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Full Paper

Anti-cancer effects of astaxanthin extract from wild-type and hyperproducing *Xanthophyllomyces dendrorhous* mutant on breast cancer cells of dissimilar estrogen receptor status

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Abstract: The effects of astaxanthin extracts from wild-type and mutant *Xanthophyllomyces dendrorhous* on MCF7 and MDA-MB-231 breast cancer cells were examined. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assays prove that the wild-type and mutant extracts are non-toxic towards non-cancerous MCF-10A cells but exhibit a growth-inhibitory effect on MCF-7 and MDA-MB-231 cells in a dose-dependent manner. The mutant extract shows a lower IC_{50} and is more effective in inhibiting MCF-7 and MDA-MB-231 cells. The IC_{50} values did not exceed the recommended limit of 30 µg/ml. MCF-7 and MDA-MB-231 cells lose their shape after treatment with the wild-type or mutant extract and apoptosis hallmarks develop in a time-dependent manner. Flow cytometry analysis signifies apoptosis induction by both extracts in MCF-7 and MDA-MB-231 cells in a cell-type-dependent manner. Cell cycle arrest is observed at the S phase and G2/M phase in MCF-7 cells treated with wild-type extract and at the G2/M phase when treated with mutant extract. In MDA-MB-231 cells, cell cycle arrest is observed at the S phase for both extract treatments. Reactive oxygen species (ROS) analysis shows 2-fold ROS accumulation in MCF-7 and MDA-MB-231 cells after treatment with both extracts. Wound healing results demonstrate that the mutant extract exhibits better migration inhibition in MCF-7 and MDA-MB-231 cells than the wild type extract. Both extracts give better inhibition on MCF-7 cells than MDA-MB-231 cells.

Keywords: astaxanthin, *Xanthophyllomyces dendrorhous*, breast cancer cells, dissimilar estrogen receptor

Maejo Int. J. Sci. Technol. **2023**, *17*(01), 24-39

INTRODUCTION

Breast cancer is the most commonly diagnosed cancer among females and the leading cause of cancer death [1]. It is a heterogenous disease where variant molecular features and therapeutic response are observed among patients and its treatment varies depending on the patient age and tumour characteristics [2]. In most breast cancer cases the expression level of estrogen receptor-α (ERα) is directly correlated with the tumour progression [3]. In general, estrogen binds with ER-α as a transcription factor complex to regulate expression of target genes and proteins that are crucial for biological functions. Estrogen has multiple functions in breast cancers including stimulating cancer growth and interfering with chemotherapeutic efficacy. The mechanism of estrogen-stimulated tumour growth has been thoroughly investigated using the MCF-7 (estrogen-receptor positive) cell model. Meanwhile, the aggressive and invasive triple negative breast cancer MDA-MB-231 (estrogen-receptor negative) cells are known to be resistant to a number of anti-cancer agents [4]. Thus, a standard breast cancer treatment approach is not suitable and current cancer treatments often induce therapeutic problems and side effects which aggravate patient discomfort. New approaches to improving tolerance and reducing damage from cancer therapy are urgently needed and natural compounds with anti-cancer properties have drawn attention from researchers.

Astaxanthin is a natural fat-soluble orange-red pigment that belongs to the xanthophyll subclass of the carotenoids [5]. The presence of the hydroxyl (OH) and keto (C=O) moieties on each ionone ring makes astaxanthin a highly antioxidative product [6]. It exhibits potent antioxidant, immunomodulating, anti-inflammatory and enzyme-inducing properties, all of which suggest its potential role in the prevention and treatment of cancer [7]. Almost 95% of astaxanthin preparations available in the market are in synthetic forms derived from petrochemical sources and less stable than the natural one [8]. The growing demand for natural products and strict regulations on synthetic chemicals have stimulated the search for natural sources of astaxanthin with potential for industrialisation. Few sources of microbial origin can compete economically with the synthetic astaxanthin, notably the green microalga *Haematococcus pluvialis* and the red yeast *Xanthophyllomyces dendrorhous*, where astaxanthin makes up as much as 83-87% of the total carotenoids produced [9]. However, so far, these products only take up a small fraction of the market due to their limited production and low level of pigment production in wild-type strains. Therefore, an astaxanthin-hyperproducing *X. dendrorhous* mutant M34 was developed in our laboratory [10] and was used in this study to overcome the problem.

