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Effects of bioactive compounds from carrots (*Daucus carota* L.), polyacetylenes, beta-carotene and lutein on human lymphoid leukaemia cells.

**Running Title: Effects of bioactive compounds from *Daucus carota* L. on lymphoid leukaemia cells**

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Abstract:
New therapies for leukaemia are urgently needed. Carrots have been suggested as a potential treatment for leukaemia in traditional medicine and have previously been studied in other contexts as potential sources of anticancer agents. Indicating that carrots may contain bioactive compounds, which may show potential in leukaemia therapies. This study investigated the effects of five fractions from carrot juice extract (CJE) on human lymphoid leukaemia cell lines, together with five purified bioactive compounds found in Daucus carota L, including: three polyacetylenes (falcarinol, falcarindiol and falcarindiol-3-acetate) and two carotenoids (beta-carotene and lutein). Their effects on induction of apoptosis using Annexin V/PI and Caspase 3 activity assays analysed via flow cytometry and inhibition of cellular proliferation using Cell Titer Glo assay and cell cycle analysis were investigated. Treatment of all three lymphoid leukaemia cell lines with the fraction from carrot extracts which contained polyacetylenes and carotenoids was significantly more cytotoxic than the 4 other fractions. Treatments with purified polyacetylenes also induced apoptosis in a dose and time responsive manner. Moreover, falcarinol and falcarindiol-3-acetate isolated from Daucus carota L were more cytotoxic than falcarindiol. In contrast, the carotenoids showed no significant effect on either apoptosis or cell proliferation in any of the cells investigated. This suggests that polyacetylenes rather than beta-carotene or lutein are the bioactive components found in Daucus carota L and could be useful in the development of new leukemic therapies. Here, for the first time, the cytotoxic effects of polyacetylenes have been shown to be exerted via induction of apoptosis and arrest of cell cycle.

Keywords: Leukaemia / Polyacetylenes / Carotenoids / Daucus carota L / Apoptosis / Cell proliferation.
1. Introduction:

Leukaemia is a major source of morbidity and mortality worldwide and is the most common childhood cancer [1], however the causes of childhood leukaemia remain mostly unclear [2]. According to the Leukaemia & Lymphoma Society about 28,500 patients are diagnosed annually in the UK with a blood cancer [3]. Approximately 40% of patients diagnosed with leukaemia survive for at least five years after diagnosis [4]. The main treatments for leukaemia are chemotherapy, radiotherapy, growth factors, and bone marrow or stem cell transplants. Treatment for leukaemia varies depending on age, level of fitness and type of leukaemia [5]. The current therapies however, show a wide range of side effects: e.g. drop in blood cell count, complete hair loss, diarrhoea, tiredness, nausea and reduced fertility [6]. Thus, new treatments with potentially different mechanisms than those presently used as chemotherapy agents could support existing treatments of leukaemia, if their use could reduce side effects without compromising efficacy. Promising sources of such new agents could be found in common foods, which contain bioactive compounds with potential anti-cancer properties.

Experimental and epidemiological evidence suggests that high intake of phytochemicals derived from fruits, vegetables and whole grains such as polyphenols and carotenoids are associated with low incidence of cancers such as lung and prostate cancer [7]. In traditional medicine, wild carrots (known as ‘Queen Anne’s Lace’) have been used as treatments for leukaemia and other cancers throughout history [8-10]. In 2002, a study demonstrated that individuals who have a high level of serum carotene or who eat more fruits and vegetables high in carotenoids, showed a lower risk of developing cancers, particularly lung cancer [11]. In addition, epidemiological studies have shown that dietary intake of α-carotene (which is almost exclusively derived from carrots) is significantly associated with reduced risk of cancer than intake of other similar phytochemicals [12].

We have previously demonstrated that carrot (Daucus carota L) juice extract contains bioactive agents which prevent leukaemia cell proliferation and induce cell death in human leukaemia cell lines preferentially to non-tumour control cells [13]. Carrot juice extract (CJE) treatment of four human myeloid and four human lymphoid leukaemia cell lines showed an inhibition in progression of cell cycle and induction of programmed cell death (apoptosis) [13]. In addition, lymphoid leukaemia cell lines were more sensitive to CJE treatment than myeloid cell lines [13].

Daucus carota L is a rich source of a number of compounds thought to have bioactive properties, including polyacetylenes and carotenoids. Polyacetylenes found within Daucus carota L include falcarniol (FaOH), falcarnidol (FaDOH) and falcarnidol-3-acetate (FaDOAc) and have demonstrated bioactive actions in a number of cell lines [14-16]. Falcarniol from Apiaceae vegetables including carrots has been shown to be cytotoxic against an acute lymphoblastic leukaemia cell line (CEM-C7H2), with an IC₅₀ of 3.5 µM [17]. Moreover, Purup and colleagues (2009), investigated the inhibitory effect of polyacetylenes from carrots (falcarniol and falcarnidol) and American ginseng (falcarniol and panaxydol) demonstrating that falcarniol was a potent inhibitor of cell proliferation of CaCo-2 cells (human epithelial colorectal carcinoma cells) [15]. Young et al (2007) demonstrated in vitro that falcarniol (from carrots) at concentrations above 10 µM decreased cell proliferation of CaCo-2 cells after 48 and 72 h [18]. Another study, tested the effect of falcarniol on the development of azoxymethane (AOM) induced colon preneoplastic lesions in male rats and found that feeding rats with freeze-dried carrots containing 35 µg falcarniol per gram, or rat feed supplemented with the same dose
of falcarinol delayed the development of tumours and aberrant crypt foci (ACF) following 18 weeks [19]. Furthermore, several in vitro and in vivo studies investigating falcarinol and falcarindiol treatments, showed cytotoxic effects on a range of cell types over a range of concentrations between 3.5 - 100 µM [15,17-19] (Table 1).

