The inhibition of Caco-2 proliferation by astaxanthin from *Xanthophyllomyces dendrorhous*

Kornchanok Wayakanon,1,* Kanjana Rueangyotchanthana,2 Praween Wayakanon3 and Chatrudee Suwannachart2,*

**Abstract**

**Purpose.** To investigate the efficiency of natural astaxanthin that has been extracted from *Xanthophyllomyces dendrorhous* in inhibiting the proliferation and viability of colorectal adenocarcinoma cell line (Caco-2; colon cancer cells).

**Methodology.** Caco-2 cells and normal human oralkeratinocytes (NOKs) were treated with different concentrations of extracted astaxanthin, ranging from 0.075 to 10 mg ml−1, for 24, 48 and 72 h. The number of cells was determined via MTS assay and the proliferating cells were investigated by bromodeoxyuridine (BrdU) assay.

**Results/Key findings.** Of the Caco-2 cells, 30–50 % remained viable, while the NOKs showed 110–120 % survival when treated with 5 mg ml−1 astaxanthin. The Caco-2 cells showed distinct structural shrinkage when treated with the same concentration of astaxanthin. Fluorescent labelling of the DNA of the proliferative cells with BrdU showed a significant decrease in the number of the proliferative Caco-2 cells when the concentration of astaxanthin was increased to 5 mg ml−1.

**Conclusion.** The natural astaxanthin from *X. dendrorhous*, at an appropriate concentration, is effective in terminating the viability of, or retarding the proliferative activity of, Caco-2 cells, without harmful effects on NOKs.

**INTRODUCTION**

Astaxanthin (3, 3′-dihydroxy-β, β′-carotene-4, 4′-dione) is a lipid-soluble carotenoid in the xanthophyll group. It shows ability to protect cells from reactive oxygen species (ROS) in the environment, and has stronger free radical antioxidant activity than vitamin E and beta-carotene [1]. It also plays an important function as an anti-inflammatory [2], anti-cancer [3] and anti-ageing agent [4], and has immunity-enhancing qualities [5]. Astaxanthin also displays immunization properties and is effective in the prevention of cardiovascular disease [6] and diabetes [7].

Astaxanthin has antioxidant properties that are greater than those of vitamin E and 10 times higher than those of other carotenoids, including β-carotene. Astaxanthin scavenges free radicals and has the ability to quench singlet oxygen (unstable energy-rich active oxygen). In the quenching process, astaxanthin remains intact, enabling it to counter more cycles of singlet oxygen quenching [8, 9].

Astaxanthin is utilized in food, and in pharmaceutical and nutraceutical products in capsule form and as tablets, oils, cosmetic creams, syrups or dry powders [10]. Astaxanthin is found in natural sources such as yeast, microalgae, salmon, trout, krill and lobster, but commercially used astaxanthin is normally obtained from the heterobasidiomycetous yeast, *Xanthophyllomyces dendrorhous*, and from microalgae and chemical synthesis [11]. However, hazardous waste is created by the chemical synthesis, which can have a detrimental affect on the environment and human health. Thus, microbial sources of astaxanthin are excellent alternative choices when considering public health safety.

**METHODS**

**Yeast culture**

*X. dendrorhous* TISTR 5953 was cultured on yeast mould (YM; Difco, USA) agar supplemented with glucose 10 % (w/v) (Sigma, USA), malt extract 3 % (w/v) (Difco, USA), yeast extract 3 % (w/v) (Difco, USA) and peptone 5 % (w/v) (Difco, USA). This cultured strain was maintained using 20 % glycerol (Sigma, USA) and kept at ~80 °C. The yeast cultures were shaken by a rotary shaker (Vision Shaking Incubator, VS-8480S, Republic of Korea) at
200 r.p.m. for 3 days at 25 °C in 500 ml Erlenmeyer flasks, each of which contained 200 ml YM broth. Pilot scale cultures were grown in a Biostat B fermenter (Sartorius, Germany) using 4 l as the working volume, at an aeration rate of 1 v.v.m. and an agitation rate of 250–300 r.p.m., with the pH level maintained between 5.5–7.0 with 1M NaOH (Merck, USA) and 1M HCl (Merck, USA). Cell pellets were harvested by centrifugation (Avanti JXN-26, Beckman Coulter, USA).