Some studies were recently conducted on the anti-cancer activity of astaxanthin in some breast cancer cell lines. Kim et al. [11] reported that astaxanthin might induce apoptosis of SKBR3 breast cancer cells by regulating the expression of apoptosis-related molecules including mutp53, PARP-1, Bcl2, SOD, MAPKs and Pontin. In other studies astaxanthin was shown to inhibit proliferation and migration of MDA-MB-231 and MCF-7 breast cancer cells [12] and decrease cell viability in T-47D and MDA-MB-231 cell lines [13]. These initial findings suggest that astaxanthin has potential anti-cancer effect, especially apoptotic effect in cancer cells. However, little is known of more detailed molecular mechanisms underlying apoptosis in the cells. This study aims to examine the *in vitro* effects of astaxanthin extracts on the viability and apoptosis of breast cancer cell lines of dissimilar estrogen receptor status.

Maejo Int. J. Sci. Technol. **2023**, *17*(01), 24-39

MATERIALS AND METHODS

Strain and Culture Conditions

The parental wild-type *Xanthophyllomyces dendrorhous* DSM 5626 strain from Deutsche Sammlung von. Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and its astaxanthin hyperproducing mutant M34, generated through N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis in our laboratory [10] were used in this study. The cultures were maintained on yeast malt agar (pH 5.5) at 4°C.

Cell Culture and Reagents

Both MCF-7 and MDA-MB-231 breast cancer cells (ATCC, USA) were cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, USA) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, USA), 100 U/mL of penicillin (Thermo Fisher Scientific, USA), and 100 µg/mL of streptomycin (Thermo Fisher Scientific, USA). The cells were maintained at 37°C in a humidity-controlled incubator with 5% CO_2.

Preparation of Astaxanthin Extracts

Pigments from the wild-type and mutant *X. dendrorhous* cells were extracted based on a modification of the method described by Sedmak et al. [14]. One gram of lyophilised cell mass was suspended in 2 mL of DMSO preheated to 55°C. After adding glass beads (diameter 1 mm), the sample was vortexed for 3 min. and 1 mL of acetone was added followed by 1 mL of petroleum ether. The mixture was vortexed for 30 sec. and 1 mL of 20% NaCl was added. The mixture was centrifuged for 5 min. and the absorbance of the supernatant was measured at OD_{474} by a UV spectrophotometer (UV-3600, Shimadzu, Japan). The spectrometric analysis showed that the mutant *X. dendrorhous* produced more pigment (594 µg/g dry cell weight) than the wild type strain (285 µg/g dry cell weight), exhibiting a 2-fold increase in pigment yield. The pigment extracts were profiled by high-performance liquid chromatography analysis and the results showed that the wild-type and mutant *X. dendrorhous* produced astaxanthin in high purity (>70%). The astaxanthin extracts were evaporated to dryness under a stream of nitrogen and used as stock astaxanthin extracts. The stock astaxanthin extracts were dissolved in 1% DMSO in Dulbecco's modified Eagle's medium and aliquots were placed in the culture medium to give the desired concentrations (0-30 µg/mL) immediately before each use. The final concentrations of DMSO did not exceed 1% (v/v) and did not affect the cell proliferation.

Cell Viability Assay

MCF-7 and MDA-MB-231 cells were seeded into 96-well plates at 5000 cells per well and incubated at 37°C, 5% CO_2 for 24 hr. The cells were then treated with astaxanthin extracts at different concentrations and incubated for 24 hr. Ten µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Invitrogen, USA) were then added and the plates were incubated in the dark. After 4 hr, the medium was removed and 200 µL of DMSO were added and the plates were read at 570 nm with a microplate reader. The experiment was repeated with normal mammary epithelial cells MCF-10A as control. The IC_{50} value of the astaxanthin extracts against both cell lines were determined by using Sigma plot version 12.5.

