Growth-inhibitory effects of the astaxanthin-rich alga *Haematococcus pluvialis* in human colon cancer cells

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**Abstract**

The growth-inhibitory effects of the astaxanthin-rich *Haematococcus pluvialis* were studied in HCT-116 colon cancer cells. *H. pluvialis* extract (5–25 μg/ml) inhibited cell growth in a dose- and time-dependent manner, by arresting cell cycle progression and by promoting apoptosis. At 25 μg/ml of *H. pluvialis* extract, an increase of p53, p21WAF-1/CIP-1 and p27 expression (220%, 160%, 250%, respectively) was observed, concomitantly with a decrease of cyclin D1 expression (58%) and AKT phosphorylation (21%). Moreover, the extract, at the same concentration, strongly up-regulated apoptosis by modifying the ratio of Bax/Bcl-2 and Bcl-XL, and increased the phosphorylation of p38, JNK, and ERK1/2 by 160%, 242%, 280%, respectively. Growth-inhibitory effects by *H. pluvialis* were also observed in HT-29, LS-174, WiDr, SW-480 cells. This study suggests that *H. pluvialis* may protect from colon cancer.

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1. Introduction

Epidemiological studies suggest an association between nutritional habits and prevention of several types of cancer. However, there is a lack of knowledge on the nature of the chemopreventive food constituents and their mechanism of action [1]. Preventive properties have been assigned to antioxidant micronutrients, including the carotenoid group. Both prospective and retrospective epidemiological studies have consistently and clearly shown that increased intake of fruits and vegetables rich in carotenoids is associated with a decreased risk of cancer [2–4]. Unfortunately, three major clinical trials of high-dose supplemental β-carotene, the carotenoid most frequently identified as protective against lung cancer, failed to demonstrate protection. In contrast, in two of these studies conducted in high-risk smokers and/or asbestos exposed workers, lung cancer incidence actually increased [10–12]. The third study in largely non-smoking US physicians did not demonstrate protection or risk [13]. In studies conducted in ferrets, one of the few laboratory models which absorb β-carotene to a comparable level as do humans, high-dose β-carotene was found to induce lung pathology. Molecular changes in the lung were consistent with a β-carotene induced deficiency of retinoic acid as a consequence of enhanced catabolism of this important regulator of cell differentiation [14]. These data suggest that the use of carotenoids without potential for conversion to vitamin A may provide protection and avoid this toxicity. Recently, the red ketocarotenoid astaxanthin, 3,3′-dihydroxy-β,β′-carotene-4,4′-dione, the main carotenoid found in aquatic animals and in seafoods, not possessing a pro-vitamin A activity, attracted considerable interest because of its po-
tumor antitumoral, antioxidant and immunomodulatory activities which are distinctly different and, at least in some cases, more potent than that of β-carotene and other carotenoids [15–21].