**Extraction of astaxanthin from X. dendrorhous**

The astaxanthin was extracted from dried X. dendrorhous by supercritical fluid critical carbon dioxide (SFC) at various conditions without co-solvent (Spe-ed SFE-2, Applied Separations, USA) for 1.5 h. The crude extract was collected and kept at 4 °C. The extracted product was analysed by high-performance liquid chromatography (HPLC).

**HPLC determination of astaxanthin**

The extracted product was investigated by HPLC (HPLC 1260, Agilent Technologies, USA, C-18 column, UV/VIS detector). The mobile phase consisted of acetonitrile, methanol, dichloromethane and H2O (79.9:10:10:0.1 v/v), with a flow rate of 1 ml min–1. The detection wavelength was performed at 474 nm.

**Culturing the colorectal adenocarcinoma cell line (Caco-2) and normal human oral keratinocytes (NOKs)**

The cell culture experiments were approved by the Naresuan University Research Ethics Committee Approval (reference: 138/57). The NOKs and i3T3s were co-cultured in Green's medium until almost all the i3T3s had been replaced by NOKs. The NOKs were cultured in Green's modified Eagle's medium (EMEM) (Hyclone, USA) with 10 % foetal bovine serum (FBS) (Hyclone, USA) and 100 IU ml⁻¹ penicillin and 100 mg ml⁻¹ streptomycin (Hyclone, USA). The NOKs were cultured in Green's medium [Dulbecco's modified Eagle's medium (DMEM) (Hyclone, USA) and Hams-F12 medium (Hyclone, USA) at a ratio of 3:1 (v/v)]. The resulting medium contained 10 % (v/v) FBS, 100 IU ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, 1.25 µg ml⁻¹ amphotericin B (Hyclone, USA), 0.1 M chola toxin (Hyclone, USA), 10 ng ml⁻¹ epidermal growth factor (Hyclone, USA), 0.4 µg ml⁻¹ hydrocortisone (Hyclone, USA), 0.18 mM adenosine (Hyclone, USA), 5 µg ml⁻¹ insulin (Hyclone, USA) (Hyclone, USA) and 2 mM l-glutamine (Hyclone, USA). Additionally, murine 3T3-swiss albino mouse embryo fibroblasts (3T3) were cultured in the complete medium (DMEM with 10 % FBS, 100 IU ml⁻¹ penicillin and 100 mg ml⁻¹ streptomycin).

Gingival tissue was obtained from consenting patients during routine dental surgery [Naresuan University Research Ethics Committee approval (reference: 138/57)]. The sample tissues were collected in Green’s medium and then submerged in 1 unit ml⁻¹ dispase with the Green’s medium at 4 °C overnight. The epithelium was peeled off from the connective tissue layer and then incubated in 0.25 % trypsin/EDTA solution at 37 °C for 30 min to retrieve the isolated epithelial cells.

Keratinocytes require additional growth support from other growth factors and extracellular matrix components from fibroblasts. The 3T3 fibroblasts were irradiated by 60 Gy gamma radiation (i3T3s) to terminate cell proliferation. The NOKs and i3T3s were co-cultured in Green’s medium until almost all the i3T3s had been replaced by NOKs. The NOKs were then fully confluent in the culturing flask [12].

