%. Cell proliferation was determined by a resazurin metabolism assay (AlamarBlue, BioSource, AH diagnostics, Aarhus, Denmark). Cell culture medium was carefully aspirated and the AlamarBlue reagent was added to all wells and measured after 2 h incubation according to the manufacturer.

Statistics. Statistical analyses were performed using the general linear models procedure (PROC GLM) of SAS Institute Inc. Different concentrations of the three polyacetylenes (Figure 2) were tested using a model including the systematic effects of the source of polyacetylenes (n = 3), concentrations of polyacetylenes (nine levels), and their interaction. The effect of the assay (n = 2) was included as a blocking factor. Different concentrations of falcarinol and falcarinon (Figure 3) were tested using a model including the systematic effect of polyacetylenes (n = 2), concentration of polyacetylenes (five levels), and their interaction. The effect of the assay (n = 2) was included as a blocking factor. Different concentrations of the two carrot extracts (Figure 4) were tested using a model...
including the systematic effect of extract (n = 2), concentration of extract (five levels), and their interaction. The effect of the assay (n = 2) was included as a blocking factor. The residual mean error was used as the error term for F-tests. All results shown in Figures 2–4 are least-squares means obtained from cultures in quadruple samples from two experiments and presented as relative to proliferation obtained in basal medium.

**RESULTS AND DISCUSSION**

The effects of falcarinol-type polyacetylenes and carrot extracts were evaluated in two different intestinal cell lines of normal and cancer origin, respectively. We have previously used both of these intestinal epithelial cell lines for identification of bioactive components in complex natural mixtures such as milk (35, 36). To be able to compare results obtained from two different cell lines, growth curves were performed for each cell line to ensure that cells were in the sigmoid phase of the growth curve when components to be investigated were added to the culture medium. Therefore, the initial number of cells was different for the two individual cell lines.

**Effect of Polyacetylenes on Intestinal Cell Proliferation.** The effects of the polyacetylenes, falcarinol, falcarindiol, and panaxydol were first evaluated in the human intestinal epithelial cell line, Caco-2, using a resazurin metabolism assay as an index of cell proliferation. Treatment of the cells with concentrations ranging from 1 ng/mL to 20 μg/mL showed a hormesis effect of all three polyacetylenes. Falcarinol, falcarindiol, and panaxydol in a concentration of 100 ng/mL increased cell proliferation by 30, 42, and 60% under basal growth conditions (0.625% FCS) (P < 0.05; Figure 2A). However, in the presence of 10% FCS, no significant increase in cell proliferation was observed with the three polyacetylenes (Figure 2B). Treatment with falcarinol at a concentration of 2.5 μg/mL, and falcarindiol and panaxydol at concentrations of 10 μg/mL significantly reduced proliferation under basal growth conditions (P < 0.05; Figure 2A). In the presence of 10% FCS, concentrations of 5, 20, and 10 μg/mL of falcarinol, falcarindiol, and panaxydol, respectively, were needed to reduce proliferation significantly (P < 0.01; Figure 2B). The relative inhibitory potency was falcarinol > panaxydol > falcarindiol with concentrations corresponding to 50% inhibition of basal response of 2.5–5 μg/mL, 5–10 μg/mL, and 10–20 μg/mL, respectively. Maximal inhibition at 20 μg/mL corresponded to approximately 80% inhibition of cell proliferation under basal growth conditions (Figure 2A). Under FCS stimulated growth conditions, concentrations corresponding to 50% inhibition were 5–10 μg/mL, 10–20 μg/mL, and >20 μg/mL, for falcarinol, panaxydol, and falcarindiol, respectively. Maximal inhibition at 20 μg/mL corresponded to approximately 90, 65, and 30% for falcarinol, panaxydol, and falcarindiol, respectively (Figure 2B).

We also determined whether falcarinol, falcarindiol, and panaxydol had similar effects in the normal human intestinal epithelial cell line, FHs 74 Int. In these cells, no hormesis effect was observed. Instead, a significantly inhibitory effect on proliferation was seen with concentrations of falcarinol, falcarindiol, and panaxydol at 5, 10, and 2.5 μg/mL, respectively (P < 0.001; Figure 2C and D) in the presence of both 0.625 and 10% FCS,
except for falcarindiol which had no inhibitory effect in the presence of 10% FCS. The relative inhibitory potency was panaxydol > falcarinol > falcarindiol with concentrations corresponding to 50% inhibition of basal response of 1–2.5 μg/mL, 2.5–5 μg/mL, and 10–20 μg/mL, respectively. When 10% FCS was present in the culture medium, concentrations corresponding to 50% inhibition were 1–2.5 μg/mL and 5–10 μg/mL for panaxydol and falcarinol, respectively (Figure 2C and D).