In addition to polyacetylenes, β-carotene is thought to be a bioactive agent within carrots and has been shown to induce apoptosis in tumor cells [20]. Treatment of U937 and HL-60 leukaemia cell lines showed a significant decrease in the number of viable cells following treatment with 20 µM commercial β -carotene [20]. Induction of apoptosis was also seen and indicated by arrest at G1 phase of cell cycle [20]. In contrast, others have demonstrated that high intake of β -carotene has no effects in the treatment or prevention of cancer. Hansen and colleagues (2003), compared the cytotoxicity of falcarinol ((9Z)-heptadeca-1,9-dien- 4,6-diyn-3-ol), obtained from carrots, with β-carotene and the results showed that falcarinol had inhibitory effects between 4.09 and 40.9 µM , while β-carotene showed no effect in the concentration range 0.001 – 186 nM [21]. Studies of smokers (aged 50–69 years) who smoked five or more cigarettes a day showed that consumption of 20 mg β-carotene supplements daily resulted in a higher risk of lung cancer compared to smokers without β-carotene supplementation, despite this the same study also confirmed a significant protective effect of baseline dietary β-carotene intake (most of which would be from carrots) [22,23]. One possible explanation for this contradictory observation could be that other compounds in fruits or vegetables which are high in β-carotene are responsible for the protective effects seen [24,25].

Lutein is another potential bioactive carotenoid, found within carrots, which has demonstrated anti-tumor activity towards lung cancer of ddY mice when given orally with a mixture of olive oil three times a week [26]. Furthermore, this carotenoid showed selective cytotoxicity and induced apoptosis in transformed mammary epithelial cells but not normal human mammary epithelial cells [27]. Semi purified lutein has also been shown to inhibit mammary cancer growth in mice, by the down regulation of Bcl-2 gene (anti-apoptotic) and up regulation of Tp53 and Bax (pro-apoptotic) mRNA [28]. They also found that mice fed a lutein-supplemented diet displayed tumours that were 30 to 40% smaller compared to mice on un-supplemented diets 50 days post-feeding [28].

Here, we aimed to identify the bioactive compounds within Daucus carota L responsible for the induction of apoptosis and inhibition of cellular proliferation in leukemic cell lines. We separated five fractions from carrot juice extracts (CJE) using solid phase extraction to isolate different groups of compounds, and investigated their effects on induction of apoptosis. Moreover we used pure bioactive chemicals from Daucus carota L (including: three polyacetylenes: falcarinol, falcarindiol and falcarindiol-3-acetate, and two carotenoids: β-carotene and lutein) to investigate their individual effects on induction of apoptosis and inhibition of cellular proliferation.
2. Materials and Methods:

2.1 Cell lines and culture

Three lymphoid leukaemia cell lines, which we previously have shown respond to whole carrot juice extracts [13], were used within this study, these were: CCRF-CEM (acute lymphoblastic leukaemia) (ATCC: CCL-119, Middlesex, UK); Jurkat (peripheral blood T cell leukaemia) (ATCC: TIB-152, Middlesex, UK); and MOLT-3 (acute lymphoblastic leukaemia patient released following chemotherapy) (ATCC: CRL-1552, Middlesex, UK).

Two million cells per 75 cm² flask (Invitrogen, Paisley, UK) in RPMI 1640 medium (Invitrogen, Paisley, UK) supplemented with 10% (v/v) fetal bovine serum, 1.5 mM L-Glutamine and 100 µg/mL penicillin/streptomycin (complete RPMI) were incubated at 37 °C with 5% CO₂. Following incubation cells from T75 cm² flasks were transferred to centrifuge tubes and centrifuged for 10 minutes at 400 g at room temperature, supernatant removed and cells re-suspended in 20 mL of complete RPMI media. The number of cells was assessed using a haemocytometer and 12 well plates (Fisher Scientific, Leicestershire, UK) seeded with 0.5 x 10⁶ cells per well.

2.2 Fraction separation from carrot juice extracts (CJE)

Solid Phase Extraction (SPE) using Strata C18E GIGA tubes (Phenomenex, Hartsfield, UK) were used to separate fractions from the carrot juice extracts used previously [13]. Firstly, tubes were pre-equilibrated by washing with 50 mL of acetonitrile then 50 mL of 0.1 % formic acid / ultra pure water (v/v) (Sigma, Poole, UK). Carrot juice extract (prepared as previously [13]) was sterile filtered through 0.22 µm syringe filters (Invitrogen, Paisley, UK) and diluted 1:1 with sterile distilled water prior to addition of 50 mL diluted juice to the C18E column, after which the unbound fraction which passed directly through the column was collected (fraction A). Then, 50 mL each of ultra pure water, acetonitrile, acetone and ethyl acetate were added to the tube to collect water (fraction B), acetonitrile (fraction C), acetone (fraction D) and ethyl acetate (fraction E), fractions respectively. Each fraction was dried using freeze drying (MODULYOD-230 freeze dryer, Thermo, UK) at -30 to -80 °C for fractions A and B or dried under a nitrogen stream at room temperature for fractions C, D and E. According to the required dilution: water, unbound and acetonitrile fractions were dissolved in distilled water; acetone fraction in methanol, and ethyl acetate fraction in DMSO (Sigma, Poole, UK).