Maejo Int. J. Sci. Technol. **2023**, *17*(01), 24-39

Cell Morphology Observation

The MCF-7 and MDA-MB-231 cells were seeded in 24-well plates and treated with the IC_{50} of astaxanthin extracts once the cells were fully attached. Treated samples were observed under an inverted light microscope at 0, 8 and 24 hr after crystal violet staining. Untreated cells were used as negative control.

Annexin-V FITC/PI Apoptosis Assessment

Annexin-V FITC/PI apoptosis assessment was performed using the FITC Annexin V apoptosis detection kit (BD Biosciences, USA). Both the untreated and treated (IC_{50}) cells at the concentration of $1x10^6$ cells/mL were harvested and washed twice with ice-cold phosphate-buffered saline before 1 mL of 1X binding buffer was added. One hundred µL of each mixture were then added with 5 µL of FITC Annexin V and propidium iodide dye and incubated for 15 min. at room temperature in the dark. Four hundred µL of 1X binding buffer were then added. Unstained cells, cells stained with FITC Annexin V only and cells stained with propidium iodide only were used to set up compensation for the assessment. The assessment was carried out within 1 hr using a flow cytometer (BD Calibur, USA).

Cell Cycle Analysis

Treated and untreated cells were harvested and washed with phosphate-buffered saline. The cells were then fixed with 70% ethanol at a cell density of 1×10^6 and treated with FxCycleTMPI/RNase staining solution (Invitrogen, USA). The cells were analysed with a flow cytometer within 1 hr.

Intracellular Reactive Oxygen Species (ROS) Analysis

ROS analysis was performed by using a fluorometric intracellular ROS kit (Sigma, USA). After $1x10^5$ cells were grown in a 96-well plate for 24 hr, the master reaction mix was then added and the plate was incubated at 37°C, 5% CO_2, for 1 hr. Astaxanthin extracts at IC_{50} were then added into the wells, which were then incubated for 24 hr at 37°C. The fluorescence intensity was measured at 490 nm and 525 nm by a fluorescence reader (Tecan, Switzerland).

Wound Healing Assay

The cell lines were seeded in 12-well plates at 200,000 cells per well and grown for 24 hr. A sterile 10-µL micropipette tip was used to make a scratch of vertical lines through the confluent cell monolayer and an image was taken as the before image. The cells were treated with IC_{50} of astaxanhtin extracts and the after images were taken at 12 hr and 24 hr. Using the before and after images, the distance that the cells migrated into the gap was analysed.

Statistical Analysis

The mean value and standard deviation (SD) were calculated from the results. One way analysis of variance (ANOVA) was applied for comparison of the mean values using Sigma Plot 12.5 version. $P< 0.05$ was regarded as significant.

Maejo Int. J. Sci. Technol. **2023**, *17*(01), 24-39

RESULTS AND DISCUSSION

Cell Viability Assay

MTT assay shows that both the wild-type and mutant astaxanthin extracts are non-toxic towards the non-tumourigenic breast cells MCF-10A as the cell viability is over 80% even after the maximum incubation period of 72 hr with a maximum dosage of 30 µg/mL (Figure 1). Wayakanon et al. [15] showed that the percentage of viable normal human oral keratinocytes clearly increased after treatment with 1.25-5 mg/mL of astaxanthin and the percentage of the viable keratinocytes decreased only when the concentration of astaxanthin reached 10 mg/mL. The effects of the wild-type and mutant astaxanthin extracts on the growth of MCF-7 and MDA-MB-231 cells are shown in Figure 2 and Figure 3 respectively. The MCF-7 and MDA-MB-231 cell viability decrease with increasing concentration of the wild-type and mutant extracts. Both astaxanthin extracts demonstrate a dose-dependent inhibition of cell proliferation of the breast cancer cell lines. Notably, both MCF-7 and MDA-MB-231 cell lines exhibit different response to the two astaxanthin extracts. A significant decrease in cell viability occurs between 14-20 µg/mL of the wild-type extract, while it occurs between 8-12 µg/mL of the mutant extract. The growth of the cancer cells is suppressed to ~20% at the end of 24 hr with 20 µg/mL of the wild-type extract and 12 µg/mL of the mutant extract.