The present study shows that falcarinol, falcarindiol, and panaxydol affect cell proliferation of human intestinal cells in a dose-dependent way and that the presence of FCS in culture medium also influences these responses. In a recent study, Caco-2 cells were chosen as the in vitro model for assessment of the bioactivity of falcarinol, but falcarindiol and panaxydol were not examined (12). However, the effect of falcarindiol was only studied in the presence of 10% FCS in the culture medium. Such high concentration of FCS in culture medium may protect intestinal cells from growth-inhibitory compounds and furthermore may include relatively high concentrations of compounds that affect the bioactivity of the tested compounds. This may explain why the hormesis effect of polyacetylenes obtained in Caco-2 cells at 0.625% FCS could not be seen in the presence of 10% FCS and that higher concentrations of polyacetylenes were needed to inhibit cell proliferation (Figure 2). These results indicate that FCS in high concentrations in culture medium protects intestinal cells against the inhibitory effects of the tested polyacetylenes. On the basis of these observations, we recommend testing the bioactivity of falcarinol-type polyacetylenes in Caco-2 cells and similar cell models in low concentrations of FCS.

The inhibitory potency was clearly different between the tested polyacetylenes in the cancer and normal intestinal cells. In Caco-2 cells, falcarinol was the most potent inhibitor of cell proliferation, while in FHs cells, panaxydol was more potent than falcarinol. The effect of falcarindiol was similar in both cell lines, although its effect was much lower with 50% inhibition of basal response at 10–20 μg/mL (Figure 2). The present results confirm the results of other studies that falcarinol and panaxydol are much more cytotoxic than falcarindiol and similar polyacetylenes of the falcarinol-type such as falcarindiol 3-acetate and 8-O-methylfallcarindiol (10). Falcarindiol has, for example, the ability to generate two active centers for nucleophilic attack, which reduces the lipophilic character of this compound and hence its reactivity, which could explain the lower reactivity of this compound compared to that of falcarinol and panaxydol. The lower reactivity and potential anticancer effect of falcarindiol compared to that of falcarinol are also in accordance with the observed nonallergenic properties of falcarindiol (23). The inhibitory activity of the tested polyacetylenes toward Caco-2 cells therefore confirm that the anticancer activity of polyacetylenes of the falcarinol-type is associated with their ability to form extremely stable carbocations, thereby acting as very reactive alkylating agents toward biomolecules.

**Effect of Oxidation of Falcarinol on Intestinal Cell Proliferation.**

The importance of a hydroxyl group in the C-3 position of falcarinol was investigated by testing the inhibitory effect of the oxidized form of falcarinol, falcarinon, on intestinal cell proliferation. Falcarinol and falcarinon, were added to Caco-2 and FHs 74 Int. cells for comparison of the effects on cell proliferation. In accordance with the results reported above, falcarinol significantly inhibited cell proliferation in Caco-2 cells at 2.5 μg/mL (P < 0.01) and in FHs 74 Int. cells at 5 μg/mL (P < 0.01) (Figure 3). Falcarinon, however, only inhibited proliferation in Caco-2 cells at the concentration of 20 μg/mL (P < 0.001) and only tended to decrease proliferation in FHs 74 Int. cells at this concentration (P < 0.10). Furthermore, the dose of 20 μg/mL only caused 40–45% reduction in cell proliferation, and there were no differences between normal and cancer cells (Figure 3). These results demonstrate that the oxidized form of falcarinol, falcarinon, is a much less potent inhibitor of cell proliferation in intestinal cells of both normal and cancer origin and strongly support the hypothesis that the mode of action of falcarinol-type polyacetylenes is due to their ability to lose water through the hydroxyl group at C-3, thereby generating reactive carbocations. This mode of action of falcarinol-type polyacetylenes makes it reasonable to assume that these compounds can affect each other’s anticancer activity in an antagonistic and/or synergistic manner. Consequently, the inhibitory effect of falcarinol and falcarindiol on Caco-2 and FHs 74 Int. cells was tested in different concentration ratios. Keeping one of the polyacetylenes constant at either 10 ng/mL or 1 μg/mL, Caco-2 and FHs 74 Int. cells were incubated with falcarinol and falcarindiol in the ratios 1:1, 1:5, 1:10, 5:1, and 10:1. These studies were again performed in a medium containing 0.625% FCS or 10% FCS. When a concentration of 10 ng/mL of falcarinol or falcarindiol was added to Caco-2 or FHs 74 Int. cells at either 0.625% or 10% FCS present in the medium, increasing the concentration of one or the other polyacetylene showed no difference from what would have been expected from the single-compound assay (data not shown).

To further determine the effect of polyacetylenes in combinations and hence their possible synergistic interactions, the effects of two

| Table 1. Effects of Different Ratios of Falcarinol and Falcarindiol on Caco-2 and FHs 74 Int. Cells in Culture Medium Containing 0.625% FCS* |
|-----------------------------------------------|
| falcarinol (μg/mL) | 0 | 1 | 5 | 10 |
| **Caco-2 cells** |
| falcarinol (μg/mL) | 0 | 1.0 ± 0.25 | 0.38 ± 0.25 | 0.18 ± 0.01 |
| 1 | 1.35 ± 0.36 | 0.71 ± 0.08 | 0.58 ± 0.04 | 0.22 ± 0.08 |
| 5 | 1.03 ± 0.32 | 0.53 ± 0.06 | 0.22 ± 0.06 | nd |
| 10 | 0.52 ± 0.17 | 0.17 ± 0.03 | nd | nd |

| FHs 74 Int. cells |
|------------------|
| falcarinol (μg/mL) | 0 | 1.02 ± 0.06 | 0.32 ± 0.35 | 0.06 ± 0.01 |
| 1 | 0.92 ± 0.01 | 0.51 ± 0.03 | 0.47 ± 0.01 | 0.36 ± 0.28 |
| 5 | 0.90 ± 0.04 | 0.34 ± 0.24 | nd | nd |
| 10 | 0.60 ± 0.14 | 0.14 ± 0.13 | nd | nd |