2.3 Treatment Regimes

2.3.1 Treatment with fractions:

The total weight of 10 mL dried whole juice was calculated together with individual weight/volume of each fraction. The weight of dried fractions equivalent to 62.5 µL whole juice was calculated to match concentrations of compounds within 6.25% CJE treatments used previously [13]. The weight equivalent to 6.25% treatment was resuspended in 1 µL appropriate solvent. Cells (0.5 x 10⁶) were then treated with 1, 2 or 4 µL of fraction solution (equivalent to compound concentration within 6.25, 12.5 or 25% whole CJE as studied previously) for 48 h. Cells were also treated with acetone fraction for additional time points: 24 and 72 h and an additional concentration equivalent to 50% whole CJE.
2.3.1.1 HPLC Analysis of carrot juice extraction and five fractions:

Dried whole juice and dried fractions (A, B, C, D and E) each corresponding to thirty milliliter juice were dissolved by adding 1 mL each of water, acetonitrile and ethyl acetate to generate polar and non-polar layers on the bottom and top of the sample respectively. The top non-polar layer contained the polyaetylenes and carotenoids from the whole juice whereas the bottom polar layer contained phenolic acids. High performance liquid chromatography (HPLC) was used to detect polyaetylenes, carotenoids and phenolic acids using previously described methods [29].

2.3.1.2 MS/MS Analysis of acetone fraction:

Tandem mass spectrometry/mass spectrometry (MS/MS) was performed on 30 mL dried acetone fraction, isolated from CJE to confirm the presence of polyaetylenes and carotenoids within this fraction. Acetone fraction was dissolved in 200 µL each of ultra pure water, ethyl acetate and acetonitrile to generate polar and non-polar layers as described for HPLC analysis. The top non-polar acetone layer, which was yellow in color, was injected manually and analysed on negative ionisation mode on a Finnigan LCQ mass spectrometer (Thermo Electron Corporation, Basingstoke, UK). MS/MS was performed to investigate the peaks corresponding to the MW of the three polyaetylenes (falcarinol MS/MS 243, falcarindiol MS/MS 259 and falcarindiol-3-acetate MS/MS 301) and β-carotene and lutein (beta-carotene MS/MS 535.8 and lutein MS/MS 567.87). The collision energy used ranged between 30 and 40 for all compounds. Data was acquired at m/z ranging from 65-400 and 140-600 for detecting polyaetylenes and carotenoids respectively.

2.3.2 Treatment with polyaetylenes:

All Polyaetylenes were purified from carrots as described previously and were obtained from Newcastle University [29] (Newcastle University, Human Nutrition Research Centre, Newcastle, UK). Polyaetylenes were dissolved in methanol (Sigma, Poole, UK) to give stock concentrations falcarinol (6.8 mM/mL), falcarindiol (1.5 mM/mL) and falcarindiol-3-acetate (5 mM/mL) and diluted in serum free media to treat half a million cells with 0, 0.5, 5, 25, 50 and 100 µM and incubated for 24 h. Methanol was added to the control cells at equivalent concentration for vehicle controls.

2.3.3 Treatment with β-carotene:

Beta-carotene (Sigma, Poole, UK) was dissolved in DMSO (Sigma, Poole, UK) to stock concentration (10 g/mL) and diluted in serum free media to treat half a million cells with 0, 0.5, 5, 25, 50 and 100 µM and incubated for 24 h. DMSO was added to the control cells at equivalent concentrations for vehicle controls.

2.3.4 Treatment with lutein:

Lutein (Extrasynthese, Genay, France) was dissolved in methanol (Sigma, Poole, UK) containing 1% v/v dichloromethane to stock concentration (5 mg) and diluted in serum free media to treat half a million cells with 0, 0.5, 5, 25, 50 and 100 µM. Methanol containing 1% v/v dichloromethane was added to the control cells at equivalent concentrations for vehicle controls.
2.4 Investigating the apoptotic cell death following the treatment with five fractions of carrot juice extract, polyacetylenes, beta-carotene and lutein:

Induction of apoptosis was assessed using Annexin V (BD, Oxford, UK) and propidium iodide (PI) staining (Sigma, Poole, UK) and examined using flow cytometry. Caspase 3 activity assay (Cambridge Bioscience, Cambridge, UK) was also used to investigate apoptosis and examined using flow cytometry.

2.4.1 Flow cytometric analysis using Annexin V/PI stain:
Following treatments cellular content from each well were transferred to Eppendorf tubes and centrifuged for 5 minutes at 400 g, 4 ºC. Supernatant was removed and cells resuspended in 100 µl Dulbecco's Phosphate-Buffered Saline (DPBS) (Invitrogen, Paisley, UK). Cells were washed twice in 100 µl binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) (BD, Oxford, UK). Cells were resuspended in 50 µl binding buffer and 5 µl of Annexin V and incubated at 4 ºC for 20 minutes in the dark. Finally, 300 µl of PI (50 µg/µL) was added and samples directly analyzed on the flow cytometer using a BD FACS Calibur instrument and data analyzed using FlowJo software (Tree Star, Treestar, Ashland, OR, USA). Live cells with undamaged membranes exclude PI, while the membranes of damaged and dead cells are permeable to PI. Thus live cells were PI and Annexin V negative, cells undergoing apoptosis were Annexin V positive and PI negative and cells that were in late apoptosis or already dead via apoptosis or necrosis were positive for both Annexin V and PI.

