# RESEARCH ARTICLE

# Wild Carrot Oil Extract is Selectively Cytotoxic to Human Acute Myeloid Leukemia Cells

**Mirna Tawil[1], Amira Bekdash[1], Mohammad Mroueh[2], Costantine F Daher[1], Ralph J Abi-Habib[1]**

## Abstract

　**Background: In this study, we used *Daucus carota* oil extract (DCOE) to target acute myeloid leukemia (AML) cells. All the AML cell lines tested were sensitive to the extract while peripheral mononuclear cells were not. Analysis of mechanism of cell death showed an increase in cells positive for annexinV and for active caspases, indicating that DCOE induces apoptotic cell death in AML. Inhibition of the MAPK pathway decreased sensitivity of AML cells to DCOE, indicating that cytotoxicity may be dependent on its activity. In conclusion, DCOE induces selective apoptosis in AML cells, possibly through a MAPK-dependent mechanism.**

**Keywords:** AML - *Daucus carota* - apoptosis - MAPK

## Introduction

　Acute myeloid leukemia (AML) is one of the most common leukemias in adults with an estimated 13,780 newly diagnosed cases and an estimated 10,200 deaths in the US in 2012 (American Cancer Society, 2012). Though a high proportion of AML patients enter complete remission following combination induction and consolidation chemotherapy, most patients eventually relapse due to persistence of chemotherapy-resistant blasts in the bone marrow (Bennett et al., 2002). Hence, alternative approaches employing novel mechanisms for targeting AML blasts are being actively sought.

　Natural products and plant extracts have been investigated extensively for potential anti-cancer activity in a number of solid and hematological malignancies, including AML, CML, Hodgkin's and non-Hodgkin's lymphomas and lymphoid leukemias (Omoregie et al., 2013; Ramkumar et al., 2013; Asmaa et al., 2014). Also, natural products and plant extracts have been investigated for their ability to protect against radiation-induced toxicity (Kma, 2014). Importantly, A number of plant-derived compounds have been shown to be active against AML cells such as icarised II, isolated from the stems and leaves of Epimedium koreanum, securinine, isolated from the root of Securinega suffruticosa, and flavopiridol, isolated from the stem bark of Amoora rohituka and Dysoxylum binectariferum (Nelson et al., 2006; Gupta et al., 2011; Kang et al., 2012). However, the potential of naturally occurring products in AML has not been fully explored yet, hence the need for identifying novel plant-derived compounds for potential anti-AML activity.

　Wild carrot, *Daucus carota* L. ssp. carota (Apiaceae), is one of many umbilliferous plants growing around the world (Van Wyk et al., 2004). The plant is edible when young but the root gets tough and woody when matured. In Lebanon, the plant is part of folk medicine for the treatment of cancer, gastric ulcer, diabetes, muscle and back pain, and for enhancing liver function and immune system. The essential oil of wild carrot obtained from the aerial part of the plant at the end of the flowering stage was reported to have antimicrobial and antifungal activities (Staniszewka et al., 2005; Rossi et al., 2007; Maxia et al., 2009). Wild carrot was also reported to possess antilithic, diuretic, and carminative properties, and has been used traditionally to treat urinary calculus, cystitis, gout, and lithuria (Barnes, 1998; Thomas et al., 2001; Rossi et al., 2007). In European folk medicine, the volatile oil from wild carrot is used as an antiseptic and anti-inflammatory remedy for cystitis and prostatitis (Hoffman, 1990). Additionally, it was demonstrated that the dichloromethane-methanol extract of the wild carrot flower possesses significant antioxidant activity (Akgul et al., 2009). Similarly, several studies have reported that a new type of sesquiterpene isolated from the fruits of wild carrot displays cytotoxic activity against a number of human gastric cancer cell lines (Wei et al., 2009).