Increasing evidence suggests that astaxanthin is a potent antitumoral agent in experimental animal models. The carotenoid protected mice from carcinogenesis of the urinary bladder by reducing the incidence of chemically-induced bladder carcinoma [22]. Dietary astaxanthin also exerted antitumoral activity in the post-initiation phase of carcinogen-induced colon [23] and oral [24] cancer models. Rats fed a carcinogen but supplemented with astaxanthin had a significantly lower incidence of different types of cancerous growth in their mouths than rats fed only the carcinogen [24]. Suppressive effects of dietary astaxanthin have also been reported in transplantable tumor cells, including methylcholanthrene-induced fibrosarcoma cells [25–27] and murine mammary tumor cells [28,29]. It has been also suggested that astaxanthin attenuates the liver metastasis induced by stress in mice thus promoting the immune response through the inhibition of lipid peroxidation [30]. Kang et al. [31] also reported that astaxanthin protects the rat liver from damage induced by CCl4 through the inhibition of lipid peroxidation and the stimulation of the cell antioxidant system. Additionally, the effects of astaxanthin have been also studied in human breast cancer cells [32], in which astaxanthin inhibits the proliferation of MCF-7 cell line, although less effectively than β-carotene and lycopene. Although several mechanisms have been proposed to explain the putative role of astaxanthin in the modulation of cell growth, including its ability to induce xenotoxic-metabolizing enzymes in the liver [33,34], to modulate immune function [25–29,35–39] and gap junctional communication [40,41] and to regulate intracellular redox status [15–21,42,43], at the moment, no many studies have directly investigated a possible influence of astaxanthin on molecular pathways involved in cell survival, proliferation and apoptosis. This study has been designed to examine the effects of an extract of Haematococcus pluvialis, which is believed to accumulate the highest levels of astaxanthin in nature, on the growth of different human colon cancer cells and to investigate some molecular pathways involved in cell cycle progression, apoptosis and cell survival. In particular, we evaluated the expression of p53 and that of cyclin kinase inhibitors (p21, p27), of cyclins (cyclin D1), of apoptosis-related proteins (Bcl-2, Bcl-xl, Bax), of cell survival-related proteins (AKT), and of apoptosis-related MAPK kinases (p38, JNK and ERK1/2). H. pluvialis could be a particularly interesting anticancer substance, since the high cost of production, isolation and purification of purified astaxanthin from natural sources or chemically synthetic methods limit its usage on cancer therapy.

2. Materials and methods

2.1. Cell culture

HCT-116 and SW480 human colon carcinoma cells (American Type Culture Collection, Rockville, MD, USA) were cultured in McCoy’s 5a and L-15, respectively. HT-29 human colon adenocarcinoma cells (American Type Culture Collection, Rockville, MD, USA) were grown in MEM medium. LS-174 human colon adenocarcinoma cancer cell line (American Type Culture Collection, Rockville, MD, USA) and WiDr human colon adenocarcinoma cancer cell line (American Type Culture Collection, Rockville, MD, USA) were cultured in RPMI 1640 medium (Gibco Biocult, Paisley, UK). Cells were maintained in log phase by seeding them twice a week at density of 3 x 10⁵ cells/ml at 37°C under 5% CO₂ air atmosphere. The medium was supplemented with 10% (v/v) fetal calf serum (Flow, Irvine, UK) and 2 mM glutamine. The medium was not further replaced throughout the experiments. Experiments were routinely carried out on triplicate cultures. After the incubation, cells were harvested and quadruplicate haemocytometer counts were performed. The trypan blue dye exclusion method was used to evaluate the percentage of viable cells.

2.2. Addition of H. pluvialis extract or purified astaxanthin to cells

Dark red lipid CO₂ extract (no traces of organic solvents) of H. pluvialis microalgae was kindly supplied by Sochim International S.P.a, Milan, Italy. It contains 10.2 wt% astaxanthin, which was a mixture of free and fatty acid ester forms (free Ax/Ax monoesters/Ax diesters = 5.0:80.0:15.0, by mol). All-trans Astaxanthin, was 10.0% and 9-cis Astaxanthin was 0.2%. The extract also contains a minor amount of other carotenoids (mostly β-carotene, 0.1%; Canthanxanthin, 0.1%; Lutein, 0.05%). From the stock solution, aliquots of the extract, suspended in THF, were rapidly added to cells to give the final concentrations of the extract indicated. Control cells received amounts of THF equal to those present in H. pluvialis-treated cells. The amount of THF added was not greater than 0.5% (v/v) and did not affect cell viability (data not shown).

Purified astaxanthin (Fluka, Chemika-bioChemika, Buchs, Switzerland) was dissolved in THF and rapidly added to cells to give the final concentration of 5 µM for 24 h.