2.4.2 Flow cytometric analysis using Caspase 3 stain:
Following treatments 200 µl of cell suspensions from each well were transferred to flow cytometry tubes and 5 µl of caspase 3 stain (0.2 mM) (Promega, Southampton, UK) added to each sample and incubated in the dark for 10 minutes. Samples were analyzed on the flow cytometer using a BD FACS Calibur instrument (BD, Oxford, UK) and data analyzed using Flow Jo software (Tree Star, Treestar, Ashland, OR, USA).

2.5 Investigating the cell proliferation inhibition following the treatment with five fractions of carrot juice extract, polyacetylenes, beta-carotene and lutein:

2.5.1 Investigating the ATP level:
Twenty five thousand cells per well were treated in white 96-wells plates (Fisher Scientific, Loughborough, UK) with acetone fraction, three polyacetylenes and two commercial carotenoids (β-carotene and lutein) at concentration's ranging from 0 to 100 µM, and after 24 h, 25 µl of cell titer-glo reagent from Cell Titer Glo Luminescent Cell Viability Assay kit (Promega, Southampton, UK) were added to each well, and incubated for 10 min at room temperature. The luminescence was measured using Wallac Victor 2 1420 luminescence detector. The ATP level, which is an indicator of metabolically active cells, was quantified to determine the number of live cells following treatment.

2.6 Investigating the cell cycle inhibition following the treatment with polyacetylenes:

2.6.1 Cell cycle analysis:
Half a million cells per well were treated with falcarinol, falcarindiol or falcarindiol-3-acetate and after 24 h
cells were transferred to Eppendorf tubes and centrifuged for 5 minutes at 400 g at 4 °C. The supernatant was removed, and cells washed twice in 100 µl cold DPBS. Cells were fixed by adding 100 µl of 80 % ethanol and stored overnight at -20 °C. Then, cells were washed twice with cold DPBS prior to addition of 300 µl of PI (Sigma, Poole UK) (50 µg/ mL) and 50 µl of RNase (0.1 unit/ mL) (Sigma, Poole UK). Samples were stained overnight at 4 °C and analyzed on the flow cytometer with BD FACS Calibur instrument and data analysed with FlowJo software using Waston (pragmatic) equation (Tree Star, Treestar, Ashland, OR, USA).

Statistical analysis
Average and Standard error of the mean (SEM) was calculated and Stats Direct was used to test whether data followed a normal distribution using a Shapiro Wilke test. Data did not follow a normal distribution, thus data was transformed using the 'logit' transformation and data statistically analyzed using one way ANOVA and Tukey post hoc tests to investigate significant differences. Results were considered statistically significant when P ≤ 0.05.
3. Results

3.1 Effect on Apoptosis

3.1.1. Effect of five fractions from carrot juice extracts (CJE) on apoptosis:
Treatment of three human lymphoid leukaemia cell lines (CCRF-CEM, Jurkat and MOLT-3) with unbound, water, acetonitrile and ethyl acetate fractions showed no effect on induction of apoptosis following 48 h at equivalent concentrations to those seen in 6.25, 12.5 and 25% whole CJE (Fig 1). Conversely, the acetone fraction (fraction D) showed a significant decrease in the number of live cells and significant increase in both apoptotic and dead cells in dose dependent manner within all three leukemic cell lines following 24 h of treatment (control v/s acetone fraction equivalent to concentration in 25% whole CJE P< 0.05) (Fig 2). In addition, treatment of CCRF-CEM and Jurkat but not MOLT-3 cells with the acetone fraction from CJE showed increased response following 48 h, however no further increase in response was seen following 72 h (Fig 2).

3.1.2 HPLC and MS/MS analysis of fractions from carrot juice extracts:
The analysis of the fractions prepared from CJE using HPLC and UV detection showed that both polyacetylenes and carotenoids were present in the acetone fraction but not in the other fractions and phenolic acids were concentrated in the acetonitrile fraction (data not shown). Moreover, MS/MS negative electrospray ionisation mode the measured masses of falcarinol, falcarindiol, falcarindiol-3-acetate, β-carotene and lutein were detected at molecular weight -1 due to loss one molecule of Hydrogen [30]. The cleavage on these compounds at different positions generated multiple smaller compounds which could be identified as fragments of the compound in question (Fig 3). In addition, mass spectrometry demonstrated the relative concentrations of these compounds where lutein was the most abundant out of the five compounds investigated within the acetone fraction. Falcarindiol-3-acetate and falcarinol were observed at similar levels and were the second and third most abundant compounds with β-carotene and falcarindiol at the lowest levels out of the five investigated.

