9-cis and all-trans beta-carotene isomers of super critical CO₂-extracted Dunaliella oil are absorbed and accumulated in mouse tissues

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\textbf{ABSTRACT}

Animals cannot produce \( \beta \)-carotene, and therefore they exclusively depend on its dietary availability. We previously showed that a 9-cis \( \beta \)-carotene-enriched diet, provided as Dunaliella powder, has a beneficial effect on atherosclerosis and diabetes mellitus in animal models and on lipid profiles, retinitis pigmentosa, and psoriasis in human trials. Therefore, the current study aimed to investigate the bioavailability of 9-cis \( \beta \)-carotene from super-critical CO₂-extracted Dunaliella oil in mice. In total, ten 12-week old mice were allocated into two groups: (1) a control-received unfortified diet; (2) a Dunaliella oil diet enriched with Dunaliella oil. Four-week dietary supplementation with Dunaliella oil led to a substantial accumulation of both all-trans and 9-cis \( \beta \)-carotene in the plasma, liver and white adipose tissue. \( \beta \)-carotene was stable in oil preparation, and the 9-cis to all-trans \( \beta \)-carotene ratio was constant (60:40, g/g) after two years of storage at 4°C. These results suggest that supercritically extracted Dunaliella oil can potentially be used as a food supplement.

\textbf{Introduction}

\( \beta \)-carotene is synthesized by plants, algae, cyanobacteria, and some yeasts, fungi, bacteria and animals (Rodriguez-Concepcion et al., 2018). Humans and most animals cannot produce carotenoids, including \( \beta \)-carotene, and therefore they depend exclusively on its dietary availability (Garcia-Closas et al., 2004). In the human body, by the action of \( \beta \)-carotene monooxygenase 1 (BCMO1) and other enzymes, \( \beta \)-carotene becomes a precursor of vitamin A and several retinoids (Wang, Krinsky, Benotti, & Russell, 1994). All-trans \( \beta \)-carotene is a precursor of all-trans retinoid acid, and 9-cis \( \beta \)-carotene has been shown to be a precursor of all-trans and 9-cis retinoic acid \textit{in vitro} and \textit{in vivo} (Hebuterne et al., 1995). It is worth mentioning that both are ligands of the nuclear retinoic acid receptor (RAR), while only 9-cis retinoic acid binds to the retinoid X receptor (RXR) (Levin et al., 1992). Thus, dietary consumption of 9-cis \( \beta \)-carotene is expected to demonstrate the pharmacological benefits of 9-cis and all-trans retinoic acid.

Clinical studies have used synthetic all-trans \( \beta \)-carotene but have failed to show beneficial effects on cancer and cardiovascular disease (Clarke & Armitage, 2002; Hennekens et al., 1996). In several experiments conducted by our research group, we demonstrated that a 9-cis \( \beta \)-carotene-enriched diet, provided as Dunaliella \textit{bardawil} powder, has a beneficial effect on atherosclerosis, fatty liver and diabetes mellitus in animal models (Harari et al., 2013a, 2013b, 2008, 2020), as well as on the lipid profile, retinitis pigmentosa and psoriasis in human trials (Rotenstreich et al., 2013; Shaish et al., 2006).

Natural \( \beta \)-carotene comprises several isomers, and 9-cis \( \beta \)-carotene is found naturally in vegetables and fruits. However, it is accumulated to the highest levels in the alga Dunaliella. Under appropriate growth conditions, Dunaliella species accumulate a massive amount of \( \beta \)-carotene (up to 10% of dry weight) in oily globules. This \( \beta \)-carotene is composed of approximately 50% all-trans (ATBC) and 50% 9-cis \( \beta \)-carotene isomers (Ben-Amotz & Fishier, 1998). It is hypothesized that the presence of 9-cis \( \beta \)-carotene minimizes the crystallization of ATBC, and therefore, their combined presence in the oily globule in the alga is crucial (Ben-Amotz, Lers, & Avron, 1988).

Dunaliella powder and oily extracts of Dunaliella are commercially available (Mazzucchi, Xu, & Harvey, 2020). However, the content of 9-cis \( \beta \)-carotene isomer is not specified in these products. Moreover, whether supercritical CO₂ extraction (SCE) or more traditional extraction methods, such as solvents, is used to produce these oily extracts is unclear. As data on \( \beta \)-carotene absorption from Dunaliella oil extracted using SCE are
lacking, the present study aimed to investigate the bioavailability of 9-cis and all-trans β-carotene from SCE-extracted *Dunaliella* oil in mice.

### Materials & methods

#### Mice

Male 12-week-old C57BL6 mice (Jackson Laboratories) were used in this study. We used these animals as several experiments showed that β-carotene, supplied as *Dunaliella* powder, is well absorbed and has beneficial effects in male mice (Harari et al., 2013a, 2013b, 2008, 2020). The mice were housed in plastic cages on a 12-hour light: 12-hour dark cycle with free access to feed and water, which were distributed evenly among the treatment groups according to their initial body weight. The mice were killed with isoflurane. The Animal Care and Use Committee of Sheba Medical Center, Tel-Hashomer, approved all animal protocols (number 1119–17-ANIM).

#### Dunaliella oil

*Dunaliella salina* oil extract was a gift from Monzon Biotech., Spain. *Dunaliella* was cultivated in an open pond in Aragon, Spain. The algal powder was produced by freeze-drying. The oil was extracted from the algal powder using supercritical technology at NATECO₂ (Germany). Specifications for the *Dunaliella* oil are presented in Table 1. The algal oil was mixed with an equal volume of refined oil from corn germ (Koipeasua) in order to produce a final *Dunaliella* oil extract that contained 15% β-carotene (g/g) and comprised ~40% all-trans and ~60% 9-cis isomers. Betatene soft gels (25,000 IU), containing 100% natural carotenoids of *D. salina*, were purchased from AlchePharma (Santa Maria, California, USA, [www.AlchePharma.com](http://www.AlchePharma.com)).

### Table 1. *Dunaliella* oil specifications.

| Ingredients                                    | 100% oil from *Dunaliella salina* |
|-----------------------------------------------|-----------------------------------|
| Description                                    | β-carotene oleoresin oil, extracted from *D. salina* biomass by CO₂-extraction process, dark red brown, characteristic odour. |
| Supplier and country of raw material           | Monzon Biotech S.L. Spain.         |
| Content of β-carotene                          | ≥ 20%                              |
| 9-cis β-carotene                               | ≥ 40% of total β-carotene         |
| Microbiology:                                  |                                   |
| Total plate count                              | Less than 1000 cfu g⁻¹             |
| *Salmonella*                                   | MPN/Negative in 25 g               |
| *E. coli*                                      | MPN/Negative in 10 g               |
| Yeast/moulds                                   | Less than 100 cfu g⁻¹              |
| Heavy metals:                                  |                                   |
| Lead (Pb)                                      | ≤ 1.0 ppm                          |
| Arsenic (AS)                                   | ≤ 1.0 ppm                          |
| Mercury (Hg)                                   | ≤ 0.1 ppm                          |
| Cadmium (Cd)                                   | ≤ 1.0 ppm                          |
| Pesticides:                                    |                                   |
| DDT                                           | ≤ 0.01 mg kg⁻¹                     |
| DDD                                           | ≤ 0.01 mg kg⁻¹                     |
| DDE                                           | ≤ 0.01 mg kg⁻¹                     |
| Mycotoxins:                                    |                                   |
| Total Aflatoxins (B1 + B2 + G1 + G2)           | ≤ 15 mcg kg⁻¹                      |
| Aflatoxins B1                                  | ≤ 5 mcg kg⁻¹                       |
| Polycyclic aromatic hydrocarbons:              |                                   |
| Polycyclic aromatic hydrocarbons               | ≤ 50 mcg kg⁻¹                      |
| Benz(a)pyrene                                  | ≤ 10 mcg kg⁻¹                      |
| Shelf life:                                    | 36 months from date of production, if stored refrigerated in original packaging |
| Transport:                                     | Room temperature                   |
| Storage:                                       | Refrigerator                       |

Cfu g⁻¹: colony-forming unit per gram; MPN: most probable number; ppm: parts per million; DDT: dichlorodiphenyltrichloroethane; DDD: dichlorodiphenyldichloroethane; DDE: dichlorodiphenyldichloroethylene.
**Study design**

In this study, ten 12-week old mice were distributed into two groups, five per group: (1) the control group received the unfortified diet and the (2) *Dunaliella* oil group received a diet enriched with *Dunaliella* oil. The mice were killed after four weeks. Plasma, liver, and white adipose tissues were harvested and stored at –20°C.

**9-cis and all-trans β-carotene determination**

β-carotene isomer concentrations in the *Dunaliella* powder, *Dunaliella* oil (Monzon and Betatene), feed, plasma, liver, and white adipose tissue were determined using HPLC. Carotenoids were isolated using reverse phase HPLC on a YMC C30 column (CT995031546QT, 150 × 4.6, 3 μm particle size; YMC Inc., USA) with methanol/methyl-tert-butyl-ether/water and 1.5% ammonium acetate as the mobile phase, at a flow rate of 1 ml min⁻¹ (Harari et al., 2020). The β-carotene was detected by monitoring its absorbance at 450 nm.

**Statistical analysis**

A t-test was used to compare the control group and the *Dunaliella*-treated group. Significance was considered as *p* < 0.05. The values in the text are means ± SE.

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**Figure 1.** Analysis of 9-cis and all-trans β-carotene in *Dunaliella* oil. A. Betatene; B. *Dunaliella* powder; C. Monzon oil; D. all-trans crystals in Betatene oil. Carotenoids were isolated using reverse phase HPLC on an YMC C30 column, as described in the Methods section. The β-carotene was detected by monitoring its absorbance at 450 nm. All-trans β-carotene retention time: A, B – 15.0 min; C – 12.5 min. 9-cis β-carotene retention time: A, B – 16.2 min; C – 14.4 min.
Results

Because 9-cis degrades faster than all-trans β-carotene upon exposure to oxygen, we first examined whether the ratio between these two β-carotene isomers was constant during long-term oil storage. HPLC analysis showed that the β-carotene is stable in Monzon oil preparation, and 9-cis to all-trans β-carotene ratio is constant (60:40, g/g) after two years of storage at 4°C (Fig 1(a,b)). In contrast, analysing 9-cis β-carotene in Betatene soft-gels (AlchePharma), showed that it comprised only 12% of the total β-carotene (Fig 1(c)). Moreover, numerous all-trans β-carotene crystals were found in Betatene oil yet were undetectable in Monzon oil (Fig 1(d)).

The four-week dietary supplementation with Dunaliella oil led to a substantial accumulation of β-carotene in plasma, liver (Fig 2), and white adipose tissue (Table 2). The β-carotene concentrations in all the tested tissues were significantly higher than those in the control tissues (Table 2). As expected, the highest β-carotene levels were detected in the liver. HPLC analysis showed that the all-trans isomer was dominant in plasma and adipose tissues, while the 9-cis was the main isomer in the liver.

Discussion

For the first time, our study showed that 9-cis and all-trans β-carotene isomers from the supercritical CO2 extract of D. salina powder are stable during long-term storage, well absorbed, and accumulated in mouse tissues. These results suggest that this oily extract could be used as a food supplement.

Two species of Dunaliella, D. bardawil and D. salina, produce large amount of β-carotene. Optimal conditions for carotene accumulation, including 9-cis β-carotene, are high light intensity, high sodium chloride concentration, low temperature and nitrate deficiency (Shaish, Ben-Amotz, & Avron, 1992). These parameters can be partially controlled in large-scale, outdoor ponds.

This oily preparation could be more beneficial than the current Dunaliella powder found on the market for several reasons: it contains higher β-carotene levels, lipid emulsions are ideal matrices for β-carotene delivery, and β-carotene is more stable in oil (Yi, Li, Zhong, & Yokoyama, 2014). 9-cis β-carotene in the alga Dunaliella, under appropriate conditions, comprises 50% of the total β-carotene. However, β-carotene degrades during inappropriate powder production conditions and storage. As 9-cis β-carotene is more susceptible to degradation than all-trans β-carotene, the ratio between 9-cis and all-trans decreases. Therefore, the ratio between the two isomers can be used as a good indicator for the quality of powder or oil preparations of D. salina.
or *D. bardawil*. Retaining β-carotene stability is also essential for preventing the formation of potentially harmful β-carotene oxidation products. Moreover, the 9-cis β-carotene isomer’s high content probably inhibits the formation of all-trans β-carotene crystals that can be formed in other preparations as specified by their producers (e.g., Betatene produced by BASF) and displayed in this study. The prevention of β-carotene crystal formation is crucial, as absorption of β-carotene provided as crystals is very low (Otani et al., 2020).

Our research group’s previous studies, as well as other groups’ studies, have shown that *Dunalieilla* powder containing 9-cis β-carotene confers beneficial effects on atherosclerosis, psoriasis, and retinitis pigmentosa (Harari et al., 2008; Shaish et al., 2006; Takahashi, Youko, Shiomi, Ayakawa, & Miyata, 2000). In the current study, we used *Dunalieilla* oil extracted from *Dunalieilla* cultivated in large-scale ponds. We assume that in future human trials high-quality oil will be used. The oil specification, including polycyclic aromatic hydrocarbons, aflatoxins, pesticides and heavy metals for human use, requires caution. We intend to investigate whether the oily extract exhibits a similar or superior effect on these diseases in future experiments.

**Highlights**

1. Supercritical CO₂ extract of *Dunalieilla* contains high levels of 9-cis β-carotene.
2. β-carotene from supercritical CO₂ extract of *Dunalieilla* is stable.
3. β-carotene is accumulated in mouse tissues.

**Disclosure statement**

The oil has future commercial value.

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