2.3. Cell cycle analysis

Cell cycle distribution was analyzed by flow cytometry, as previously described [44]. Aliquots of 10⁶ cells were harvested by centrifugation, washed in PBS, fixed with ice-cold 70% ethanol. The cells were incubated at 4°C for 30 min and then centrifuged at 2500g for 10 min. The pellet was resuspended in 0.5 ml PBS and 0.5 ml DNA-Prep stain (Coulter Reagents, Miami, FL, USA), containing 1 g/l RNase and 50 g/l propidium iodide. All samples were incubated for 30 min in the dark at 4°C. The DNA content of cells stained with propidium iodide was measured with a FACS instrument (EPICS XL-MCL Flow Cytometer, Coulter Electronics, FL, USA), by using Multicycle AV software.

2.4. Apoptosis detection

The percentage of apoptotic cells was determined by TUNEL [45]. Briefly, cells were centrifuged, fixed with ace-
2.5. Western blot analysis of cyclin D1, p53, p21\(^{WAF-1/CIP-1}\), p27, Bax, Bcl-2, Bcl-xL, Akt, pAkt, p38 and p-p38, ERK1/2, pERK1/2, JNK, and p-JNK expression

Cells (10 \(\times\) 10^6) were harvested, washed once with ice-cold phosphate buffered saline (PBS) and gently lysed for 30 min in ice-cold lysis buffer (1 mM MgCl\(_2\), 350 mM NaCl, 20 mM HEPES, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM Na\(_2\)P\(_2\)O\(_7\), 1 mM PMSF, 1 mM aprotonin, 1.5 mM leupeptin, 1 mM Na\(_3\)VO\(_4\), 20% glycerol, 1% NP40). Cell lysates were centrifuged for 10 min at 4°C (10,000 g) to obtain the supernatants, which were used for Western blot analysis. The anti-Cyclin D1 (clone 72-13G, catalog no. SC-450), anti-p21\(^{WAF-1/CIP-1}\) (clone F-5, catalog no. 6246), anti-p27 (clone N-20, catalog no. SC-527), anti-Bax (clone P-19, catalog no. SC-526), anti-Bcl-xL/S (clone L-19, catalog no. SC-1041), anti-p38 (clone C-20, Cat. No. SC-535), anti-Akt-1 (clone B-1, catalog. no. SC-5298, S.C.B. Inc.), anti-p-Akt1/2 (clone Thr 308-R, catalog. no. SC-16646-R, S.C.B., Inc.), anti-p-p38 (clone D-8, Cat. No. 7973), anti-ERK1/2 (clone K-23, Cat. No. SC-94), anti-p-ERK1/2 (clone E-4, Cat. No. SC-7383), anti-JNK (clone C-17, Cat. No. SC-474), anti-p-JNK (clone G-7, Cat. No. SC-6254) monoclonal antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. The anti-p53 (clone DO-1, Cat. No. SC-126) and the anti-Bcl-2 (clone: Bcl-2/100/D5) monoclonal antibodies were purchased from YLEM, Rome, Italy. The blots were washed with PBS and exposed to horseradish peroxidase-labelled secondary antibodies (Amersham Pharmacia Biotech, Arlington Heights, IL) for 45 min at room temperature. The immunocomplexes were visualized by the enhanced chemiluminescence detection system (ECL\(^{TM}\) Western blotting Analysis System, Amersham, Buckinghamshire, UK) and quantified by densitometric scanning.