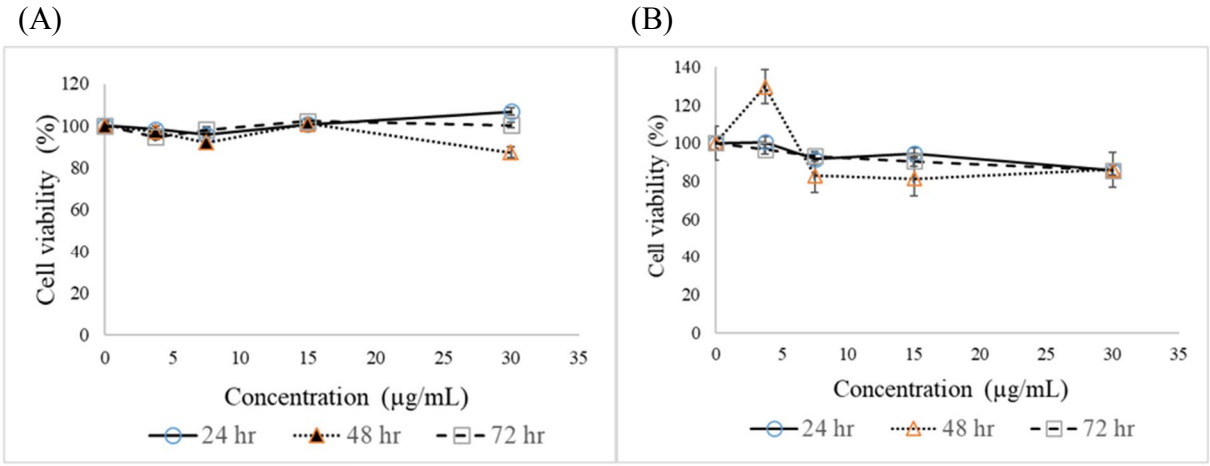

Figure 1. Cell cytotoxicity assay on MCF-10A normalised cell line with (A) wild-type and (B) mutant M34 astaxanthin extracts for 24 hr, 48 hr and 72 hr

For both cell lines, the concentrations of the wild-type and mutant astaxanthin extracts higher than 18 µg/mL and 10 µg/mL respectively are considered to be effective in inducing cell growth inhibition. A study by Atalay et al. [16] found that astaxanthin treatment affected the cell proliferation in a concentration-dependent manner. Astaxanthin at 10 µg/mL and 15 µg/mL decreased proliferation by 1% and 19% respectively while astaxanthin at 30 µg/mL was toxic and led to a dramatic reduction in the proliferation of MCF-7 cells (63%). Karimian et al. [13] showed that astaxanthin significantly decreased the viability of T-47D and MDA-MB-231 breast cancer cells in a dose-dependent manner after 24 hr.