<table>
<thead>
<tr>
<th>Experimental test</th>
<th>Pressure (MPa)</th>
<th>Temperature (°C)</th>
<th>Concentration of astaxanthin (p.p.m.)</th>
<th>Concentration of astaxanthin (mg g⁻¹ of dried cell)</th>
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<tr>
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<td>4.60±0.51*</td>
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<td>42.12±5.02abc</td>
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<td>3.28±0.13d</td>
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<tr>
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<td>50</td>
<td>44.48±2.52ab</td>
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</table>

The superscript letters represent statistically significant differences (P<0.05) within the column.
Studying the effect of astaxanthin on cell viability and proliferation ability in Caco-2 cells and NOKs

The Caco-2 cells and NOKs ($10^4$ cells) were treated with different concentrations of astaxanthin (10, 5, 2.5, 1.25, 0.625, 0.31, 0.15 and 0.075 mg ml$^{-1}$) for 24, 48 and 72 h. The viability of the cells was determined by observing the morphology of cells and performing the MTS assay (Thermo Fisher Scientific, USA). The cell proliferation was observed via the bromodeoxyuridine (BrdU) assay (Thermo Fisher Scientific, USA). The percentage of proliferating cells was quantified by counting the BrdU-labelled cells and normalized with the number of total cells in the same pictures. The BrdU-labelled pictures from five areas of each slide were randomly taken under ×200 magnification under a fluorescent microscope (Olympus BX60, Tokyo, Japan). There were nine slides for each group.

Data analysis

The concentration of the extracted astaxanthin from X. dendrorhous, the percentages of the vital NOKs and Caco-2 cells, and the percentage of proliferating Caco-2 cells were evaluated by the Kruskal–Wallis and Mann–Whitney U tests ($P<0.05$).

RESULTS

Extraction of astaxanthin from X. dendrorhous

The best astaxanthin extraction results were obtained from supercritical fluid extraction at a pressure of 30 MPa and a temperature of 35 °C without co-solvent (4.60±0.51 mg g$^{-1}$ of dried cells) and with 4.57±0.30 mg g$^{-1}$ of dried cells at a pressure of 35 MPa and a temperature of 50 °C (Table 1). Although the two sets of extraction conditions showed no significant difference ($P>0.05$) of astaxanthin concentration in their results, high temperatures can affect astaxanthin antioxidant activity and so conditions of 30 MPa pressure and a temperature of 35 °C were chosen for further experimentation.

The vitality of Caco-2 and NOKs after treatment with astaxanthin

The number of viable Caco-2 cells and NOKs after treatment with astaxanthin for 24, 48 and 72 h was detected by MTS assay. The percentage of viable NOKs clearly increased (between 110–130 % survival) after treatment with 1.25–5 mg ml$^{-1}$ of astaxanthin. When the concentration of astaxanthin reached 10 mg ml$^{-1}$, the percentage of viable NOKs decreased sharply (with approximately 20 % survival). For the viability of Caco-2 cells, the percentage of viable cells

![Fig. 1.](image-url)
hovered between 70–80 % after treatment with an astaxanthin concentration of between 0.075 and 2.5 mg ml$^{-1}$. When the concentration increased to between 5 and 10 mg ml$^{-1}$, the percentage of viable Caco-2 cells decreased significantly to between 10 and 50 %.

The MTS assay showed that the number of NOKs treated with 10 mg ml$^{-1}$ astaxanthin decreased significantly at all time points (Fig. 1a). The number of viable Caco-2 cells treated with 5 and 10 mg ml$^{-1}$ astaxanthin also decreased significantly at all time points (Fig. 1b), while the Caco-2 cells also had a higher mortal sensitivity to the astaxanthin than the NOKs.

**Median lethal dose (LD$_{50}$) of astaxanthin**

LD$_{50}$ is defined as the concentration of substance required to kill 50 % of the members of a population. The LD$_{50}$ for the NOKs was equal at all time points at 8.13 mg ml$^{-1}$ concentration of astaxanthin (Fig. 1c). The LD$_{50}$ for the Caco-2 cells was 5.01 mg ml$^{-1}$ after 24 h of exposure, 4.17 mg ml$^{-1}$ at 48 h and 3.39 mg ml$^{-1}$ at 72 h (Fig. 1d).