2.6. Extraction and analysis of astaxanthin

The extraction of astaxanthin was performed from cells (10 \(\times\) 10^6) as described by Palozza et al. [46]. Briefly, cell pellet was resuspended in 1 ml ethanol, containing BHT (100 \(\mu\)g/ml), and homogenised by ultra turrax (on ice, 1 min, 12,000 rpm). Then, 1 ml of PBS and 3 ml of n-hexane were added and shaken. After centrifugation (5000 rpm, 2 min) the hexane layer was transferred into another tube and was evaporated (N\(_2\), 30 ± 1°C) to dryness. The extraction procedure with 3 ml of n-hexane was performed three times on each sample to ensure total removal of astaxanthin. The sample was dissolved in 100 \(\mu\)l methanol and a 20-\(\mu\)l aliquot was analyzed by reverse phase HPLC with spectrophotometric detection on a Perkin–Elmer LC-295 detector at 450 nm [46]. The column was packed with Alltech C18 Adsorbosphere HS material, 3-\(\mu\)m particle size, in a 15 x 0.46-cm cartridge format (Alltech Associates, Deerfield, IL). A 1-cm cartridge precolumn, containing 5-\(\mu\)m C18 Adsorbosphere packing was used. The mobile phase was 85% acetonitrile/15% methanol at a flow rate of 1 ml/min. At 8 min, the mobile phase was 60% acetonitrile/10% methanol/30% isopropanol. Ammonium acetate, HPLC grade, 0.01%, was added to the mobile phase.

2.7. Statistical analysis

Three separate cultures per treatment were utilized for analysis in each experiment. Values were presented as means ± SEM. One-way analysis of variance (ANOVA) was adopted to assess any differences among the concentrations (Figs. 1, 2A, 3A, 3B, 4D, 4F, 4H, 5B, 5D, 5E, 5F, 6B, 6D, 6E, 6F, Table 1). When significant values were found (P < 0.05), post hoc comparisons of means were made using Fisher’s test. Multifactorial two-way analysis of variance (ANOVA) was adopted to assess any differences among the treatments and the times (Fig. 2B). When significant values were found (P < 0.05), post hoc comparisons of means were made using the Tukey’s Honestly Significant Differences test. Differences were analyzed using Minitab Software (Minitab, Inc., State College, PA, USA).

![Fig. 1](image-url) Cell association and/or incorporation of astaxanthin in HCT-116 cells treated for 24 h with different concentrations of *Haematococcus pluvialis* extract. Values are means ± SEM of three different experiments. Values not sharing the same superscript were significantly different (P < 0.05) (Fischer’s test). N.d. = not detectable.
3. Results

3.1. Uptake of astaxanthin from H. pluvialis in HCT-116 cells

Results of the cellular uptake of astaxanthin from H. pluvialis extract are shown in Fig. 1. After 24 h of H. pluvialis treatment, astaxanthin was incorporated and/or associated into HCT-116 cells in a dose-dependent manner. Although it has to be underlined that H. pluvialis extract also contains a little amount (less than 0.5%) of other carotenoids (β-carotene, lutein canthaxanthin) besides astaxanthin (data not shown), no traces of them have been detected in HCT-116 cells, at the concentrations indicated.

3.2. Effects of H. pluvialis on the growth of HCT-116 cells

The effects of the H. pluvialis extract on the growth of human colon adenocarcinoma HCT-116 cells are shown in Fig. 2. Panel A shows the growth-inhibitory effects of different doses of the extract (0–25 μg/ml) in cells treated for 24 h and panel B shows the growth-inhibitory effects of the extract at the maximum dose tested (25 μg/ml) in cells treated for different periods of time (0–72 h). The treatment with H. pluvialis was able to inhibit the growth of HCT-116 cells in a dose- and a time-dependent manner. A significant (P < 0.05) inhibition was found at doses of the extract starting from 5 μg/ml. On the other hand, no significant difference in cell number were found between untreated and vehicle control cells (data not shown), suggesting that THF at the concentration up to 0.5% did not modify cell growth.