Maejo Int. J. Sci. Technol. **2023**, *17*(01), 24-39

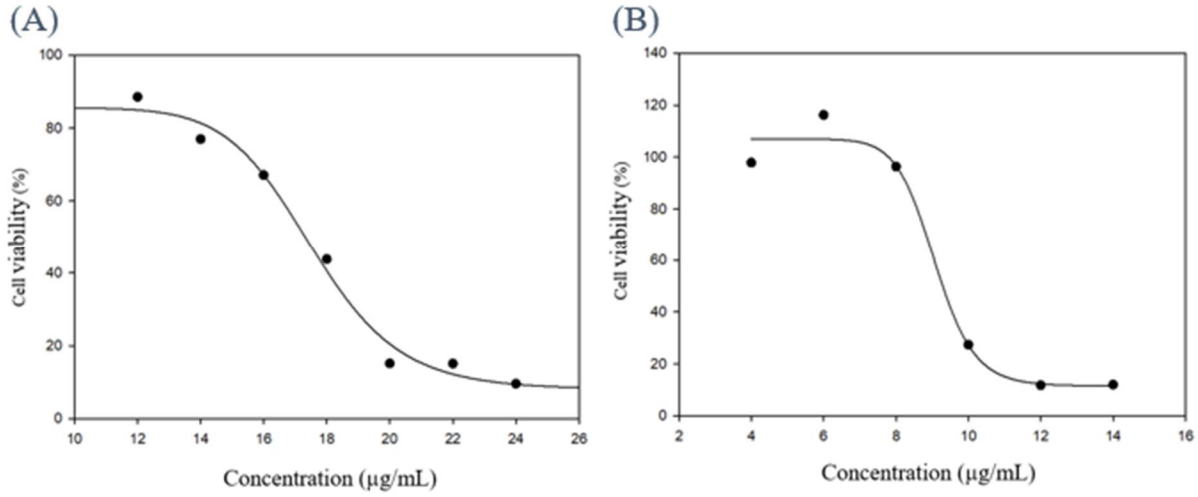

Figure 2. Effect of (A) wild-type and (B) mutant astaxanthin extracts on viability of MCF-7 cell line using MTT assay

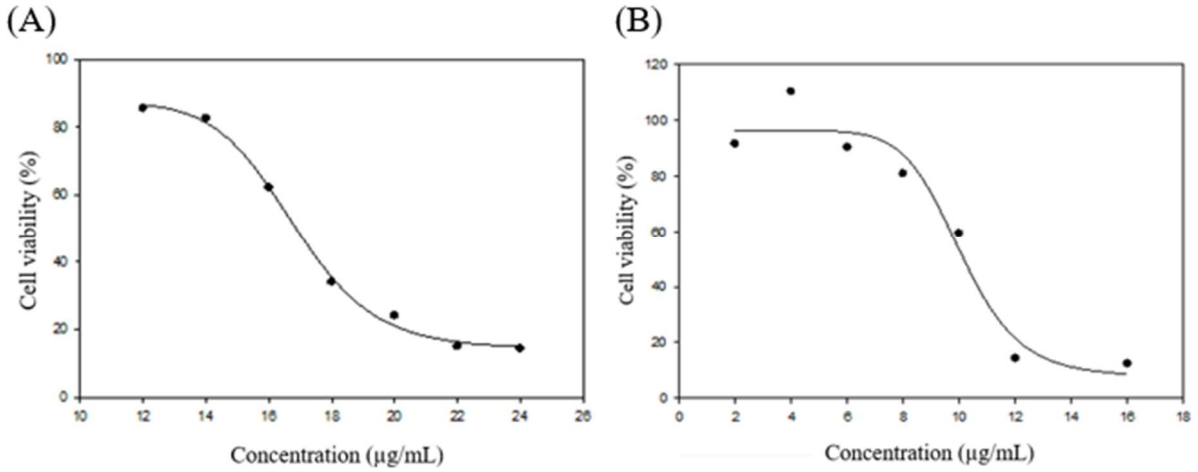

Figure 3. Effect of (A) wild-type and (B) mutant astaxanthin extracts on viability of MDA-MB-231 cell line using MTT assay

Table 1 indicates that the mutant astaxanthin extract shows a lower IC_{50} than the wild-type extract and is more effective in inhibiting both MCF-7 and MDA-MB-231 cell lines. However, the difference between the two cell lines toward the wild-type and mutant extract treatments is not obvious. According to the American National Cancer Institute guidelines, the limit of activity for crude extracts at 50% inhibition (IC_{50}) of proliferation is fixed at less than 30 µg/mL after the exposure time of 72 hr [17]. The IC_{50} values obtained in this study did not exceed the required limit and the extracts exhibited desirable effects by inhibiting the breast cancer cell lines in a dose-dependent manner but not the non-malignant MCF-10A cells. This shows that they could be potential agents for breast cancer treatment which is independent of the estrogen receptor status, yet with limited influence on the normal cell line.