**The alteration of NOK and Caco-2 cell morphology after treatment with astaxanthin**

The morphology of the untreated NOKs and Caco-2 cells showed various tetragonal features, with the cell membrane clearly in contact with the adjacent cells. After treatment with astaxanthin, NOKs and Caco-2 cells showed altered cell morphology and were observed to have experienced structural shrinkage. The shape of the treated NOKs had changed from being tetragonal to being round after treatment with astaxanthin at different concentrations and at all time points (Fig. 2). The Caco-2 cells also had rounded features after treatment with 5 mg ml$^{-1}$ at all time points (Fig. 3). The cell membranes

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**Fig. 2.** The morphology of NOKs when treated with 0–10 mg ml$^{-1}$ astaxanthin for 24–72 h. The morphology of the NOKs in all groups became rounded after treatment with astaxanthin at all times. This was clearly seen in comparison with the control.
of both of the cells with a round structure were no longer in contact with each other.

**Determination of the proliferation of NOKs and Caco-2 cells by BrdU assay after treatment with astaxanthin**

BrdU can be incorporated into freshly synthesized DNA, allowing it to be utilized to investigate the proliferation of Caco-2 cells following treatment with astaxanthin. The newly synthesized DNA of the proliferating cells was localized by colouring the cells green with FITC by immunocytochemistry (Fig. 4). As the concentration of astaxanthin increased, the number of proliferating Caco-2 cells decreased. In particular, at a 5 mg ml$^{-1}$ concentration of astaxanthin, the number of BrdU labelled Caco-2 cells decreased distinctly (to less than 25–30\%) ($P<0.01$) (Table 2).

**DISCUSSION**

The supercritical fluid extractions were performed under different experiment conditions to ascertain the most suitable conditions for astaxanthin extraction. Although none of the conditions showed significant differences ($P>0.05$), we chose astaxanthin extraction at a pressure of 30 MPa and a temperature of 35°C as the most suitable conditions. These conditions produced a high concentration of astaxanthin at 4.60±0.51 mg g$^{-1}$ of dried cells. This suggests that the increase of cell disruption at 30 MPa is a consequence of the supercritical fluid effect of carbon dioxide, which penetrates the cell wall and disrupts the carotene–protein bonds [13]. Temperature is the most influential environmental factor, as it leads to an unstable astaxanthin structure. Given this, 35°C was chosen as an appropriately low temperature to avoid the problem of astaxanthin degradation that can occur at high temperatures [14].

The cytotoxicity of astaxanthin on keratinocytes and intestinal cancer cell lines was investigated by determining the number of viable cells and observing the cell morphology after the cells had been treated with astaxanthin. These cytotoxic doses for NOKs and Caco-2 cells resulted in changes to the morphology of the cells after treatment that were similar to the changes observed from the MTS assay. Both kinds of cells had the same characteristics when treated with these cytotoxic doses, i.e. they experienced shrinkage and rounding of the cells. After the observation of the cell morphology, the effect of the cytotoxicity of astaxanthin on both cells was quantified by the MTS assay.
The percentage of viable cells was calculated from the non-treated group to give us a standard for comparison. Astaxanthin at a concentration of 10 µg mL⁻¹ was the cytotoxic dose given to the NOKs (with approximately 25% survival), while 5 µg mL⁻¹ seemed to be the cytotoxic dose for Caco-2 cells (with approximately 35% survival). Our results showed that the most effective concentration of astaxanthin for killing Caco-2 cells is between 5 and 10 µg mL⁻¹, and that this concentration is not harmful to NOKs.

Surprisingly, the percentage of viable NOKs clearly increased (between 110–130% survival) after treatment with 1.25–5 µg mL⁻¹ astaxanthin. Within this range of concentrations, astaxanthin might be able to either increase the proliferation rate or extend the longevity of NOKs. This aspect needs further investigation.