　Recently, we reported that the aqueous and methanolic extracts of the wild carrot umbels exhibit anti-inflammatory and antiulcer activities (Wehbe et al., 2009). We also found that Daucus carota oil extract (DCOE) has anti-tumor activity in a DMBA/TPA skin carcinogenesis model in mice in addition to anti-cancer and antioxidant activity against a wide range of tumor types including human

*[1]Department of Natural Sciences, School of Arts and Sciences, [2]Department of Pharmaceutical Sciences, School of Pharmacy, Lebanese American University, Beirut, Lebanon  *For correspondence: ralph.abihabib@lau.edu.lb*

colon (HT-29, Caco-2) and breast (MCF-7, MDA-MB-231) cancer cell lines (Zeinab et al., 2011; Shebaby et al., 2013). However, the effects if DCOE have not been investigated yet in hematological malignancies. In this study, we investigate the potential for targeting human AML cell lines with DCOE and determine its underlying mechanism of action.

## Materials and Methods

*Preparation of daucus carota oil extract:*

Daucus carota oil extract (DCOE) was prepared using Methanol/Acetone (1:1) extraction of the plant umbel as described previously (Zeinab et al., 2011). Briefly, mature umbels of Daucus carota (Linnaeus) ssp. carota were collected in August from their natural habitat in Lebanon. No specific permission was required to collect the plant from the particular locations in which it grows. The field studies consisted of collecting mature Daucus carota umbels only, hence did not involve endangered or protected species. The plant was identified according to the characteristics described in 'Handbook of Medicinal Herbs'.8 Stripped umbels were air dried in the shade and then cut into small pieces for oil extraction. Leaves were soaked in methanol/acetone (1:1) and the extract was then filtered and evaporated to dryness under reduced pressure. The residue was centrifuged, and the oil was dried over anhydrous sodium sulfate. The final yield (3.47%) was stored in a closed amber bottle at 4°C until used.

*Cells and cell lines*

Human AML cell lines HL60, U937, ML1, ML2, Mono-Mac-1, Mono-Mac-6, KG-1, MV-4-11, TF1-vRaf, TF1-vSrc and TF1-HaRas were grown as described previously (Abi-Habib et al., 2004).

Human peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque gradient as described previously (Tanios et al., 2013). Peripheral blood samples were collected from healthy adult individuals based on approval by the Committee on Human Subjects in Research (CHSR) of the Lebanese American University and following written informed consent by the donors. Briefly, 10 ml of blood were diluted 3-fold in dilution buffer (phosphate buffered saline, 2 mM EDTA), layered carefully over ficoll-paque and centrifuged at 4500 rpm for 20 min. The layer corresponding to PBMCs was isolated, transferred to 45 ml of dilution buffer, centrifuged twice at 3000 rpm for 20 min at 20°C and the resulting pellet re-suspended in 10 ml growth media.

*Proliferation inhibition assay (cytotoxicity)*

Sensitivity of AML cell lines and normal human PBMCs to Daucus carota oil extract (DCOE) was determined using a proliferation inhibition assay as described previously (Abi-Habib et al., 2005). Briefly, aliquots of 104 cells/well, in 100 µl cell culture medium ($10^5$ cells/well in 100 $\mu$l media for PBMCs), were plated in a flat-bottom 96-well plate (Corning Inc. Corning, NY). Then, 50 µl DCOE in DMSO, or DMSO alone, in media were added to each well to yield concentrations ranging from 100 $\mu$g/mL to 0.5 ng/mL. Following a 48 h

incubation (24 h incubation only for PBMCs) at 37°C/5% $CO_2$, 50 µl of XTT cell proliferation reagent (Roche, Basel, Switzerland) were added to each well and the plates incubated for another 4 h. Absorbance was then read at 450 nm using a microplate reader (Thermo Fisher Scientific, Waltham, MA). Nominal absorbance and percent maximal absorbance were plotted against the log of concentration and a non-linear regression with a variable slope sigmoidal dose-response curve was generated along with IC50 using GraphPad Prism 5 software (GraphPad Software, San Diego, CA).

*Cell cycle analysis*

The impact of DCOE treatment on the cell cycle of AML cells was determined using Propidium Iodide (PI)-staining, as described previously (Kassab et al., 2013). Briefly, cells incubated with 2 different concentrations of DCOE (40 and 5 $\mu$g/mL) or media alone for 24 and 48 h at 37°C/5% $CO_2$, were harvested and fixed in 70% ethanol for a minimum of 24 h, at -20°C. Cells were then incubated in 500 µl PI staining solution (50 $\mu$g/ml) for 40 min at 37°C. Samples were then read on a C6 flow cytometer (BD Accuri, Ann Arbor, MI) and total cell DNA content was measured on FL2-H. Percent of cells in G0/G1, S and G2/M phase was determined in control cells and in cells treated with the 3 different concentrations of DCOE following gating on width versus forward scatter.