Maejo Int. J. Sci. Technol. **2023**, *17*(01), 24-39

Table 1. Cell cytotoxicity effect of wild-type and mutant astaxanthin extracts on MCF-7 and MDA-MB-231 cell lines. Values are expressed as mean ± SD. (n=3).

Cell line	IC_{50} (µg/mL) Wild-type extract	R^2 value	IC_{50} (µg/mL) Mutant extract	R^2 value
MCF-7	17.55±0.42	0.9878	9.08±0.40	0.9852
MDA-MB-231	16.81±0.19	0.9972	10.06±0.63	0.9565

Cell Morphology Observation

One of the most efficient methods for killing cancer cells is to induce apoptosis, which is generally characterised by distinct morphological characteristics including cell shrinkage, loss of organelles in the cytoplasm, chromatin cleavage, nuclear condensation, DNA fragmentation, membrane blebbing and formation of pyknotic bodies of condensed chromatin [18]. From Figures 4A and 4B, the untreated MCF-7 and MDA-MB-231 cells maintain their original morphology and close contact to each other when the incubation is prolonged to 24 hr. In contrast, they lose their original shapes and exhibit fewer cellular contacts in a time-dependent manner after 24 hr of the wild-type and mutant astaxanthin extract treatments. Morphological changes are noticed at different time points. At 8 hr, the cells tend to shrink and condensation of nuclei is observed. With the mutant astaxanthin extract treatment, cell blebbling and condensation of cell chromatin are observed at 8 hr. At 24 hr, the MCF-7 cells under both wild-type and mutant extract treatments display remarkable morphological changes with cell shrinkage, breakage into single cells or small clusters of cells, and breaking of cells into small fragments. The MDA-MB-231 cells first appear spindle-shaped and are well spread on the culture dish (Figure 4B).

Compared to the untreated cells, the micrographic changes of distinct cell structural shrinkage were observed throughout the time with both astaxanthin extract treatments. Cell blebbling was noticed at 8 hr with wild-type astaxanthin extract treatment. Finally, when treated with both astaxanthin extracts, the cells shrunk and detached from the surface and suspension cells (dead cells) were identified. The hallmarks of apoptosis in the form of cell shrinkage, chromatin condensation and membrane blebbing were seen developed in a time-dependent manner in MCF-7 and MDA-MB-231 cells upon treatment with both astaxanthin extracts, illustrating the induction of apoptosis. The cells shrank in size, the cytoplasm became denser, and the organelles became more tightly packed as a result of cell shrinkage. A similar transformation of the MCF-7 and MDA-MB-231 cells such as cell shrinkage and rounding of cells were reported in the induction of *X. dendrorhous* yeast extract fermentation products [19]. Astaxanthin was shown to inhibit cell proliferation and change the cellular morphology of SKBR3 cells, whereby the untreated cells were closely attached to one another but the astaxanthin-treated cells exhibited fewer cellular contacts in a dose-dependent manner [20].

