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Falcarinol, a C_{17}–polyacetylene, in *Daucus carota* L. subsp. *Carota*: first report in calli of wild carrot

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Abstract

The C_{17}- polyacetylene, falcarinol is one of the most important bioactive compounds known for its roles as an anti-inflammatory, anti-fungal, anti-platelet-aggregatory and anti-tumor. The current study explored the potential of using *In vitro* culture techniques to produce falcarinol in calli of some *Daucus* genotypes growing in Syria. The current work adopted a protocol for growing seeds from wild and cultivated carrots, and producing calli *in vitro*. Results show, for the first time in literature, the possibility of qualitative and quantitative detection of falcarinol in calli from wild and cultivated carrots stored at -30 C° using LC-MS/MS and HPLC.

Keywords: *Daucus carota* L.; carrot; falcarinol; LC-MS/MS; HPLC

1. Introduction

Carrot (*Daucus carota* L., *Apiaceae*), a biennial crop, is an important vegetable crop worldwide with impressive content of phytochemicals [1] and a total world production in 2021, along with turnip, of about 41 million tonnes [2]. Carrots play a great role in human nutrition and health with pharmacological evidences from carrots have been reported in management of cognitive dysfunctions [3], reduction of cancer cells invasion [4], wounds healing activity [5], and as a defense agent against plant pathogens [6]. There are four main types of phytochemicals that contribute to carrots dietary and therapeutic value; phenolic compounds, carotenoids, polyacetylenes, and ascorbic acid [7].

Carrots among other Apiaceae family members such as parsley, celery, parsnip and fennel are known to be rich source of polyacetylenes with the C_{17}-polyacetylenes (C_{17}-PAs) falcarinol (FaOH) and falcarindiol (FaDOH) on top [8,9]. Falcarinol induces allergic skin reactions and is reactive towards amino groups in proteins forming a hapten protein complex (antigen) [10]. The C_{17}-polyacetylene, falcarinol have been shown to have several bioactivities including anti-inflammatory [11], anti-fungal [12,13], anti-platelet-aggregatory [14], and anti-tumor [15].

Isolation of metabolites from naturally grown plants is widely spread by conventional methods, except the fact that this process can be limited by environmental and seasonal constraints [16]. Plant tissue culture techniques offer a surpass solution to efficient metabolite production in a short period of time and under controlled environmental conditions [16,17]. Callus culture, hairy roots, protoplasts culture and micropropagation are top tissue culture techniques to produce secondary metabolites [17]. Tissue culture techniques have been applied in carrot biotechnology research in fields of multiplication, conservation, mutant screening, and essential oils production [18-24]. Several studies investigated secondary metabolites in carrots [5,15,25-29], and rather few studies looked in producing essential oils in carrots calli [21,22].

This study investigated the potential of *in vitro* techniques in producing the polyacetylene FaOH in calli originated from wild and cultivated carrot subspecies.

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2. Materials and Method

2.1. Plant material

Seeds from the wild *Daucus carota* L. subsp. *carota* species growing in Syria were collected and identified according to Al-Safadi [30]. Additionally, seeds from cultivated carrot cultivar (*D. carota* L. subsp. *sativus*) were also tested. Seeds collection sites and information about altitudes and rain fall are described in Jawdat et al. [29].

2.2. Callus induction

Under sterile conditions seeds were soaked in distilled water with few drops of a detergent for 30 min with continuous agitation. The seeds were then rinsed three times in sterile distilled water. Seeds were later washed with water and 70% alcohol for two minutes with agitation. This was followed by rinsing seeds three times in sterile distilled water. Seeds were transferred to 5% sodium hypochlorite for 2 min with continuous agitation. Seeds were finally rinsed several times with sterile distilled water. The seeds were placed on the surface of ½ MS medium pH 5.8 [31] with 7.5 g/L agar and 15 g/L sucrose without growth regulators in test tubes at 25 °C and 16 hrs light.

To induce callus formation, roots (~10 cm) of 6-week-old plants were cut into 1 cm-long pieces and placed on 9 cm petri dishes containing 35 mL modified MS medium at the rate of 4 pieces per petri dish. The medium contained 30 g/L sucrose, 7.5 g/L agar and supplemented with vitamins: thiamine, myo-inositol, nicotinic acid, and pyridoxine HCl (0.01, 0.1, 0.001, 0.001 g/L respectively) [19]. Growth regulators were, also, added as follows: 0.001 g/L of Kinetin and 0.003 g/L of 2, 4-D. The cultures were incubated at 27 °C in dark conditions. Cultured explants and growing callus were transferred 9 days later to fresh medium and incubated under the above-mentioned conditions. Three weeks later, the callus was transferred again to a fresh medium treated as above to obtain enough callus for analysis. Calli were collected in 50 mL falcon tubes and stored at – 30 °C for later analysis.

2.3. Extraction of Falcarinol

Callus samples were extracted following method of Pferschy-Wenzig et al. [32] using ethyl acetate. Twenty grams of callus samples were mixed with 60 mL of ethyl acetate and mixture was incubated in ultrasonic bath for 10 min at room temperature overnight. Extracts were collected and callus samples were re-extracted with 60 mL of ethyl acetate for 3 hours. Total filtrates were dried with Sodium sulfate, Na_2SO_4, and the remaining solvent was removed under a stream of N_2. Samples were then each dissolved in 1 mL of acetonitrile and tetrahydrofuran (9:1) and filtered through microfilters (0.45 µm).

2.4. Qualitative and quantitative detection of Falcarinol using LC-MS/MS and HPLC

Extracts were analyzed for the presence of FaOH by LC-MS/MS (Liquid Chromatography with Tandem Mass Spectrometry) following SIM (Single Ion Monitoring) approach by turning the instrument in the ESI (Electrospray Ionization) positive mode with the following parameters: nebulizer gas 20 psi, drying gas flow 9 mL/min, drying gas temperature 350 °C, and capillary voltage 3000 V.

Quantitative analysis was conducted using HPLC (High Performance Liquid Chromatography). External standard of FaOH (Merck) was prepared in 1 mg/mL stock using ethyl acetate and standard series of 1, 5 and 10 µg/mL was prepared. Standard and samples were analyzed for FaOH content by HPLC instrument (Agilent 1260) with DAD (diode array detector) using column Eclipse XDB C18, 4.6 *150 mm, 3.5 µm, with the following parameters: column temperature 25 °C, mobile phase acetonitrile: water (50:50), flow rate 0.8 mL/min, wavelength 205 nm, injection volume 10 µl.

3. Results and Discussion

Growing evidence is accumulating to demonstrate that chronic inflammations are linked to cancer development worldwide [33,34]. *Apiaceae* and *Araliaceae* plant families are known to have cytotoxic effects, anti-inflammatory and anti-cancer properties [13,35,36]. FaOH is a cytotoxic, anti-inflammatory and anti-cancer polyacetylenic oxylipin, commonly found in *Apiaceae*, the carrot family [37]. These properties are behind the current study on investigating the potential of continual production of this bioactive compound *in vitro*.

Several studies have been published on protocols for the production of essential oil and bioactive compounds in carrots *in vitro* [22,38,39]. However, smaller number of studies, mainly in cultivated carrots, have focused on FaOH production. A study by Dunemann and Böttcher in 2021 [9]

demonstrated that hairy roots cultures of seven differently colored cultivated carrots are potential for the accumulation of polyacetylenes, specifically FaOH. The 2021 study showed that higher content of FaOH is present in the periderm and that purple cultivated carrots have the highest content compared to other cultivated carrots [9].

The current study adopted a successful protocol (Figure 1) to produce calli from wild and cultivated carrots [22] for FaOH content analysis. The current study, up to our knowledge, is the first report on FaOH content in plant calli.

Figure 1. Wild carrot callus regeneration *in vitro*. (A) Six weeks *D. carota* subsp. *carota* plantlet grown from seeds collected in Damascus country side. (B) Six-week-old roots transferred to callus regeneration medium to be cut in 1 cm pieces. (C) Three weeks old wild carrot calli

There are several available methods for polyacetylenes extraction from roots of plants from the *Apiaceae* family, and majority of these methods include extraction of roots puree using EtOH [8,25]. Some protocols use lyophilized samples and data demonstrated that lyophilization process does not affect FaOH content when compared to fresh samples [32]. The current study followed the later protocol of extraction using callus samples stored at – 30 ºC for periods range between 3 months to several years.

The current study confirmed a qualitative detection of FaOH in calli of wild carrots at 268 m/z fragment using LC-MS/MS (Figure 2) and it is in agreement with the study of Pferschy-Wenzig et al. [32]. The later study determined FaOH by extracting the main ion species $[M+H–H2O+MeCN]^+$ at m/z 268 from lyophilized roots of 27 carrot genotypes, followed by quantitative analysis using external and internal standards and results ranged between 0.70 to 4.06 mg/100g fresh weight [32]. This ion has been also selected to use for quantification of FaOH by other groups using SIM approach [40,41].

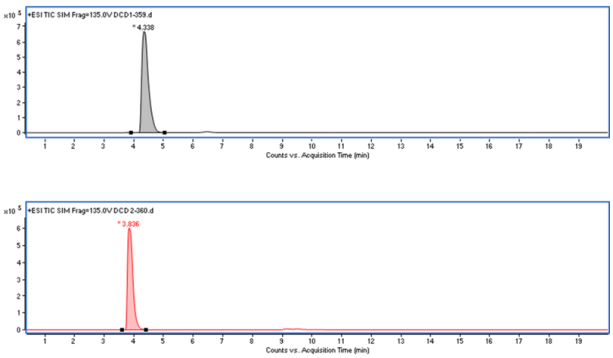

Figure 2. Qualitative detection of FaOH at 268 m/z by LC-MS/MS using SIM method. Each chromatogram represents callus sample from wild *D. carota* subsp. *carota*

Following qualitative detection of FaOH in calli of wild carrots, HPLC-UV/VIS quantitative analysis of FaOH content was conducted (Figure 3). Samples of calli generated, in the current study, from wild (*D. carota* subsp. *carota*) and cultivated (*D. carota* subsp. *satvius*) carrots, stored at -30 °C for varied periods ranging from 3 months to 5 years, showed a general decrease in FaOH content with longer storage periods. Calli stored for 3 months, 1, 3, 4, and 5 years gave 0.76, 0.29, 0.04, 0.13, and 0.03 µg/g FaOH, respectively. Stability of FaOH has been an issue when attempting preservation of this bioactive compound in food products and supplements. Most polyacetylenes are sensitive to UV and temperature which can be challenging for isolation and quantification processes [13,35]. Pure FaOH is not stable and data showed that this bioactive compound is rapidly decomposed when stored in solvents such as DMSO, n-hexane and ethanol [42]. FaOH was found to decompose rapidly in DMSO at room temperature with half-life of less than 24 hours [43]. Decomposition was delayed but not prevented when this bioactive compound was frozen to – 80 ºC in argon and protected from light [42]. Decomposition of FaOH is inevitable and this can be the reason behind the lower concentrations of FaOH in our older stored calli samples in -30 ºC.

Results of the current study showed higher content of FaOH in calli originated from cultivated carrot subspecies *sativus* with a mean of 0.41 ± 0.14 µg/g compared to a mean of 0.17 ± 0.14 µg/g in calli of wild carrot subspecies *carota*; means represent three replicates of calli, 5 grams each. Up to our knowledge, all available literature has investigated content of FaOH in cultivated carrots (*D. carota* subsp. *sativus*) [9,44], and this study is the first report on FaOH content in calli originated from wild

carrots. The current study demonstrated the potential of using regenerated calli as a sustainable and cost-effective source of falcarinol. Means to induce higher concentrations of falcarinol in calli are yet to be investigated.

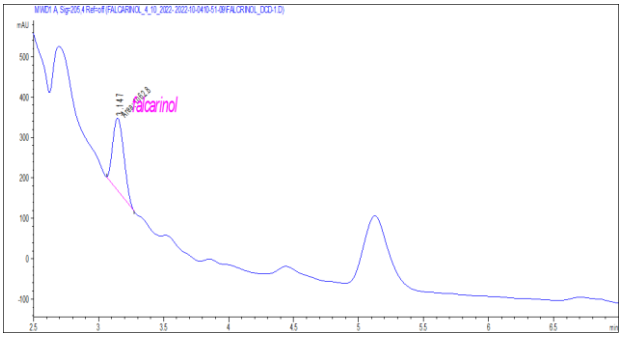

Figure 3. Chromatogram generated by HPLC quantitative analysis of FaOH content in one calli replicate of wild *D. carota* subsp. *carota*

4. Conclusion

Falcarinol, an anti-inflammatory and anti-cancer bioactive compound, is detected for the first time in calli of wild and cultivated carrots. Stored calli at –30 ºC for long periods of time showed a decrease in falcarinol concentrations. Hence, regenerative calli can be considered as a sustainable and cost-effective source of falcarinol.

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