Analysis of cell cytotoxicity

Determination of apoptotic versus non-apoptotic cell death was carried out using an Annexin V-fluorescin Isothiocyanate (Annexin V-FITC) and Propidium Iodide (PI) labeled apoptosis/necrosis detection kit (Abcam, Cambridge, MA) and a FITC-conjugated active caspase inhibitor (ApoStat Apoptosis Detection Kit, R&D Systems, Abingdon, England), as described previously (Kassab et al., 2013). Briefly, cells were incubated with either 100 µl of medium alone (control cells) or medium containing two different concentrations of DCOE (as described above under cell cycle analysis) for 6 and 24 h (for active caspase staining) and for 6, 24 and 48 h (for annexin V/PI staining) at 37°C/5% $CO_2$. Cells were then harvested and incubated with a FITC-conjugated annexin V antibody (2.5 mg/ml) and PI (5 mg/ml) in antibody binding buffer for 45 min at 37°C or incubated with 0.5 $\mu$g/ml of apostat for 30 min then harvested. Cells were then read using a C6 flow cytometer. Annexin V/PI data was analyzed on FL1-H versus FL2-H scatter plot and active caspases were detected on FL1-H. Unstained cells were used as negative control. Cells with positive annexin V staining, negative PI staining and positive active caspase staining were considered apoptotic, while cells positive for both annexin V and PI staining and negative for active caspase staining were considered non-apoptotic.

*Inhibition assays*

AML cells were incubated with DCOE (40 $\mu$g/ml) in the presence or absence of the small molecular weight mitogen-activated protein/extracellular signal-regulated kinase kinase 1/2 (MEK1/2) inhibitor U0126 (Cell Signaling Technology, Danvers, MA). Briefly, 104 cells/

well were plated in 100 µl of medium in a flat-bottom, 96-well plate. Then 100 µl of either medium alone (control cells) or medium containing DCOE (40 µg/mL), U0126 (20 and 50 µM) or a combination of the above were added. Cells were then incubated for 48 h at 37°C/5% $CO_2$ followed by the addition of 50 µl of XTT cell proliferation reagent (Roche, Basel, Switzerland). Cells were incubated for another 4 h and absorbance was read at 450 nm using a 96-well plate reader (Thermo Fisher Scientific, Waltham, MA). Data was analyzed using GraphPad Prism V software (GraphPad Software, San Diego, CA). Total absorbance and percent absorbance of controls were compared between the different treatment groups.

*Intracellular staining and flow cytometry analysis*

Activation of the MEK1/2-ERK1/2 pathway in AML cell lines was assessed by determining the presence or absence of phospho-MEK1/2 using flow cytometry as described previously (Kassab et al., 2013). Approximately $3 \times 10^6$ cells were fixed in 70% ethanol for 15 min. Cells were then incubated with a 1/100 dilution of anti-phospho-ERK1/2 (Ser 217/221) rabbit monoclonal antibodies (Cell Signaling Technology, Danvers, MA) in antibody binding buffer for 1 h at 37°C, followed by a 30-minute incubation with a 1/100 dilution of a FITC-conjugated mouse anti-rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Fixed cells stained only with FITC-conjugated mouse anti-rabbit polyclonal antibody were used as isotypic control. Samples were then analyzed using a C6 flow cytometer (BD Accuri, Ann Arbor, MI). Positivity for the presence of phospho-ERK1/2 was determined using the ratio of fluorescence intensity (RFI) between the mean fluorescence intensity (MFI) of the stained cells and the MFI of the isotypic control. RFI ≥ 2 was considered positive.