3.3. Effects of H. pluvialis on cell cycle progression and apoptosis in HCT-116 cells

To elucidate possible mechanism(s) responsible for the reduction of cell number by H. pluvialis extract in HCT-116 colon cancer cells, we first examined whether such a reduction was associated with cytostatic effects due to changes in cell cycle progression. Table 1 shows the cell cycle distribution of HCT-116 cells incubated in the absence or in the presence of varying concentrations of H. pluvialis. extract for 24 h. In the absence of the extract, most of HCT-116 cells (about 50%) were in S phase due to the high proliferative state. However, after a 24-h addition of H. pluvialis extract, we observed a net dose-dependent increase in the percentage of cells in G0/G1 phase, which was maintained throughout the treatment (72 h) (data not shown). The G0/G1 accumulation was accompanied by a corresponding reduction in the percentage of cells in S phase. In addition, the presence of a distinct sub-G1 peak (subdiploid DNA content), suggestive of the presence of apoptotic cells, was found following H.P. treatment at concentrations starting from 15 μg/ml. Such a peak increased in a dose-dependent manner.

Apoptosis induction by H. pluvialis extract in HCT-116 cells was further studied by evaluating the the percentage of apoptotic cells measured by TUNEL method (Fig. 3A) and the activation of caspase-3 (Fig. 3B). In cells treated with H. pluvialis, apoptosis was induced in a dose-dependent manner, as evidenced by both the methods. The pro-apoptotic effects were observed at concentrations of H. pluvialis starting from 15 μg/ml.

3.4. Effects of H. pluvialis on proteins involved in cell cycle progression, survival and apoptosis in HCT-116 cells

In an attempt to explore the effects of H. pluvialis on cell cycle progression, we measured the expression of the G0/G1 phase-related cyclin D1 (Fig. 4A and B), the expression of p53 (Fig. 5C and D) and those of the cyclin kinase inhibitors p21 WAF1/CIP1 (Fig. 5E and F) and p27 (Fig. 5G and H) in HCT-116 cells incubated for 24 h. H. pluvialis-treated cells exhibited a dose-dependent decrease of cyclin D1 and a dose-depen-
dent increase in p53, and p21^{WAF-1/CIP1} and p27 as compared with the respective untreated controls. These findings were consistent with the blockage of cell cycle in G0/G1 phase.

We also examined Akt expression and its phosphorylation in HCT-116 cells (Fig. 5A and B). Treatment of HCT-116 cells with *H. pluvialis* for 24 h decreased phosphorylation of Akt in a dose-dependent manner, with substantial inhibition already evident at the dose of 15 µg/ml.

Moreover, we evaluated the effect of *H. pluvialis* on apoptosis-regulating proteins (Fig. 5C), examining the expression of Bax which acts as apoptosis promoter (Fig. 5D), and that of Bcl-2 (Fig. 5E) and Bcl-XL (Fig. 5F), which are known to suppress programmed cell death, in HCT-116 cells treated for 24 h. The treatment with *H. pluvialis* significantly (P < 0.05) reduced the expression of both Bcl-2 and Bcl-XL and increased that of Bax. Such effects were consistent with the pro-apoptotic effects of the extract observed in these cells.

3.5. Effects of *H. pluvialis* on MAP kinases in HCT-116 cells

We also examined the expression of the p38, JNK, and ERK1/2 MAP kinases (Fig. 6). These kinases have been reported to be implicated in apoptosis induction. *H. pluvialis* induced a remarkable increase in the level of the phosphorylated forms of p38 (p-p38), JNK (p-JNK), and ERK1/2 (p-ERK12) at concentrations starting from 15 µg/ml, suggesting a role of these proteins in the pro-apoptotic effect of *H. pluvialis*.

3.6. Effects of *H. pluvialis* on the growth of other colon cancer cell lines

The growth inhibitory effects of *H. pluvialis* were also studied in other colon cancer cell lines, including HT-29, LS-174, WiDr, SW-480 cells (Table 2). In all these cells, the extract was given at the concentration of 25 µg/ml for 24 h. The extract inhibited cell growth in all the cell lines analysed, suggesting that it can act as a potent antitumoral agent in colon cancer cells. The percentage of cell growth inhibition ranged from 60.0 ± 3.0 in HCT-116 cells to 37.8 ± 2.1 in WiDr cells.