3.1.3 Effect of polyacetylenes (falcarinol, falcarindiol and falcarindiol-3-acetate) from carrots on apoptosis:
Falcarinol and falcarindiol-3-acetate significantly decreased the percent of live cells and induced apoptosis in CCRF-CEM, Jurkat and MOLT-3 lymphoid leukemic cells after treatment with 25, 50 and 100 µM following 24 h stimulation to a greater extent than falcarindiol. In contrast, lower concentrations of falcarinol induced a small increase in viability within Jurkat cells, although this did not reach significance (Fig. 4). Moreover, these cell lines displayed differing sensitivity levels when treated with falcarinol: CCRF-CEM (acute lymphoblastic leukaemia) was the most sensitive cell line, with 45% of cells live following 24 h treatment and 0 % alive following 48 h treatment with 25 µM (Fig 4 and Table 2). Similarly, after 24 h treatment with 100 µM falcarindiol-3-acetate CCRF-CEM cell line was the most affected with 0% alive. While Jurkat cell line (peripheral blood T cell leukaemia) and MOLT-3 cell line (acute lymphoblastic leukaemia patient released following chemotherapy) showed 20% and 45% cells remaining viable respectively (Fig 4). The inhibitory potency effect of different polyacetylenes from Daucus carota L was falcarindiol-3-acetate > falcarinol > falcarindiol (Fig 4 and Table 2).

3.1.3. Effect of Carotenoids (β-carotene and lutein) on apoptosis:
Treatment with β-carotene on CCRF-CEM cells at concentrations of 25 µM, 50 µM and 100 µM showed a small but significant decrease in the number of live cells, whereas Jurkat and MOLT 3 cells were only affected by the
highest concentration of $\beta$-carotene (100 $\mu$M) (Fig 4). Beta-carotene treatments failed to reach $AP_{50}$ even following 100 $\mu$M treatments. Lutein treatments failed to induce apoptosis in any cell line at any concentration (Fig 4 and Table 2).

3.2 Effect on cell proliferation

3.2.1. Effect of acetone fraction from carrot juice extracts (CJE) on cell proliferation:

The effect of the acetone fraction on cell proliferation following treatment with concentrations equivalent to concentrations of compounds found within 12.5%, 25% and 50% of whole CJE after 24, 48 and 72 h showed a significant decrease in the ATP level within cultures of CCRF-CEM, Jurkat and MOLT-3 leukaemia cell lines indicating decreased cell number ($P \leq 0.05$). CCRF-CEM and MOLT-3 cells showed no significant difference in ATP levels within treatments between time points, whereas Jurkat cells showed increased response following 72 h compared to 24 and 48 h treatments (Fig 5).

3.2.2. Effect of polyacetylenes (falcariolin, falcarindiol and falcarindiol-3-acetate) from Daucus carota L on cell proliferation:

Leukaemia cell lines showed differential response when treated with 0.5 $\mu$M, 5 $\mu$M, 25 $\mu$M, 50 $\mu$M and 100 $\mu$M polyacetylenes at 24 h. All three cell lines showed greatest sensitivity to falcariolin, with significant inhibition in ATP levels at concentrations $\geq 5$ $\mu$M in CCRF CEM cells, $\geq 25$ $\mu$M in Jurkat cells and $\geq 5$ $\mu$M in MOLT 3 cells. CCRF-CEM and Jurkat cells showed greater sensitivity to falcarindiol while the MOLT-3 cell line was more affected after treatment with 100 $\mu$M falcarindiol-3-acetate than falcarindiol (Fig 6 and Table 3).

3.2.3. Effect of beta-carotene and lutein on cell proliferation:

All leukaemia cell lines showed a small decrease in the ATP level when assessed at 24 h after the 100 $\mu$M treatments with $\beta$-carotene although this only reached significance in Jurkat cells ($P \leq 0.05$) (Fig 6). Conversely an increase in ATP levels was observed within Jurkat cells treated with doses of 50 $\mu$M $\beta$-carotene or less although this did not reach significance ($P > 0.05$). Treatment of all three cell lines with any concentration of lutein did not induce any effect on ATP levels (Fig 6 and Table 3).

3.2.4. Effect of polyacetylenes (falcariolin, falcarindiol and falcarindiol-3-acetate) from Daucus carota L on cell cycle:

Lymphoblastic leukaemia cell lines showed differential response to different polyacetylene types following 24 h. However, all three cell lines showed a significant decrease in G0/G1 and a significant increase in S phase of the cell cycle when treated with 25 $\mu$M of falcariolin (Data shown for CCRF-CEM only Fig 7A). Moreover, the CCRF-CEM cell line showed an increase in sub-G0 population, which is an indicator for apoptotic cells, with 25 $\mu$M falcariolin (Fig 7 B). In contrast, falcarindiol and falcarindiol-3-acetate showed no effect on CCRF-CEM when treated with 5 and 25 $\mu$M and assessed at 24 h (Fig 7A).

The results of this study demonstrated that falcariolin and falcarindiol-3-acetate are the most active type of polyacetylenes in carrots that induce apoptosis to a greater extent than falcarindiol (Fig 4). In addition, falcariolin inhibited cell proliferation of CCRF-CEM with an IC$_{50}$ of 12 $\mu$M, Jurkat with an IC$_{50}$ of 28 $\mu$M and MOLT-3
with an IC_{50} of 46 µM. However, both carotenoids \( \beta \)-carotene and lutein showed only small effects on cell proliferation and apoptosis in all examined lymphoid leukaemia cell lines even after treatment with high concentrations.
4. Discussion:

The present study isolated different compounds by solid phase extraction (SPE) from CJE and identified a number of bioactive chemicals within *Daucus carota* L. Crude extracts from *Daucus carota* L have previously been shown to be cytotoxic to leukaemia cells, inhibiting proliferation and inducing apoptosis in leukaemia cell lines preferentially to non-tumour control cells [13]. Here, we investigated which bioactive compounds found within *Daucus carota* L are responsible for this potential anticancer activity.