## Results

*Cytotoxicity of Daucus carota oil extract*

DCOE was cytotoxic to all 11 AML cell lines with IC50 values ranging from 1.0 to 26.2 µg/mL and a percent

**Table 1. Sensitivity of Human AML Cell Lines to DCOE**

| Cell line | DC crude oil extract (IC50;µg/mL) |
|---|---|
| U937 | 1 |
| KG-1 | 1 |
| HL60 | 5.5 |
| TF1-VSrc | 7.3 |
| Mono-Mac-6 | 12.4 |
| Mono-Mac-1 | 13.2 |
| TF1-VRaf | 14.4 |
| MV-4-11 | 14.9 |
| ML1 | 19.6 |
| TF1-HaRas | 26 |
| ML2 | 26.2 |
| Normal PBMCs | >100 |

cell kill at highest concentration ranging from 70 to 96% (Figure 1). To exclude any contribution of DMSO, the excipient used to re-suspend the extract, to the cytotoxicity of DCOE, we tested the effects of DMSO alone, at an equivalent volume, on the panel of AML cells. DMSO alone did not cause any significant cytotoxicity, hence excluding its contribution to the observed cytotoxicity of DCOE to human AML cell lines (Table 1).

Normal human peripheral blood mononuclear cells (PBMCs) were not sensitive to DCOE with an IC50 > 100 µg/mL and a percent cell kill at the highest concentration ≤ 50%) (Figure 1).

*Cell cycle effect of daucus carota oil extract*

In order to determine whether DCOE induces cell cycle arrest in AML cells, we determined the cell cycle status of a subset of AML cell lines (ML1, ML2 and U937) following 24 and 48 h incubation with two different concentrations of extract. None of the cell lines tested showed any effect of DCOE treatment on cell cycle at either concentration and at both 24 and 48 h incubation (Figure 2A and B). The fraction of cells in both the G0/G1 and G2/M phases decreased with increasing concentrations of DCOE. This was accompanied by an increase in the fraction of cells in the pre-G0/G1 phase (>95% of total events) at the
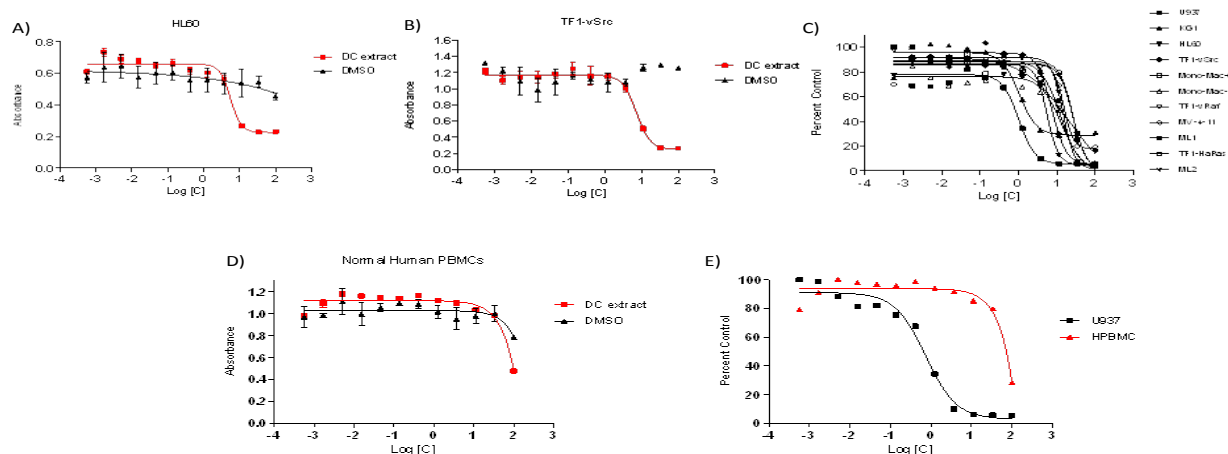