3.7. Effects of purified astaxanthin on the growth of HCT-116 cells

The effects on cell growth, apoptosis and expression of cell cycle- and apoptosis-related proteins were also studied in HCT-116 cells treated with purified astaxanthin (Fig. 7). In such experiments, the purified carotenoid was used at the concentration of 5 µM, which was comparable to the concentration of astaxanthin present in the *H. pluvialis* extract added to the cells at 25 µg/ml and present in the extract at 10%. A 24-h treatment with purified astaxanthin was able to inhibit cell growth (Fig. 7A) and to induce apoptosis, measured as caspase-3 activation (Fig. 7B), by affecting the expression of cell cycle-related proteins, including cyclin D1, p53, p21 and p27 (Fig. 7C) and that of apoptosis-related proteins, including Bax, Bcl-2 and Bcl-XL (Fig. 7D), similarly to the treatment with *H. pluvialis* extract. However, at the same astaxanthin concentration (5 µM), the effects of *H. pluvialis* extract were more pronounced than those observed by using purified astaxanthin.
4. Discussion

Our data demonstrate that *H. pluvialis* extract, very rich in astaxanthin, may act as potent growth-inhibitory agent *in vitro*, confirming previous observations of an antitumoral effect of astaxanthin *in vivo* [22–25,28,29]. Growth-inhibitory effects of purified astaxanthin have been reported in different tumor cells, including colon [23], oral [24] fibrosarcoma [25–27], breast [28,32,34], prostate [47] cancer cells and embryonic fibroblasts [41,48].

The inhibition of HCT-116 cell growth by *H. pluvialis* was associated with a slowing of cell cycle progression at the G0/G1 phase. Such finding is in agreement with previous reports showing that purified astaxanthin decreased cell proliferation of nonlesional and tumoral squamous [24] epithelium, as revealed by AgNOR enumeration and BrdUrd-labeling index and arrested cell cycle progression at G0/G1 phase.

Our finding is also in agreement with recent data on another xanthophyll, the β-cryptoxanthin, which has been reported to inhibit the growth of A549 cells, a non-small-cell lung cancer cell line and BEAS-2B cells, an immortalized human bronchial epithelial cell line in a dose-dependent manner, through an arrest of cell cycle at the G1/G0 phase [49]. In the present study, the arrest of cell cycle progression at the G0/G1 phase seems to involve a down-regulation of cyclin D1, which has been implicated in the control of this phase of cell cycle and a concomitant increase in p53 and in cyclin kinase inhibitors, including p21^{WAF-1/CIP-1} and p27. It is well known that cyclin D1 is an oncogene and it is over-expressed in several cancer cell lines [50]. It is interesting to note that other carotenoids, including canthaxanthin [51] and lycopene [52,53] have been reported to inhibit tumor cell growth by an arrest in cell cycle progression and a concomitant decrease in cyclin D1 expression. Concomitantly, we recently observed that both p53 and p21^{WAF-1/CIP-1} can be modulated by

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**Fig. 5.** AKT, Bax, Bcl-2 and Bcl-XL expression in HCT-116 cells treated with varying *Haematococcus pluvialis* (H.P.) extract concentrations for 24 h. Panels A and C: Representative Western Blot analyses; panels B, D, E, and F: Densitometric analysis of three different determinations. Values are expressed as protein/actin ratio. Values not sharing the same superscript were significantly different (*P* < 0.05) (Fischer’s test).
beta-carotene. In particular, we found that the carotenoid was able to induce an enhanced expression of p21WAF-1/CIP-1 and by a concomitant arrest of cell cycle progression at the G0/G1 phase [54]. Recently, b-Cryptoxanthin, consistently with the inhibition of the lung cancer cell growth, has been reported to suppress the protein levels of cyclin D1 and cyclin E, and to up-regulate the cell cycle inhibitor p21 [49]. Moreover, fucoxanthin has been shown to inhibit the proliferation of cancer cells by a mechanism involving an arrest of cell cycle [55] and an up-regulation of p21WAF-1/CIP-1 [56].