LC₅₀ is a term that is used in in vitro studies and refers to the concentration of drug that kills half of the tested population [15]. The LC₅₀ value is frequently of low reliability [17].

In our study, NOKs and Caco-2 cells were submerged in astaxanthin solution throughout the testing periods, except for the duration of the MTS assays, in which situation the astaxanthin solution was discarded prior to the incubation of the cells, for approximately 3 h, in the MTS solution. This period of 3 h without astaxanthin solution appears to be the recovery period for both kinds of cells. Notwithstanding this period of recovery, the morphology of the cells had clearly changed.

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**Table 2.** The percentage of proliferating Caco-2 cells after treatment with different concentrations of astaxanthin

<table>
<thead>
<tr>
<th>Concentration of astaxanthin (µg mL⁻¹)</th>
<th>Treatment with astaxanthin (hours)</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td></td>
<td>57.69 ± 6.24</td>
<td>51.27 ± 6.56</td>
<td>43.91 ± 6.56</td>
</tr>
<tr>
<td>1.25</td>
<td></td>
<td>57.91 ± 4.71</td>
<td>51.07 ± 6.59</td>
<td>43.93 ± 4.38</td>
</tr>
<tr>
<td>2.5</td>
<td></td>
<td>61.67 ± 4.38</td>
<td>39.21 ± 3.58</td>
<td>50.81 ± 3.83</td>
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<tr>
<td>5</td>
<td></td>
<td>25.30 ± 5.73</td>
<td>8.57 ± 3.19</td>
<td>14.16 ± 0.59</td>
</tr>
</tbody>
</table>

Lower case characters represent statistically significant differences (P<0.05) within the row.

Upper case characters represent statistically significant differences (P<0.05) within the column.

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![Fig. 4. BrdU-detected proliferation of Caco-2 cells after treatment with astaxanthin at 1.25–5 µg mL⁻¹ for 24–72 h. The proliferation of cells in the Caco-2 samples after treatment with 5 µg mL⁻¹ astaxanthin was lower than that of the control group, indicating that the treatment retarded cell proliferation.](image-url)
changed during the 24 h of treatment with the cytotoxic dose, implying that the recovery phase might not affect the LC_{50} value.

The BrdU assay allowed us to detect the proliferating cells by identifying the actively replicating DNA [18], which presented as a fluorescent green nucleus of the BrdU-incorporated Caco-2 cells. The density of green-nucleus Caco-2 cells treated with 5 mg ml^{-1} astaxanthin was obviously reduced compared to the other concentrations, indicating that 5 mg ml^{-1} is the most effective concentration of astaxanthin for retarding the proliferation of Caco-2 cells. However, the BrdU did not stain the nuclei of the NOKs in any of the cells, including the control group. The explanation for this may be that at the beginning of our experiments, the NOKs and i3T3s were co-cultured to support the proliferation of NOKs. During this process, the i3T3s provided many essential growth factors, such as keratinocyte growth factors (KGFs), hepatocyte growth factors (HGFs), heparin-binding EGF-like growth factors (HB-EGFs) and platelet-derived growth factors (PDGFs) for the NOKs [19]. After the NOKs’ confluence with the i3T3s, they were trypan-blue stained and then grown independently without i3T3s for 24 h before receiving the astaxanthin treatment. Under these conditions, the NOKs were able to survive, but they may have proliferated minimally, resulting in the BrdU labelling not being seen in them.

**Conclusion**

When administered at an appropriate concentration, natural astaxanthin from *Xanthophyllomyces dendrorhous* is effective in retarding the proliferation of colon cancer cells (Caco-2) without having harmful effects on normal epithelial cells.

**Funding information**

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**Ethical statement**

The Ethical Committee of Naresuan University approved the cell culture experiment, and the subjects gave informed consent.

**References**