**Figure 1. Non-Linear Regression Curves of DCOE (square) and DMSO (triangle) on Human AML Cell Lines.** Two representative cell lines HL60 (A) and TF1-vSrc (B) are sensitive to DCOE. C) Compilation of DCOE non-linear regression curves on all AML cell lines tested. D) Non-linear regression curves of DCOE (square) and DMSO alone (triangle) on human normal PBMCs. E) Comparison of non-linear regression curves of DCOE on U937 cells and human normal PBMCs
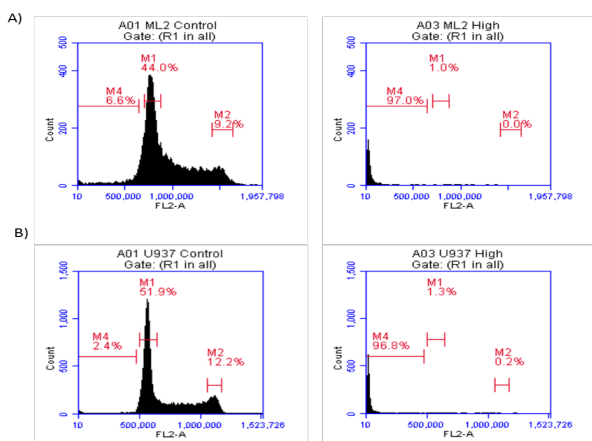
**Figure 2. Cell Cycle Analysis of the ML2 (A) and U937 (B) AML Cell Lines Following Treatment with DCOE.** Control cells are represented in the left panels and cells treated with 40 $\mu$g/mL DCOE for 48 h in the right panels. Cells are gated on width versus forward scatter (R1). Cells in G0/G1 are gated M1, G2/M are gated M2 and pre-G0/G1 (dead) are gated M4
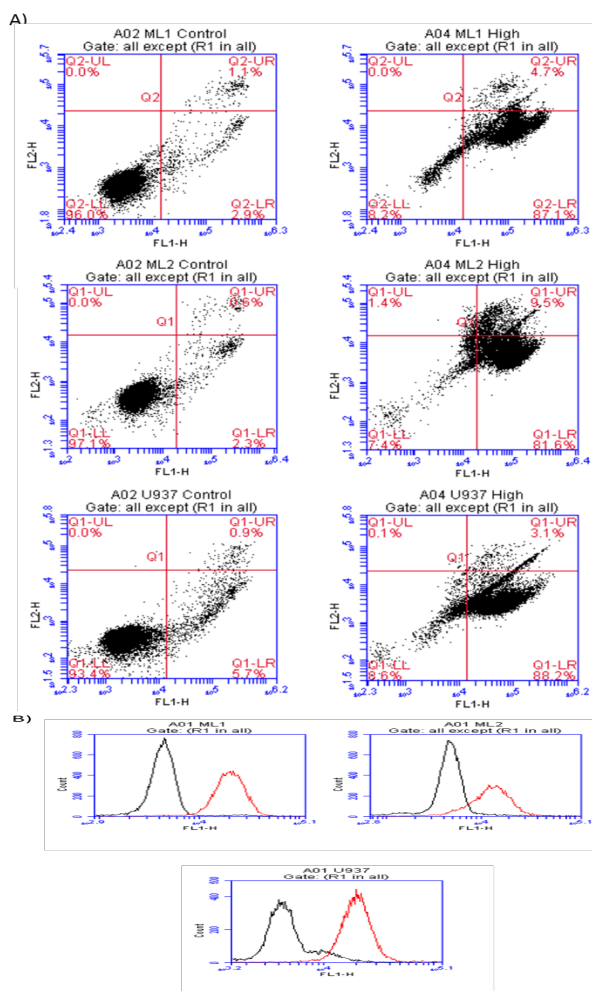


**Figure 3. Analysis of DCOE-Mediated Cytotoxicity in ML1, ML2 and U937 Cells Using Annexin V/PI (A) and Active Caspase Staining (B).** All 3 cell lines incubated with 40 $\mu$g/mL DCOE for 48 h (right panel) stained positively with annexin V (FL1-H) and negatively with PI (FL2-H). Incubation of DCOE-treated ML1, ML2 and U937 cells with a cell permeable, FITC-conjugated active caspase inhibitor revealed high levels of caspase activation following 24 h incubation with the extract (red)

highest concentrations of the extract at both 24 and 48 h incubation, indicating that treatment induces complete cytotoxicity in the totality of treated cells.