Solid Phase extraction enabled separation of potential agents into different fractions, separating the polar non-retained compounds including sugars, acids, and proteins into the unbound and water fractions, polyphenolic agents in the acetonitrile fraction and polyacetylenes and carotenes in the acetone fraction. Interestingly the only fraction which demonstrated any significant biological activity was the acetone fraction which was shown to contain both polyacetylenes and carotenes. These compounds were then investigated individually to identify which of these induced apoptosis and inhibited proliferation in lymphoid leukaemia cell lines. The acetonitrile fraction which contained phenolic acids failed to induce significant apoptosis within any of the three cell types investigated, treatments were at the concentrations equivalent to those present in the whole juice extracts investigated previously and thus was not investigated further. The negative results found on phenolic fractions (Acetonitrile fraction) could be due to low levels of phenolic acids within this cultivar of *Daucus carota* L, indeed orange carrots contain lower levels of phenolic acids than other coloured varieties such as purple carrots [31], thus it is possible that phenolic fractions from these richer sources of phenolic acid cultivars may show biological activity.

The current study demonstrated that pure commercial β-carotene showed minimal effects on induction of apoptosis or inhibition of cell growth following 24 h. Similarly Hansen et al., (2003) showed no inhibitory effect for β-carotene between 0.001 - 100 μg mL⁻¹, where as falcariolin showed a very potent inhibitory effect in the concentrations 1 and 10 μg mL⁻¹ on mammary epithelial cells [21]. However, Upadhyaya et al., (2007) demonstrated a significant toxic effect of β-carotene at a dose of 20-100 μM towards U937 (histiocytic lymphoma) and HL-60 (Human promyelocytic leukaemia cells) when assessed after 12 h [20]. They also reported a number of morphological abnormalities with inclusion of apoptotic bodies and DNA fragmentation in both cell lines [20]. The reasons for these conflicting results are unclear, whether the source of β-carotene used in these previous studies was not pure, and thus may have contained additional agents such as polyacetylenes, or it is possible that the differing effects are due to variation in sensitivity of different cell types.

In addition to β-carotene, lutein is another type of carotenoid, which was expressed at high concentrations in the acetone fraction from CJE. However within the three human lymphoid leukaemia cell lines investigated here no effect was seen on apoptosis or cell proliferation in concentrations from 0.5 μM to100 μM following 24 and 48 h. On the other hand, an *in vivo* study on mouse mammary cancer has demonstrated that dietary lutein (semi purified) inhibits cancer growth by decreasing the expression of anti-apoptotic gene Bcl-2 and up regulating pro-apoptotic genes: Tp53 and Bax [28]. These mice fed with lutein also showed 35% smaller tumor size on day 50 compared with mice fed with an un-supplemented diet [28]. Lutein (semi-purified) treatment has also been shown to induce apoptosis selectively in transformed human mammary cells but not within normal mammary cells after 4 days [27]. The difference in observations between our results and the results from these studies
could indicate a difference in sensitivity between mammary cancers and leukaemia. Alternatively as the lutein treatment within the studies on mammary cancers was not pure, it is possible these extracts were contaminated with other bioactive agents such as polyacetylenes which could have provided the effects that were attributed to lutein.

In this study, we demonstrated that falcarinol, and falcarindiol-3-acetate isolated from Daucus carota L significantly decreased the percentage of live cells and increased the number of both apoptotic and dead cells within three human lymphoid leukaemia cell lines (CCRF-CEM, Jurkat and MOLT-3) at concentrations 25-100 µM to a greater extent than falcarindiol, which agrees with previous studies on alternative cell types (Table 1) [15,17-19]. However, total cell number significantly increased following treatment with low concentrations of falcarindiol-3-acetate within Jurkat and MOLT 3 cells, although this did not reach significance. Similarly, treatment with falcarinol (0.5 and 1 µM) showed an increase in cell number of CaCo-2 after 72 h [18]. The increase in cell proliferation at low concentrations, known as hormesis, is a characteristic feature of bioactive compounds [32,33], and has been observed in all studies on other cell types where appropriate ranges have been studied [15, 18, 21, 25, 34]. Interestingly within the cell types studied here we only demonstrated a hormesis effect for falcarindiol-3-acetate. Purup et al (2009) showed that falcarinol, isolated from carrots, showed a more potent effect on inhibition of cell proliferation in a human cancer intestinal cell line (CaCo-2) than falcarindiol-type polyacetylene [15]. Another study by Young and colleagues (2007), showed that the polyacetylene falcarinol, extracted from carrots, could cause a decrease in cell proliferation and increase apoptosis in CaCo-2 (human colon carcinoma) cells at high concentrations (≥20 µM) after 72 h [18]. Here we showed an IC₅₀ of falcarinol of 12, 15 and 35 µM within CCRF-CEM, Jurkat and MOLT-3 leukaemia cell lines respectively, this concentration range collaborates the dose responses of falcarinol in a number of previous studies (Table 1) [15,17-19].