**Table 1**

Effects of *Haematococcus Pluvialis* extract (H.P.) on cell cycle progression in HCT-116 cells.

<table>
<thead>
<tr>
<th>Treatment H.P. (µg/ml)</th>
<th>G0/G1</th>
<th>S</th>
<th>G2</th>
<th>sub-G1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>31.0 ± 1.0a</td>
<td>49.9 ± 1.4c</td>
<td>14.7 ± 1.5a</td>
<td>4.4 ± 0.4a</td>
</tr>
<tr>
<td>5</td>
<td>34.5 ± 1.2b</td>
<td>46.6 ± 1.4c</td>
<td>14.3 ± 1.2b</td>
<td>4.6 ± 0.4c</td>
</tr>
<tr>
<td>15</td>
<td>39.8 ± 1.4c</td>
<td>40.8 ± 1.5b</td>
<td>13.4 ± 1.3c</td>
<td>6.0 ± 0.5b</td>
</tr>
<tr>
<td>25</td>
<td>41.5 ± 1.3c</td>
<td>37.8 ± 1.1a</td>
<td>13.3 ± 1.1a</td>
<td>7.4 ± 0.6c</td>
</tr>
</tbody>
</table>

The values were the means ± SEM of five experiments. Within the same column, values not sharing the same letter were significantly different (P < 0.05, Fisher’s test).

**Table 2**

Growth-inhibitory effects of *Haematococcus pluvialis* extract (H.P.) in colon cancer cells.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Growth Inhibition (% of Inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT-116</td>
<td>60.0 ± 3.0</td>
</tr>
<tr>
<td>HT-29</td>
<td>0.7 ± 3.5</td>
</tr>
<tr>
<td>LS-174</td>
<td>53.0 ± 3.1</td>
</tr>
<tr>
<td>WiDr</td>
<td>37.8 ± 2.1</td>
</tr>
<tr>
<td>SW-480</td>
<td>40.5 ± 2.2</td>
</tr>
</tbody>
</table>

*Haematococcus pluvialis* extract was added to the cells at the concentration of 25 µg/ml for 24 h. The values were the means ± SEM of three experiments.

**Fig. 6.** JNK, ERK1/2, p38 expression in HCT-116 cells treated with varying *Haematococcus pluvialis* (H.P.) extract concentrations for 24 h. Panels A, C, and E: Representative Western Blot analyses; panels B, D, and F: Densitometric analysis of three different determinations. Values not sharing the same superscript were significantly different (P < 0.05) (Fischer’s test).
cancer cells. However, several studies show that carotenoids are able to act as apoptosis inducers, by modulating different molecular pathways involved in the apoptotic process, as recently reviewed [7]. In particular, xanthophylls, such as canthaxanthin and zeaxanthin have been reported to act as potent apoptosis inducers [51,57]. It has been demonstrated that \(\beta\)-carotene is able to decrease the expression of Bcl-2 and Bcl-XL in colon cancer cells [58] and to diminish that of Bcl-2 in HL-60 cells [59]. Moreover, recent data suggest that carotenoids modulate Bid, Bad, Bcl-XL and Bax expression in different experimental models [7]. Recently, it has been suggested that the cleavage of the antiapoptotic protein Bcl-XL could be an important event during \(\beta\)-carotene-induced apoptosis, suggesting the presence of an extensive feedback amplification loop in \(\beta\)-carotene-induced apoptosis [60].

A sustained expression of the epidermal growth factor (EGF) receptor has been suggested to play a key role in the development of carcinogenesis [61] and in apoptosis induction [62]. In particular, Muto et al. suggested that \(\beta\)-carotene may prevent cervical carcinogenesis through an apoptosis induction mediated by the down-regulation of EGF receptor in pre-malignant cervical dysplastic cells [62]. It is noteworthy that the non-provitamin A carotenoid astaxanthin has been also found to be active as \(\beta\)-carotene in down-regulating EGF-binding, suggesting that such a mechanism is independent of the conversion to retinoids [62] and can be implicated in the pro-apoptotic effects of \(H. pluvialis\).

Several studies have linked AKT signalling pathway to changes in cell ability to undergo apoptosis. In our study, HCT-116 cells exhibited high levels of phosphorylated AKT, as a common feature of several cancer cells. \(H. pluvialis\) extract induced, at concentrations starting from 15 l\(g/\)ml, a decrease in phosphorylated AKT, which is consistent with the pro-apoptotic effects of the extract. Recent evidence suggests that carotenoids may modulate AKT pathway in cancer cells [53,63]. A recent in vitro study suggests that the modulation of AKT pathway may have a key role in the pro-apoptotic effects of lycopene under smoke conditions [54]. In fact, while RAT-1 fibroblasts exposed to cigarette smoke condensate (TAR) exhibited high levels of phosphorylated AKT, cells exposed to a combination of TAR and lycopene strongly decreased them. Moreover, the exposition of RAT-1 fibroblasts to TAR alone suppressed Bad-mediated apoptosis by inducing the phosphorylation of Bad at Ser136. Conversely, lycopene was able to completely prevent the phosphorylation of Bad induced by TAR, confirming in vitro the results obtained in vivo by Liu et al. [64]. In addition, \(\beta\)-carotene acted as a potent antitumoral agent in cav-1-positive cells, but not in cav-1-negative cells and inhibited AKT phosphoryla-
tion which, in turn, stimulated apoptosis by increasing the expression of β-catenin and c-myc and the activity of caspases, including caspases-3, -7, -8, -9 [63].

The major mitogen-activated protein kinases (MAPKs), c-JUN N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK) are three kinases that have been shown to regulate apoptosis [65]. Moreover, it has been reported that MAPK signalling cascades play an important role in oxidative stress-induced apoptotic cell death [66]. Therefore, we measured the level of p38, JNK and ERK1/2 in HCT-116 cells following the treatment with H. pluvialis for 24 h. The extract induced a remarkable increase in the expression of the phosphorylated forms of p38 (p-p38), JNK (p-JNK), and ERK1/2 (p-ERK12) at concentrations starting from 15 μg/ml, suggesting a role of these proteins in the pro-apoptotic effect of H. pluvialis. This observation is not surprising, since previous data have shown that carotenoids may exert a prooxidant and pro-apoptotic role in cancer cells [7]. The modulation of cell cycle- and apoptosis-related proteins by astaxanthin, present in H. pluvialis, also occurs by using other oxygenated (52 and 57) and non-oxygenated (7, 54, 58–60, 62, and 63) carotenoids. This suggests that such a modulation is a key-event in the growth-inhibitory effect of carotenoids in cancer cells and occurs by a mechanism independent of the conversion to retinoids.

The present work could have important implications for future studies regarding chemopreventive effects of the astaxanthin-rich alga H. pluvialis and its mechanism of action in colon cancer. In particular, we have demonstrated that the H. pluvialis extract acted as a potent inhibitor of cell growth in several colon cancer cell lines. It may mediate its protective effects through a decrease in the expression of cyclin D1 and an increase of p53 and some cyclin kinase inhibitors, including p21WAF1/CIP-1 and p27, which arrest cell cycle progression. Moreover, it may promote apoptosis through a down-regulation of the phosphorylation of AKT, changes in apoptosis-related proteins, including Bax, Bcl-2 and Bcl-XL and in MAP kinases signalling. Finally, the observation that at the same astaxanthin concentration, the effects of H. pluvialis extract on cell growth and apoptosis were more pronounced than those of purified astaxanthin, strongly supports the use of such an extract in human supplementation.

5. Conflict of interest

All authors disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations.

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