*Analysis of cell death*

To determine the type of cell death observed following treatment of AML cells with DCOE, we tested for caspase activation and annexin V/PI staining, in a subset of cell lines (ML1, ML2 and U937), treated with two different concentrations of extract (40 and 5 $\mu$g/mL) for 6, 24 and 48 h. In all three cell lines, a dose-dependent increase in the percentage of cells with positive annexin V staining and negative PI staining was observed, at all the time points tested, indicating apoptotic cell death (Figure 3). Staining for active caspases, following treatment with DCOE, revealed a dose-dependent increase in caspase activation, in all AML cell lines tested, following treatment with the extract for 6 and 24 h (Figure 3). The percentage of cells staining positive for the presence of active caspases increased from 2.4%, 3.2% and 10.1% in non-treated cells to 88%, 67.7% and 92.8% in cells treated with 40 $\mu$g/mL DC crude oil extract, for the ML1, ML2 and U937 cell lines, respectively, at 24 h post treatment. The increase in the percentage of cells showing positive annexin V staining and negative PI staining, in addition to caspase activation, indicates that DCOE induces caspase-dependent, apoptotic cell death in AML cell lines (Table 2).

*Inhibition of MAPK pathway*

In order to determine the impact of the inhibition of the Ras-Raf-MEK1/2-ERK1/2 pathway on the sensitivity of AML cell lines to DCOE, we tested the cytotoxicity of the extract on a subset of AML cell lines (ML2, U937 and TF1-vSrc) in the presence of two different concentrations of the small molecular weight MEK1/2 inhibitor U0126. Co-Incubation of AML cell lines with both DCOE (40 $\mu$g/mL) and U0126 (20 and 50 $\mu$g/mL) induced a statistically-significant, dose-dependent decrease in cytotoxicity compared to DCOE alone (40 $\mu$g/mL). Addition of 20 $\mu$M U0126 increased the percentage of surviving cells by a range of 13% to 21% compared to the extract alone, while addition of 50 $\mu$M U0126 induced a more significant increase in the percentage of surviving cells ranging from 23% to 35%, compared to DCOE alone, following a 48 h incubation (Figure 4). Furthermore, in one of the cell lines
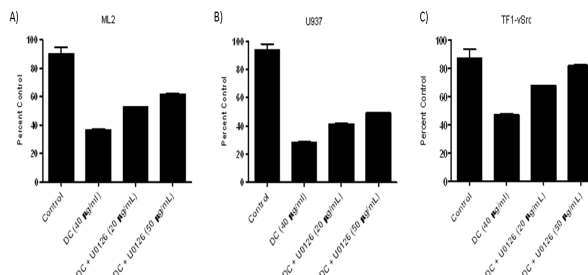


**Figure 4. Sensitivity of AML Cell Lines to DCOE Alone and in Combination with U0126.** AML cell lines ML2 (A), U937 (B) and TF1-vSrc (C) are sensitive to DCOE alone. Co-incubation with the small molecular weight MEK1/2 inhibitor induces a statistically significant, dose-dependent decrease in the sensitivity of AML cells to the extract.
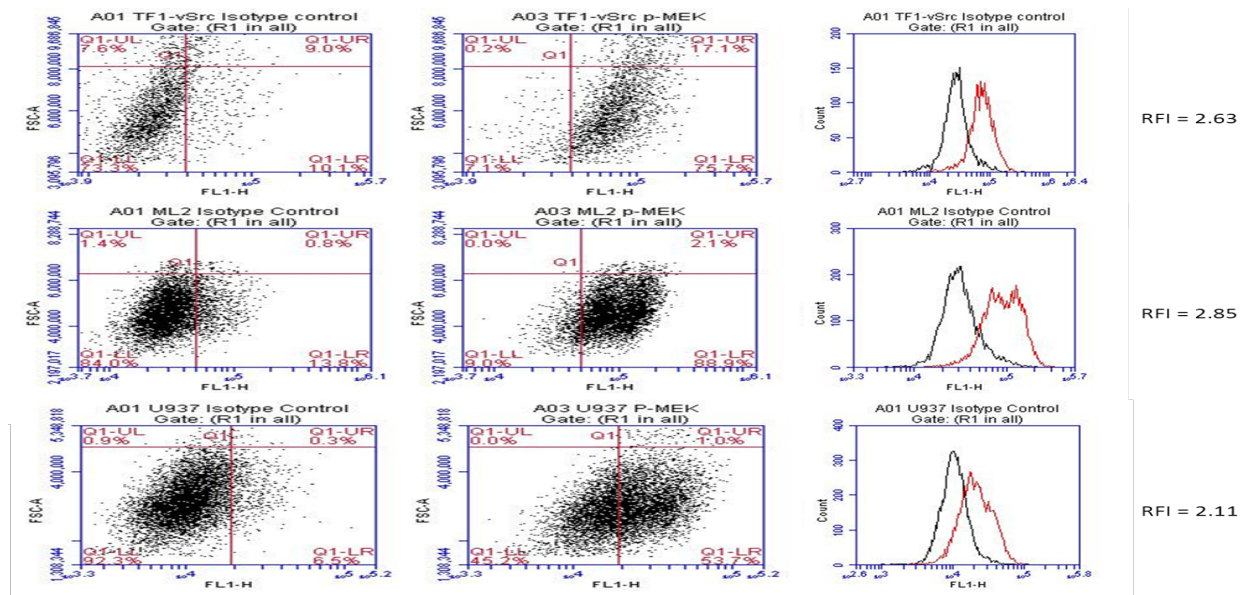
**Figure 5. Single Cell, Intracellular Staining of Phospho-MEK1/2 Using Flow Cytometry.** ML2 (A), TF1-vSrc (B) and U937 (C) cells, which were rescued by U0126, were positive for phospho-MEK1/2 as evidenced by a ratio of fluorescence intensity (RFI) > 2 between cells stained for phospho-MEK1/2 and cells incubated with the isotype control (black). Cells are gated on width versus forward scatter (R2 and R3)