Few studies to date have investigated the effects of polyacetylenes on cell proliferation. Previous work on carrot extracts containing 2.5 µg/mL of falcarinol and 10 µg/mL of falcarindiol on both a normal human epithelial cell line (FHs 74 Int.) and a human colon cancer cell line (CaCo-2) showed significant inhibition of cell proliferation under basal growth conditions [15]. Here, we found that all three types of polyacetylenes showed an inhibition on cell proliferation, indicated by reduced ATP level, when assessed at 24 h. However, the cell lines showed various levels of sensitivity to different polyacetylenes: CCRF-CEM and Jurkat were affected following treatment with falcarinol and falcarindiol; whereas: MOLT-3 cells were more affected after treatment with falcarinol: falcarindiol-3-acetate and falcarindiol respectively.

Here, we investigated, for the first time, the potential of polyacetylenes to arrest cells at stages of cell cycle. The treatment of three lymphoid leukaemia cell lines (CCRF-CEM, Jurkat and MOLT-3) with 25 µM falcarinol showed a significant cell accumulation at S phase of the cell cycle when assessed at 24 hours, whereas the other polyacetylenes failed to show responses on cell cycle. Similarly, an in vitro study showed S phase arrest on CCRF-CEM, Jurkat and MOLT-3 cell lines when treated with (25%) whole carrot juice extraction for 24 h [13]. Arrest of cells within S phase can indicate disruption of DNA repair mechanisms and prevention of cellular proliferation which could pose an additional therapeutic approach to reducing tumour cell burden together within induction of cell death.
However the hormesis effects seen following treatment with falcarindiol-3-acetate could indicate that the concentrations attained via consumption may not be sufficient to generate the anti-cancer actions and could potentially be detrimental to cancer prevention. Identification of the concentrations attained via consumption and circulation via the blood and targeting to the bone marrow will be essential to determine if the concentrations of the agents required to induce the anti-cancer effect could be attained via consumption. Previous studies, have indicated that plasma concentration of polyacetylenes only reach 0.01 µM suggesting that normal consumption can not lead to the doses required to generate cytotoxic properties [25]. Feeding studies of falcarinol in a rat colon cancer model, demonstrated biological effects with a dose of 35 µg falcarinol per gram feed were equal to the effects of entire carrots at the same dose [19]. Corroborating our study which suggests falcarinol is the major bioactive species within carrots, and suggests bioactive levels of falcarinol can feasibly be attained via consumption [19]. Alternatively these agents could be extracted and delivered pharmaceutically. The acute LD$_{50}$ of falcarinol within mice after injection has been shown to be around 400 µM [35], thus the AP$_{50}$ of falcarinol of 18-68 µM is promising for targeted toxicity to cancerous cells in particular if they act synergistically with other drugs allowing each drug to be used at a lower dose causing fewer side effects than when used on its own, such as that suggested for sulphoraphane [36,37,38]. Future studies will focus on the elucidation of the mechanisms of action of these polyacetylenes particularly in relation to induction of apoptosis and cell cycle arrest. Together with investigation into whether they can work synergistically with current chemotherapeutic agents will aid our understanding of the usefulness of these agents in leukaemia therapies.

The significance of the our study lies in the observation that the polyacetylenes falcarinol and falcarindiol-3-acetate types of polyacetylenes isolated from Daucus carota L induced apoptosis and inhibited cell cycle in human lymphoid leukaemia cells, and falcarindiol induced apoptosis in CCRF-CEM cells only whereas, the two types of carotenoids; β-carotene and lutein showed little effect on any of the three examined cell lines. Suggesting that the polyacetylenes rather than carotenoids demonstrate effects on apoptosis and proliferation on cancer cells, this would resolve the paradox where increased carrot consumption is associated with reduced cancer incidence despite the failed associations with beta-carotene. The combined effect of polyacetylenes on induction of apoptosis and inhibition of cell cycle indicates that these agents could be beneficial in the treatment of leukaemia.
Abbreviations used
CJE, carrot juice extract; DPBS, Dulbecco's Phosphate-Buffered Saline; IC₅₀, half maximal inhibitory concentration; HPLC; High-performance liquid chromatography; SPE, solid phase extraction; PI, propidium iodide; UV, ultraviolet.

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The authors wish to thank Dr. Gordon McDougall (Plant Products and Food Quality, The James Hutton Institute, Invergowrie, Dundee DD2 5DA, Scotland) for advice on Solid Phase Extraction and Dr. Sarah Haywood-Small for help on Flow Cytometry. The study was funded by The Saudi Arabian Ministry of Health.
Figure Legends:

Figure 1: Apoptotic induction by CJE fractions generated by Solid phase extraction within three leukaemia cell lines (CCRF-CEM, Jurkat and MOLT-3). Cells treated for 48 h with unbound, water, acetonitrile, acetone and ethyl acetate fractions at concentrations equivalent to compound concentrations found in 6.25, 12.5 and 25% whole CJE, Apoptosis investigated using Annexin V/PI staining. Data are presented as means ± standard error. * = P ≤ 0.05.

Figure 2: Induction of apoptosis by acetone fraction generated by SPE of carrot juice extracts within three leukaemia cell lines (CCRF-CEM, Jurkat and MOLT-3). Cells treated for 24, 48, 72 h with acetone fractions at concentrations equivalent to compound concentrations found in 12.5, 25, and 50% whole CJE, Apoptosis investigated using caspase 3 activity assay and analysed by flow cytometry. Live cells normalized to vehicle controls and presented as means ± standard error. * = P ≤ 0.05.