*Cells were cultured for 72 h, and cell proliferation was determined by a resazurin metabolism assay. Mean ± standard deviation is shown for each combination of the two tested polyacetylenes. Data are presented relative to cell proliferation obtained in medium without polyacetylenes for two individual experiments. Significant synergistic inhibitory effect on cell proliferation compared to single-compound assay (P < 0.01). nd: not determined.
carrot EtOAc extracts were studied in Caco-2 and FHs 74 Int. cells. Concentrations of falcarinol, falcarindiol, and falcarindiol 3-acetate in carrot extracts are shown in Table 2. Carrot extract 1 contained approximately 50% of the falcarinol in carrot extract 2, but approximately 4 and 2 times more falcarindiol and falcarindiol 3-acetate, respectively, than carrot extract 2. Both extracts had significant inhibitory effects on intestinal cell proliferation in the highest concentration of 0.5 and 1% of extract in culture medium (Figure 4). In Caco-2 cells, carrot extract 2 tended (P < 0.10) to be a more potent inhibitor of proliferation than carrot extract 1, as significant inhibition was obtained also with 0.1% extract giving an approximate 20% decrease in proliferation (Figure 4A). In FHs 74 Int. cells, there was no difference between the effects of the two extracts (Figure 4B), but the effect at a given concentration was larger than that in Caco-2 cells. At the highest concentrations of falcarinol, cell proliferation was reduced to 6% of proliferation in FHs 74 Int. cells, while in Caco-2 cells, cell proliferation was reduced to only 36% (Figure 4).

The more potent inhibitory activity of carrot extract 2 than of carrot extract 1 in Caco-2 cells (Figure 4A) is in accordance with the approximately 3 times higher concentration of falcarinol in carrot extract 2, as falcarinol is a more potent inhibitor than falcarindiol (Figure 2). Comparing the effects of the carrot extracts with the effects of falcarinol or falcarindiol alone, we showed that the mixture of polyacetylenes found in the extracts was more potent in inhibiting cell proliferation. As an example, the 1% carrot extract 1 prepared from a stock solution by diluting 100 times in cell culture medium (Table 2) contained falcarinol, falcarindiol, and falcarindiol 3-acetate in concentrations of 2.81, 5.96, and 1.96 μg/mL, respectively, and caused a reduction in cell proliferation to 36%. For comparison, a concentration of 2.5 μg/mL of falcarinol decreased proliferation to 68%, while falcarindiol caused no decrease in cell proliferation (Figures 2 and 4, and Table 2). Concentrations in falcarinol of 5 μg/mL or falcarindiol of 10–20 μg/mL were required to obtain a decrease in cell proliferation corresponding to 36%. However, addition of carrot extract 2 to Caco-2 cells did not decrease cell proliferation more than carrot extract 1 despite a concentration in falcarinol of 6.46 μg/mL. These studies suggest that falcarinol in combination with the relatively high concentrations of falcarindiol and falcarindiol 3-acetate in carrot extract 1 has a synergistic effect on Caco-2 cell proliferation. In FHs 74 Int. cells, a similar synergistic effect can be suggested as carrot extract 1 or 2 both caused a decrease in cell proliferation to 30 and 6% in concentrations of 0.5% and 1%, respectively, of the extracts. To obtain such decreases in cell proliferation of FHs 74 Int. cells by falcarinol alone, a concentration of 10 μg/mL would be necessary.

The synergistic effect observed between falcarinol and other falcarinol-type polyacetylenes in carrots are contradictory to the antitumor effect previously observed for a carrot diet compared to a falcarinol diet in rats with induced (pre)neoplastic lesions (9). The present results therefore clearly suggest that other metabolites in carrots to some extent may interact with falcarinol and other polyacetylenes in carrots, thereby affecting their effectiveness in vivo.

Table 2. Concentrations of Falcarinol, Falcarindiol, and Falcarindiol 3-Acetate in EtOAc Extracts from the Carrot Cultivars cv. Bolero (Carrot Extract 1) and cv. Purple Haze (Carrot Extract 2)*

| Carrot extract | Falcarinol (μg/mL) | Falcarindiol (μg/mL) | Falcarindiol 3-acetate (μg/mL) |
|----------------|--------------------|---------------------|-------------------------------|
| 1              | 281.1              | 596.0               | 195.8                         |
| 2              | 645.7              | 152.3               | 107.1                         |

* Concentrations are given in stock solutions of which different concentrations were prepared in cell culture medium.
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