**Table 2. Percent of AML Cells Positive for Annexin V Staining Following Treatment with DCOE**

| Cell line | Time Point | Non-treated cells | Cells treated with 5 $\mu$g/mL | Cells treated with 40 $\mu$g/mL |
|---|---|---|---|---|
| ML1 | 6 h | 4.90% | 5.40% | 48.20% |
|  | 24 h | 4.50% | 5.50% | 66.80% |
|  | 48 h | 2.90% | 5.10% | 87.10% |
| ML2 | 6 h | 8% | 42.80% | 42.70% |
|  | 24 h | 3.90% | 23.80% | 68.20% |
|  | 48 h | 2.30% | 45.90% | 81.60% |
| U937 | 6 h | 5.50% | 6.20% | 40.50% |
|  | 24 h | 4.30% | 4.60% | 68.80% |
|  | 48 h | 5.70% | 5.00% | 88.20% |

**Table 3. Percent Survival of AML Cells Treated with DCOE Alone (40 $\mu$g/mL) or in Combination with U0126 (20 and 50 $\mu$M)**

| Cell line | DC crude oil extract | DC extract+ 20 $\mu$M U0126 (p<0.0001) | DC extract+ 50 $\mu$M U0126 (p<0.0001) |
|---|---|---|---|
| U937 | 29% | 42% | 50% |
| TF1-VSrc | 47% | 68% | 82% |
| ML2 | 37% | 53% | 62% |

tested (TF1-vSrc), addition of the 50 $\mu$M U0126 led to the complete reversal of DCOE cytotoxicity with no statistical significance observed between survival of control cells and cells treated with the combination of DCOE and 50 $\mu$M U0126 (p= 0.425) (Table 3). These results indicate that cytotoxicity of DCOE to AML cells is dependent on the activity of the Ras-Raf-MEK1/2-ERK1/2 pathway with the inhibition of this pathway significantly reducing the cytotoxic effects of the extract.

*Analysis of MAPK activation*

We examined the activation level of the MAPK pathway by determining the phosphorylation status of phospho-MEK1/2, in the subset of three AML cell lines used in the MAPK inhibition assay (ML2, U937 and TF1-vSrc). All three cell lines were positive for phospho-MEK1/2 (RFI= 2.85, 2.63 and 2.11 for ML2, TF1-vSrc and U937, respectively), demonstrating the presence of an active MAPK pathway in these cells (Figure 5). These findings match the results observed earlier in which co-incubation with U0126 partially or completely rescued cells from DCOE-induced cytotoxicity and indicate that the activity of DCOE may require the presence of an active MAPK pathway.

## Discussion

Investigating natural products and plant extracts for potential anti-cancer activity has been steadily rising over the last decades. In fact, several plant-derived natural compounds, or their derivatives, have been approved for the treatment of a wide range of solid and hematological malignancies including vinblastine used for the treatment of bladder cancer, breast cancer and Hodgkin's lymphoma, vincristine used for the treatment of lymphoid leukemias and lymphomas and etoposide used for the treatment of lung cancer, testicular cancer and certain types of Leukemias and lymphomas (Bates et al., 2013; Watanabe et al., 2013; Trifilio et al., 2013). In addition, several novel plant-derived natural products with significant anti-cancer activity against a wide array of tumor types have been recently isolated, identified and characterized including, among others, noscapine, bruceantin and silvestrol (Cuendet et al., 2004; Madan et al., 2013; Alachkar et al., 2013). One of the hematological malignancies for which novel treatments are urgently needed, and in which the investigation of plant-derived compounds has been relatively limited thus far is acute myeloid leukemia (AML).

In this study, we have shown that DCOE possesses a robust anti-AML activity as evidenced by its cytotoxicity to a panel of 11 human AML cell lines. DCOE was found

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to be highly cytotoxic to all eleven cell lines tested. The observed anti-leukemic effects of DCOE match the effects previously seen with this extract against colon and breast cancer cell lines and confirm its wide-range anti-cancer activity (Zeinab et al., 2011; Shebaby et al., 2013). Furthermore, we have shown that DCOE selectively targets AML cell lines while sparing normal blasts with human PBMCs being more than 5-fold less sensitive to the cytotoxic effects of the extract compared to AML cells. This is encouraging since it indicates that the potential active compound in Daucus carota oil extract may be selectively cytotoxic to AML cells and, subsequently, possess a large therapeutic window in this disease. Moreover, we have demonstrated that the anti-proliferative effects observed following treatment of AML cells with DCOE are entirely due to the cytotoxicity of the extract to AML cells with no contribution of cell cycle arrest, since cell cycle analysis revealed the absence of cell cycle arrest following treatment. The cytotoxicity of DCOE and the total absence of cytostatic effects support the robust anti-leukemic activity observed with this extract and are in line with previous findings using DCOE in a number of solid malignancies including breast and colon cancer (Shebaby et al., 2013). We have also shown that treatment of AML cells with DCOE induces apoptotic cell death. This was demonstrated by positive annexin V staining, persistence of membrane integrity (negative PI staining) and the presence of active caspases following treatment with DCOE. These findings are in line with results obtained by other groups testing novel plant-derived natural products in AML, such as Icariside II, shown to induce apoptosis in the U937 AML cell lines (Gupta et al., 2011).

In addition, we have tried to decipher some of the molecular mechanisms underlying the cytotoxicity of DCOE to AML cells by investigating the effects of the combination of DCOE and the small molecular weight MEK1/2 inhibitor U0126. We originally hypothesized that the combination of DCOE and U0126 may show additive/synergistic effects in AML, particularly since we have previously shown that the majority of AML cell lines are sensitive to the inhibition of the MAPK pathway (Kassab et al., 2013). However, our results showed that the combination of DCOE and U0126 was antagonistic with cells being less sensitive to DCOE in the presence of U0126, regardless of their sensitivity to U0126 alone. In addition, we have shown that all cells rescued by co-incubation with U0126 have an active MAPK pathway as evidenced by the presence of phosphorylated MEK1/2. Though unexpected, these results indicate that the Ras-Raf-MEK1/2-ERK1/2 pathway is active in AML cells and that its inhibition decreases the sensitivity of AML cells to DCOE and even lead to complete rescue in certain cell lines. These findings indicate that the cytotoxicity of DCOE to AML cells may be dependent on the activity of the MAPK pathway with a decrease in activity associated with a lower sensitivity to the extract. Since the inhibition of the MAPK pathway may lead to a decrease in cell proliferation, this might indicate that DCOE-induced cytotoxicity to AML cells could be proliferation dependent. Additional studies will be carried out to investigate in further detail the mechanisms of

DCOE cytotoxicity and the underlying mechanisms of its potent anti-AML activity.

In conclusion, we have shown that the Daucus carota oil extract is selectively cytotoxic to human AML cell lines and induces caspase-dependent apoptotic cell death. Furthermore, we have shown that DCOE-induced cytotoxicity may be dependent on the activity of MAPK pathway.

## Acknowledgements

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