Figure 3: Negative ionization mode MS/MS on acetone fraction isolated from CJE. MS/MS performed for falcarinol (M - H)− ion at 243 (A), falcarindiol at 259 (B), falcarindiol-3 acetate at 301 (C), beta-carotene at 535 (D) and lutein at 567 (E).

Figure 4: Apoptotic induction within three leukaemia cell lines (CCRF-CEM, Jurkat and MOLT-3). Cells treated for 24 h with falcarinol, falcarindiol, falcarindiol-3-acetate, β-carotene or lutein. Apoptosis investigated using caspase 3 activity assay and analysed by flow cytometry. Live cells normalized to vehicle controls and presented as means ± standard error. * = P ≤ 0.05.

Figure 5: Cellular proliferation investigated by measuring ATP levels within three leukaemia cell lines (CCRF-CEM, Jurkat and MOLT-3). Following treatment for 24, 48 and 72 h with acetone fractions at concentrations equivalent to compound concentrations found in 12.5, 25, and 50% whole CJE, ATP levels determined using Cell Titer Glo assay. ATP levels normalized to vehicle controls and presented as means ± standard error. * = P ≤ 0.05.

Figure 6: Cellular proliferation investigated by measuring ATP levels within three leukaemia cell lines (CCRF-CEM, Jurkat and MOLT-3). Following treatment for 24 h with falcarinol, falcarindiol, falcarindiol-3-acetate, β-carotene or lutein. ATP levels determined using Cell Titer Glo assay. ATP levels normalized to vehicle controls and presented as means ± standard error. * = P ≤ 0.05.

Figure 7: Accumulation of CCRF-CEM cells in S Phase following 24 h treatment with falcarinol. No effect was observed with the other polyacetylenes. Proportion of cells within each stage of cell cycle analysed within CCRF-CEM following 24 h of treatment with three polyacetylenes types (falcarinol, falcarindiol and
falcarnidol-3-acetate (A). Cell cycle histograms displayed for CCRF-CEM treated with 0, 0.5 and 25 μM falcarnol analysed with FlowJo software using Waston pragmatic model (B)
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cells</th>
<th>IC$_{50}$ concentration</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Falcarinol</td>
<td>CEM-C7H2</td>
<td>3.5 µM</td>
<td>Zidorn et al, 2005</td>
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<td>Falcarinol</td>
<td>CaCo-2</td>
<td>10.2 µM</td>
<td>Purup et al, 2009</td>
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<td>Falcarinol</td>
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<td>30.1 µM</td>
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<td>HRT-18</td>
<td>42.3 µM</td>
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<td>Falcarinol</td>
<td>CaCo-2</td>
<td>50- 100 µM</td>
<td>Young et al, 2007</td>
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<td>HT2919</td>
<td>60 µM</td>
<td>Zidorn et al, 2005</td>
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<tr>
<td>Falcarindiol</td>
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<td>Falcarindiol</td>
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<td>Falcarindiol</td>
<td>HT2919</td>
<td>&gt; 100 µM</td>
<td>Zidorn et al, 2005</td>
</tr>
</tbody>
</table>

**Table1:** Falcarinol and falcarindiol effects on cell lines and animal models from published literature [15,17-19]
<table>
<thead>
<tr>
<th></th>
<th>FAL</th>
<th>FAOH</th>
<th>F3A</th>
<th>Beta-carotene</th>
<th>Lutein</th>
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<tr>
<td>CCRF-CEM</td>
<td>26 µM</td>
<td>45 µM</td>
<td>23 µM</td>
<td>Not reached 50%</td>
<td>Not reached 50%</td>
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<tr>
<td>Jurkat</td>
<td>18 µM</td>
<td>No reached 50%</td>
<td>30 µM</td>
<td>Not reached 50%</td>
<td>Not reached 50%</td>
</tr>
<tr>
<td>MOLT-3</td>
<td>68 µM</td>
<td>No reached 50%</td>
<td>38 µM</td>
<td>Not reached 50%</td>
<td>Not reached 50%</td>
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</table>

**Table2:** This table summarises the AP50 inhibition effect (causing apoptosis of 50% of the cells) for falcarinol, falcarindiol, falcarindiol-3-acetate, β-carotene and lutein treatment when assessed at 24 hours.
### Table 3

This table summarises the IC₅₀ inhibition effect for falcarinol, falcarindiol, falcarindiol-3-acetate, β-carotene and lutein treatment when assessed at 24 hours.

<table>
<thead>
<tr>
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<th>FAL</th>
<th>FAOH</th>
<th>F3A</th>
<th>Beta-carotene</th>
<th>Lutein</th>
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<tbody>
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<td>CCRF-CEM</td>
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<tr>
<td>Jurkat</td>
<td>15 µM</td>
<td>45 µM</td>
<td>77 µM</td>
<td>Not reached 50%</td>
<td>Not reached 50%</td>
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<tr>
<td>MOLT-3</td>
<td>35 µM</td>
<td>No effect</td>
<td>71 µM</td>
<td>Not reached 50%</td>
<td>Not reached 50%</td>
</tr>
</tbody>
</table>


Figure 1:

**CCRF-CEM**

Unbound Water Acetonitrile Acetone Ethyl acetate

**Jurkat**

Unbound Water Acetonitrile Acetone Ethyl acetate

**MOLT-3**

Unbound Water Acetonitrile Acetone Ethyl acetate
Figure 2:
Figure 3:
Figure 4
Figure 5:
Figure 6:
Figure 7: