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Apoptosis Comparison Effects Between Synthetic and Natural B-Carotene from *Dunaliella salina* on MDA-MB-231Brest Cancer Cells

Jorge Olmos[1]*, Rocío Gómez[1] and Viviana P. Rubio[2]

[1]*Molecular Microbiology Laboratory, Department of Marine Biotechnology, Centro de Investigación Científica y de Educación Superior de Ensenada (CICESE), Ensenada, B.C., México*

[2]*Marine Science Faculty, Universidad Autónoma de Baja California (UABC), Ensenada, B.C., México*

Abstract

Dunaliella salina is genus most important species for β-carotene production. Several investigations have demonstrated that *D. salina* produces more than 10% of its dry mass. β-carotene is an important pro-vitamin A source and can also act as a lipid radical scavenger and as a singlet oxygen quencher. Vitamin A Deficiency (VAD) has been related with cancer, for this reason synthetic and natural β-carotene has been used for prevention and treatment of the disease. Synthetic β-carotene is cheaper than natural molecule but only contains *all-trans*-β-carotene (ATβC), while 9-cis-β-carotene (9CβC) and ATβC are both produce by *D. salina*. Meta-analysis of controlled trials using high levels of synthetic β-carotene supplementation in smoker's individuals, mention that instead to prevent and control lung cancer, treatment increases risk and percentage of positive cases. Results obtained in cancer cell lines and animal models using β-carotene from Dunaliella, prevented and controlled diseases proliferation. In this work, effects of synthetic and natural β-carotene from *D. salina* were evaluated on MDA-MB-231 breast cancer cells, and even when apoptosis induction results were obtained with both sources, natural β-carotene generates considerable higher cell rates mortality.

Keywords: *D. salina;* β-carotene; ATβA; 9CβA; MDA-MB-231

Introduction

Breast cancer is most frequently diagnosed cancer globally and main cause of death among women [1,2]. Breast cancer is a complex disorder that can occur because of vitamin deficiencies and due process including oxidative stress and lipid peroxidation. Biological mechanisms exist to support anticancer properties of natural antioxidant pro-vitamin A molecule (β-carotene) in animals and overall impression is that intake of it, play a substantial role in prevention of cancer [3-5]. However, anticancer effects are not so positive in humans using synthetic β-carotene molecule [6-8].

Dunaliella genus includes a reduced number of species that when exposed to elevated luminous intensities, high saline concentrations and limited conditions of oxygen and nitrogen accumulate great amounts of β-carotene [9,10]. *D. salina* and *D. bardawil* are the most important species of the genus for β-carotene production. Several investigations have demonstrated that both species produces more than 10% of its dry mass [11,12]. The commercial production of β-carotene that comes from *Dunaliella* is the third most important microalgae industry since 1986 [13]. β-carotene products derived from *Dunaliella* are; 1) *Dunaliella* powder for animal feed production, 2) *Dunaliella* powder for human food elaboration and 3) extracts of pure β-carotene for medical and pharmaceutical use, with prices oscillating between US $ 300 to US $ 3000 per kg, respectively [13]. Synthetic β-carotene is easier to obtain and cheaper than β-carotene from *Dunaliella*, reason why synthetic molecule dominates almost 95% of the market.

All-trans-retinoic-acid (ATRA) β-carotene derived molecule from synthetic and natural sources are 100% equals in structure and function [14]. ATRA is been considered the most potent biologically active metabolite derived from vitamin A, because prevent and rescue cancer anomalies induced by VAD in adult animals [15,16]. Subsequent studies anticipated a strong rationale for use of retinoids in treatment and prevention of cancer [17]. β-carotene from *D. salina* contains 47% of 9CβC and 53% ATβC (Figure 1) [18]. Antiperoxidative*9-cis*-retinoic-acid (9CRA) activity is greater compared to that shown by ATRA, preventing further malignant cells development and cardiovascular diseases [19,20]. Cytochrome P450RAI (CYP26) metabolizes ATRA but not 9CRA isomer increasing its half-life [21,22]. ATRA and 9CRA ligands interact with nuclear retinoic acid receptors (RARs) and retinoic X receptors (RXRs); ATRA binds to RARs while 9CRA can bind to both RARs and RXRs [23,24]. DNA promoter retinoic acid respond elements (RAREs) combinations tightly regulate gene expression, through interaction with homo and heterodimers constituted by RARs and RXRx previously bound to ATRA and 9CRA [25].

In this work *D. Salina* strain isolated from Baja California peninsula of México was identified both morphologically and molecularly via *18S rDNA*conserved and specific primers [26,27]. Natural β-carotene from optimally grown cells was solvent extracted and quantified. β-carotene was assessed for its anticancer property in MDA-MB-231 human breast cancer cell line, showing 70% of effective apoptosis with respect to synthetic β-carotene that only induced 30% in a MTT analysis.

Experimental Section

Dunaliella environmental sampling and growth conditions

Locations in Baja California peninsula of México were selected for water-sample collection [11]. Environmental samples were obtained using 50 ml plastic tubes from red hypersaline waters between May

***Corresponding author:** Jorge Olmos, Department of Marine Biotechnology, Centro de Investigación Científica y de Educación Superior de Ensenada (CICESE), Ensenada, B.C., México, E-mail: jolmos@cicese.mx

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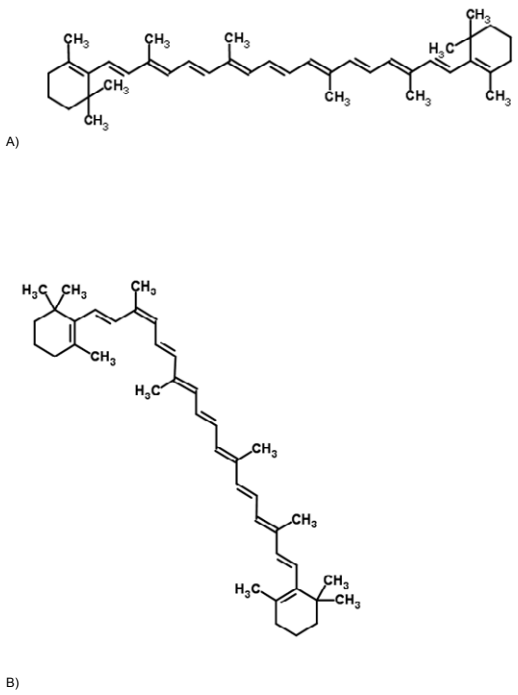

A) CSID:4444129, http://www.chemspider.com/Chemical-Structure.4444129.html
B) CSID:8004366, http://www.chemspider.com/Chemical-Structure.8004366.html

Figure 1: A) ATβC and B) 9CβC.

and September 2006. Red flagellated microalgae strains contained in samples were morphologically and molecularly identified as *Dunaliella,* before purification and growth. Samples containing *D. salina* were serially diluted up to 10^{-4} and 0.1 ml spread on 2% De Walne's agar medium [3, 28]. Distinct colonies developed on plates were transferred to β-carotene inducing medium supplemented with 1.0, 1.5 and 2 M of NaCl and kept at 24 ± 1°C in thermostatically controlled chamber, illuminated with four cool fluorescent lamps to generate an irradiance of 200 µE m-2 s-1, under 12 h/12 h light/dark photo period.

DNA Purification and PCR Amplification

Isolation of chromosomal DNA from *Dunaliella* strains was carried out and species identification of isolates was developed using MA1 [5'-CGGGATCCGTAGTCATATGCTTGTCTC-3'] and MA2 [5-GGAATTCCTTCTGCAGGTTCACC-3'] conserved and DSs (*D. salina*) [5'-GCAGGAGAGCTAATAGGA-3'] specific oligonucleotide reported by Olmos and coworkers [5, 11]. MA1 and MA2 conserved oligonucleotides amplify *18S rDNA* complete sequence. DSs oligonucleotide is specific for β-carotene "*D. salina var Teod*" hyper producer strain [11,12]. PCR reactions were carried out in a total volume of 100 µl containing 50 ng of chromosomal DNA in TE (Tris-EDTA) buffer, pH 8 and 200 ng MA1 and MA2 conserved primers. The amplification was carried out using 25 cycles with a Tm of 52°C to all reactions. One cycle consisted of 1 minute at 95°C, 1 minute at 52°C and 2 minute at 72°C. PCR with specific primer was combined with MA2 conserved primer (DSs-MA2) and reactions were carried out under same conditions.

β-Carotene HPLC identification and quantification

One ml sample was centrifuged at 2000 rpm for 5 min, supernatant was discarded and pellets were homogenized and disrupted in one ml of 100% cold methanol. Samples were incubated on ice for one hour in the dark, centrifuged at 10000 rpm 5 min, filtered and kept at -20°C. 20 µl methanol β-carotene extracted sample was injected into a Hewlett Packard 1100 model HPLC apparatus, using a reverse phase VYDAC 201 TP52 C-18 column of 4 mm internal diameter and 150 mm length, with 5 µm particle size. The detector was set at 340 nm and 450 nm for identification of 9CβC and ATRA respectively. Elution was performed with an isocratic mixture of acetonitrile:methanol:tetrahidrofuran:water (70:15:10:5) at 0.5 ml/min. Synthetic ATβC was obtained from Sigma and used as standard.

Purification of β-Carotene from *D. salina*

Thirty days old *D. salina* grown under laboratory condition was centrifuged at 2000 rpm for 5 min and pellet was collected. Five gram pellets were taken, washed with distilled water and centrifuged again to remove salt. Collected pellets were dissolved with 10 ml of methanol and macerated with mortar and pestle in an ice bath until the pellet became colorless, sample was centrifuged again, pellet was discharged and methanol was evaporated at room temperature. β-carotene resultant amorphous dry powder was dissolved in olive oil and concentrations of 10 µg/ml were used for following anticancer studies.

Effect of Natural β-Carotene from *D. salina* on MDA-MB-231

Human keratinocytes cells (HaCat) and human Brest cancer cells (MDA-MB-231), were cultured in RPMI-1640 medium supplemented with 10% inactivated fetal bovine serum (v/v) (GIBCO), and 1% antiant commercial antibiotic (invitrogen) in a humidified incubator with 5% CO_2 at 37°C. The MTT method described by Mosmann was used to quantitatively detect living cells [29]. In brief, 5×10^3 cells/well were charged onto 96 well plates. After 24 h, supplemented medium was discarded, non-supplemented medium was added and cells were incubated 24 h more. 10 µg/ml solution of synthetic and purified β-carotene from *D. salina,* were added to HaCat and MDA-MB-231 cells that were incubated 2 h at the same conditions. Medium was discarded and 100 µl of fresh medium was added with 10 µL of MTT (5 mg/ml). After 2 h of incubation medium was eliminated and 100 µl of cold isopropanol was added to dissolve crystals formed. Absorbance was quantified with a microplate reader at 570 nm. Growth inhibition percentage was calculated using DMSO cells treated as positive control and as negative control RPMI medium was used.

DNA fragmentation assay in MDA-MB-231 cells

Cleavage of DNA was analyzed as described previously [30]. MDA-MB-231 cells were grown and treated with a 10 µg/ml β-carotene solution. Cells were washed twice with ice-cold PBS and resuspended in lysis buffer containing 10 mMTris-HCl (pH 8.0), 20 mM EDTA, 0.5% Triton X-100 and incubated for 30 minutes on ice. After centrifugation at 10000 rpm for 10 min at 4°C DNA was extracted with phenol chloroform, precipitated at −20°C overnight with 0.1 volume 3 M sodium acetate and 2.5-volume ethanol. DNA was pellet at 10000 rpm for 5 min and 4°C, rinsed with 70% ethanol, resuspended in TE buffer (pH 8.0) contained 30 µg/ml of RNase and incubated for 6 h at 37°C. DNA was run on 2% agarose gel with ethidium bromide staining and visualized under UV and documented [3].

Results and Discussion

First description of a unicellular biflagellate red colored alga living in concentrated brines of Montpellier on Mediterranean coast of France was reported in 1838 by Dunal, who described occurrence of

the organism we know today as *Dunaliella salina* [31]. Descriptions of *Dunaliella* (*Chlorophyta, Chlorophyceae, Chlamydomonadales, Dunaliellaceae*) as a new genus, was presented in year 1905 by Teodoresco from Bucharest using Romanian salt lake samples and by Clara Hamburger from Heidelberg using samples from Cagliari, Sardinia [32, 33]. *Dunaliella* do not have a cell wall only is enclosed by a thin elastic plasma membrane, in this sense species of the genus present a vast morphological variability with respect to environmental conditions were algae growth [34-36]. *Dunaliella* can be ellipsoid, ovoid to almost spherical, pyriform or fusiform (Figure 2).

Motile cells are biflagellate, with flagella inserted at anterior end of the cell with length varying between species. There is a single large posterior chloroplast occupying most of the cell volume. It is either cup-, dish or bell-shaped and contains a pyrenoid in the thickened basal part in all species except some of the freshwater species [37].

Taxonomic studies among *Dunaliella* have identified several new species since Teodorescos identification in 1905 [36]. However, high plasticity in green stage and almost indistinguishable differences in red phase make identification and differentiation of species very difficult and time consuming [26]. *Dunaliella* genus includes a reduced number of species that when exposed to elevated luminous intensities, high saline concentrations, oxygen and nitrogen limitation accumulates great amounts of β-carotene [9,10]. *D. salina* and *D. bardawil* are most important species for β-carotene production; several investigations have demonstrated that both produce more than 10% of its dry mass [27]. In this sense, *Dunaliella* isolates were morphological and molecularly identified. Figure 2, shown *Dunaliella* strain isolated from locations reported by Olmos and coworkers [11], picture presents a microalga growing on a brine lagoon containing high levels of β-carotene and two flagella, characteristics from a *Dunaliella* hyperproducer strain. Thus, Intron-Sizing-Method was applied to make an easy, fast and precise identification of the isolates [11,12]. In natural samples "*D. salina var Teod*" was identified due its *18S rDNA* fingerprinting profile presented a 2100 bp PCR product using MA1-MA2 conserved oligonucleotides and a 700 bp product by using *D. salina* specific and conserved oligonucleotides (DSs-MA2) (Figure 3).

Once isolated strain was identified, it was grown using an inducer medium formulated with addition of three NaCl concentrations (Table 1), to evaluate its influence in growth and β-carotene production (Figure 4). *D. salina* isolate reached stationary phase 15 days after initiation of growth culture, approximately 1×10^6 cells were obtained in all NaCl conditions and β-carotene production analyzed thirty days after initiation, was slightly greater in 1.5 M of NaCl (data not shown).

9CβC and ATβC production in green (15 days) and red (30 days) phases were evaluated with an HPLC methodology developed. In this sense, *D. salina* isolated strain produces 9CβC:ATβC ratio of 70:30 under conditions established (Figure 5a). However, results obtained by Sheu and coworkers shown a *D. salina* strain producing β-carotene 9CβC:ATβC ratio of 47:53 [18]. Additionally, it is important to point out that 9CβC is preferentially produced in red phase, due in green phase 9CβC:ATβC ratio was inversely proportional (Figure 5b). Results differences obtained between Sheu and this work maybe was due by medium, culture conditions and/or timing of evaluation. Other factors involved in 9CβC:ATβC ratio differences could be HPLC methodology and/or *Dunaliella* strain used.

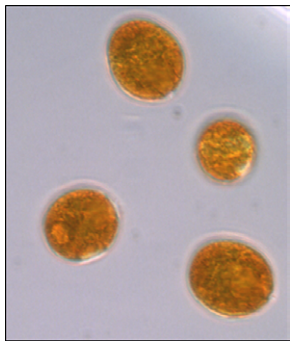

Figure 2: *Dunaliella salina* isolated strain.

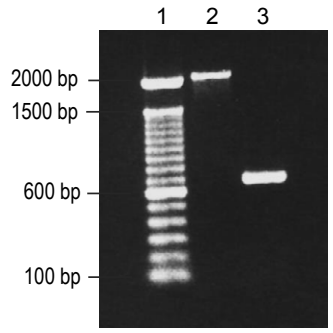

Figure 3: *D. salina* specific identification. Lane 1, Molecular Weight Marker. Lane 2, PCR product using MA1-MA2 conserved primers. Lane 3, PCR product using DSs-MA2 specific-conserved primers.

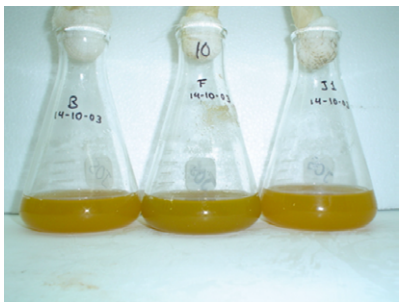

Figure 4: *D. salina* growth under 1, 1.5 and 2 M of NaCl, respectively.

Component	Concentration
$CaCl_2$	0.10 μM
H_3BO_3	61.7 μM
$MgSO_4$	1.67 mM
TRISMA	0.48 mM
$NaNO_3$	1.17 mM
NaH_2PO_4	53.3 μM
$FeCl_3$	13.3 μM
EDTA	13.3 μM
$ZnSO_4$	100 nM
Na_2MoO_4	33 nM
$MnCl_2$	1.21 μM
$CoCl_2$	53 nM
$CuSO_4$	46.67 nM
Thiamine	3.5221 μM
Biotin	0.024 μM
Cyanocobalamin	4.38 μM
NaCl	1.0-1.5-2.0 M

Table 1: Culture medium with 1, 1.5 and 2 M of NaCl.

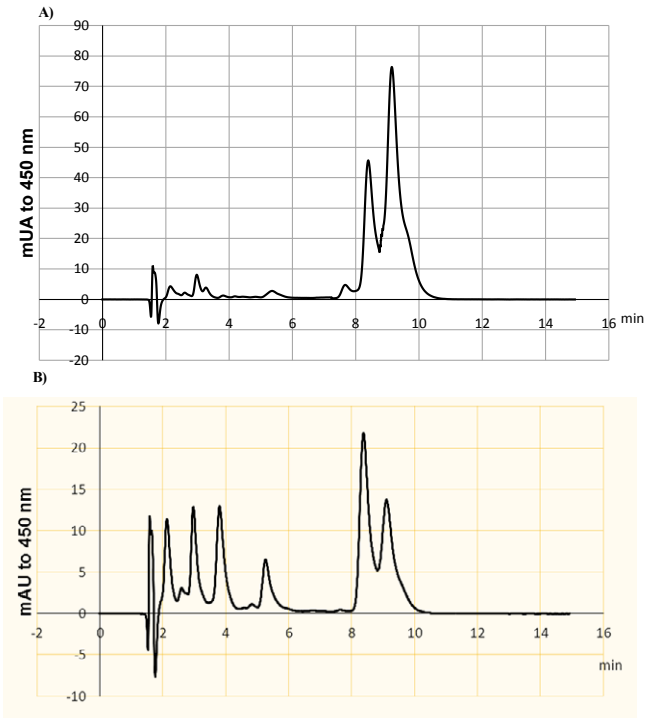

Figure 5: A) β-carotene red phase extraction and B) β-carotene green phase extraction.

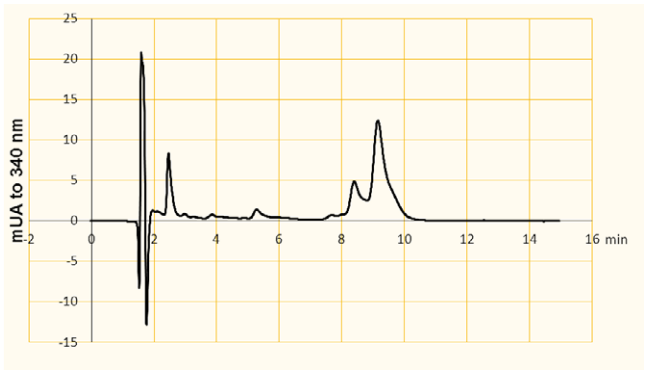

Figure 6: ATβC small peak (8.5 min) and 9CβC higher peak (9.5 min).

Moreover, β-carotene production level reached by our isolate was around 20 mg/l meaning that formulated medium succeed in its overproduction, as well as in 9CβC induction. Furthermore, β-carotene isomers identification was made by using 340 λ, due 9CβC presents a better absorption than ATβC at this wavelength [38]. The obtained results corroborate findings made by Briton and coworkers, where *D. salina* 9CβC peak showed higher levels than ATβC (Figure 6). Additionally, ATβC from *D. salina* presented same retention time as synthetic molecule obtained from Sigma-Aldrich (data not shown). Supporting these findings Grune and coworkers mention that both molecules presented the same structure-function and by consequence the same retention time [14]. Differences between 9CβC:ATβC ratio from synthetic and natural samples, was not possible to calculate due synthetic just contains ATβC molecule.

Metaanalysis of controlled trials using synthetic β-carotene in Finland (**ATβC**) and synthetic β-carotene and retinol in USA (**CARET**), showed that high levels (20-30 mg/day) of supplemental β-carotene instead to prevent and control lung cancer, increases risk and percentage of positive cases [6]. In this sense, Palozza and coworkers found that under low oxygen pressure (15 mmHg pO_2) β-carotene behaved as antioxidant, inhibiting cigarette smoke condensate (tar) to induced lipid peroxidation [8]. Nevertheless, β-carotene progressively acted as a prooxidant in a dose-dependent manner under 100-760 mmHg pO_2 range [8]. β-carotene auto-oxidation, measured as formation of 5,6-epoxy- β,β-carotene, was faster at high than at low pO_2 and carotenoid was more rapidly consumed in tar presence [39]. In contrast to **ATβC** and **CARET** studies, the Physicians' Health Study was conducted among mainly nonsmokers; 50 mg of β-carotene was given every other day and no effect was found on lung cancer risk in either smokers or nonsmokers [40]. β-carotene serum concentration (210–300 μg/dL) in trials where lung cancer incidence increased was markedly higher than in Physicians' Health Study (120 μg/dL), where no increase in lung cancer risk was seen from β-carotene supplementation [40]. Additionally, normal range of serum β-carotene derived from dietary intake is around 5–50 μg/dL [41]. In this sense, 10 μg/ml of synthetic and natural β-carotene were supplemented on experiments developed in this work, using MDA-MB-231 Brest cancer and HaCat cell lines (Figure 7a and 7b**).** Due β-carotene low levels used in these assays and low culture plates pO_2, pro-oxidant activity was discarded in these experiments. Obtained results in MDA-MB-231 Brest cancer cells shown that β-carotene from *Dunaliella* induced 70% cells death with respect to synthetic molecule that only presented 30% in MTT assays (Figure 7a). These results are in agreement with cell and animal model trials published, where induction of apoptosis at different levels was carried out by *D. salina* extracts [3-5,18]. Nevertheless, no one of the mentioned authors had worked with MDA-MB-231 Brest cancer cells neither HaCat, only Prakash and coworkers made some

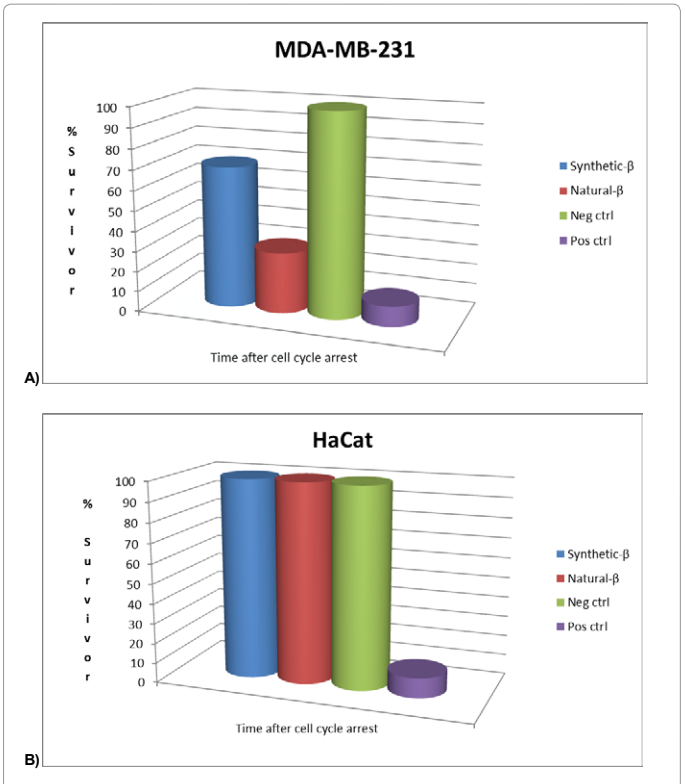

Figure 7: Synthetic and Natural β-carotene used to treat; A) MDA-MB-231 and B) HaCat cells.

β-carotene inhibition test in MDA-MB-231 cells, but non-results were reported [42]. Breast cancer is the most frequently diagnosed cancer globally and main cause of death among women [1,2]. For these reasons, Brest cancer is one of the most important targets to natural and synthetic molecules development. Major carotenoids in *D. salina* include ATβC and 9CβC; specifically *9-cis* isomer had demonstrated a great antiperoxidative effect due to higher reactivity of *cis* bond compared to *trans* [5]. In addition, 70% MDA-MB-231 apoptotic induced cells using natural β-carotene was in accordance to higher 9CβC levels produced by *D. salina* isolate (Figure 7a and 5a). On the other hand, MDA-MB-231 synthetic β-carotene treated cells reached 30% mortality only (Figure 7a). Thus, obtained results suggest that 9CβC is the most influential isomer in apoptosis induction in MDA-MB-231 Brest cancer cells. Moreover, non-deleterious effects in HaCat cells were induced by β-carotene treatment, meaning that used concentration was not harmful to tested cells (Figure 7b). Additionally, it is important to point out that failed human clinical trials in Finland and USA (**ATβC** and **CARET**), were developed using high levels of synthetic β-carotene and without inclusion of 9CβC [6]. In this sense, clinical trials development using natural β-carotene with high levels of *9-cis* isomer are recommended, due results obtained in this work and other reports published [3-5,18]. For this reason, a culture medium specifically formulated to overproduce 9CβC in *D. salina,* as the one developed in this work is required. Additionally, timing to *D. salina* β-carotene harvesting is also of extreme importance to obtain the greatest 9CβC concentration (Figure 5a and 5b). In addition, *D. salina* species-specific identification is highly recommended to obtain desired results.

With respect to DNA fragmentation assays better degradation results were obtained in MDA-MB-231 Brest cancer cells treated with natural instead of synthetic β-carotene (data not shown), results expected due better apoptosis induction obtained using natural molecules (Figure 7a) and by previously reported works using *D. salina* extracts [3-5,18]. Evasion of apoptosis is considered a hallmark of human cancers; apoptosis cell death modality is executed by caspases, which are up-regulated by factors like retinoids [43-45]. Although, caspases activity is regulated primarily at post-translational level its overexpression sensitizes cells for apoptosis [43-45]. In addition, CYP26 expression is regulated by ATRA and 9CRA throughout RARs and RXRs nuclear receptors. Furthermore, an important function of RARs and RXRs receptors is auto-regulate their own activity by controlling CYP26 transcription that in turn, controls ATRA ligand concentration by its degradation but without affecting 9CRA [22,23,25,46]. In this sense, caspase and CYP genes expression is modulated by ATRA and 9CRA ligands; however, *9-cis* activate both RARs and RXRs nuclear receptors, while ATRA only activate RARs regulators [23]. Therefore, data mentioned above could explain why 9CβC:ATβC (70:30) ratio obtained from *D. salina*, induces apoptosis more efficiently than ATβC from synthetic origin. Additionally, presence of both isomers seems to be imperative for a correct RXR:RAR heterodimer genes regulation and expression [25].

Conclusion

Retinoid regulates a wide variety of essential biological processes, such as vertebrate embryonic morphogenesis and organogenesis, cell growth arrest, differentiation and apoptosis, as well as, homeostasis and their disorders [15,47-49]. VAD is been related with cancer, although controlled trials using synthetic β-carotene increased the disease in smokers. 9CRA and ATRA are the most potent biologically active metabolites derived from natural β-carotene (9CβC:ATβC); a pro-vitamin A molecule isolated principally from *Dunaliella* species. However, synthetic β-carotene only generates ATRA and non-9CRA is detected in human bodies. Commercial price differences between both isomers are of great concern, reason why synthetic β-carotene (ATβA) is been used preferentially for a long period. 9CRA and ATRA regulates gene expression throughout interactions with nuclear retinoic acid receptors (RARs) and retinoic X receptors (RXRs), being RXRs the most important and represented in human bodies. DNA promoter retinoic acid respond elements (RAREs) combinations tightly regulate gene expression through homo and heterodimerization between RXRx and RARs, after interaction with 9CRA and ATRA. In this sense, presence of both retinoids is indispensable to an accurate gene expression and apoptosis induction as was demonstrated in this work. Pending tasks are related to know about precise ratio needed between 9CRA:ATRA and the concentration that must be apply, to prevent and/or to cure cancer in humans. Meta-analysis already demonstrated that application of high levels of synthetic ATRA one of the two components of the ratio, is not adequate to prevent neither to cure cancer. Utilization of natural sources of β-carotene like *D. salina* is more expensive at the beginning, but at the end could be cheaper preventing this kind of diseases.

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