Maejo Int. J. Sci. Technol. **2023**, *17*(01), 24-39

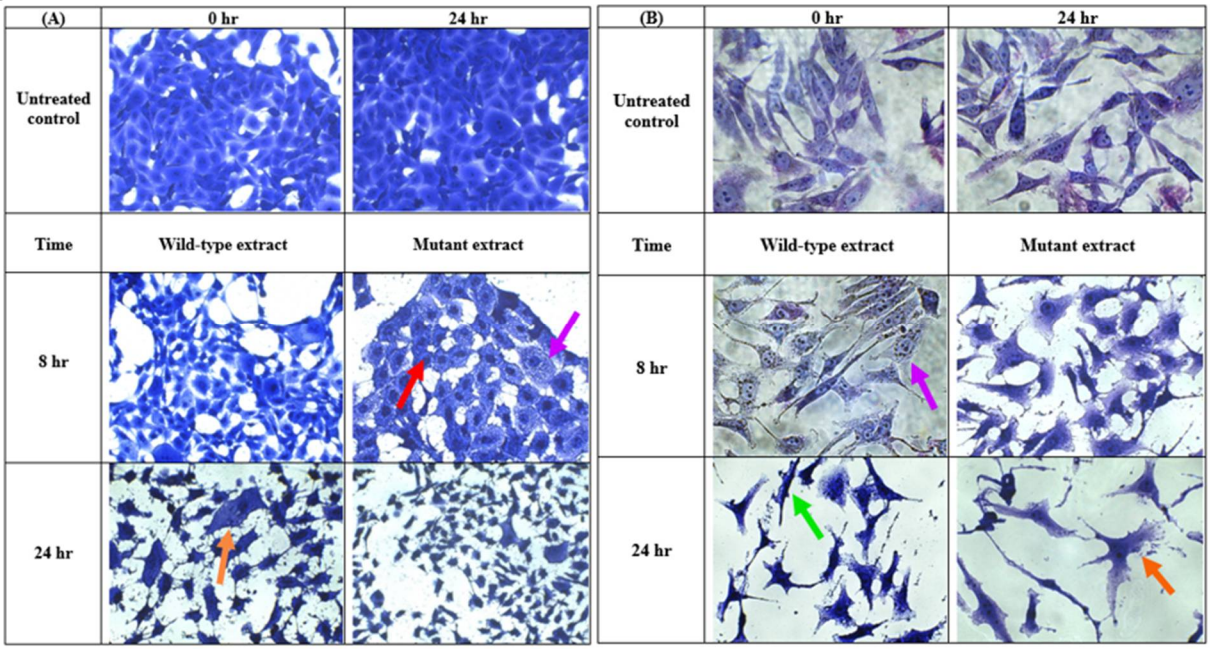

Figure 4. Morphology changes between 0-24 hr in (A) MCF-7 cells and (B) MDA-MB-231 cells after treatment with IC_{50} dosage of wild-type and mutant astaxanthin extracts (200x magnification). Yellow arrow indicates cell blebbling; red arrow indicates condensation of chromatin; orange arrow indicates loss of cell membrane integrity; green arrow indicates cell total shrinkage.

Annexin-V FITC/PI Apoptosis Assessment

Apoptosis induction in cancer cells has become a treatment priority because cancer chemotherapeutics causes apoptotic cell death as part of its pharmacological impact. The results demonstrate clear detection of three populations (viable, apoptotic, necrotic) based on treatment. Untreated cells with single positive and double positive staining (LR and UR quadrants) are most likely undergoing normal senescence. Figure 5 shows that MCF-7 and MDA-MB-231 cells treated with the wild-type and mutant astaxanthin extracts clearly demonstrate a shifting in quadrants from viable to apoptotic region. From Table 2, untreated controls show the viable cell percentages of 92 and 94 for MCF-7 and MDA-MB-231 cells respectively after 24 hr. For MCF-7 cells, 52% and 20% of viable cells remain when treated with the wild-type and mutant astaxanthin extracts respectively, while for MDA-MB-231 cells 64% viable cells remain when treated with both astaxanthin extracts.

After 24 hr treatment with wild-type astaxanthin extract, 34% of MCF-7 cells are in early apoptosis while 11% are in late apoptosis (Table 2). Under the same condition, the mutant astaxanthin extract induces 47% early apoptosis and 32% late apoptosis in MCF-7 cells. In MDA-MB-231 cells similar early and late apoptosis of 18% and 15% respectively occur after treatment with either the wild-type or mutant astaxanthin extracts. Interestingly, apoptotic effects of the astaxanthin extracts seem to be cell-type dependent. The percentages of apoptosis (early and late combined) of MCF-7 cells treated with wild-type (46.28%) and mutant (78.5%) extracts are significantly higher than those of MDA-MB-231 cells treated with the same extracts (~33%).

Maejo Int. J. Sci. Technol. **2023**, *17*(01), 24-39

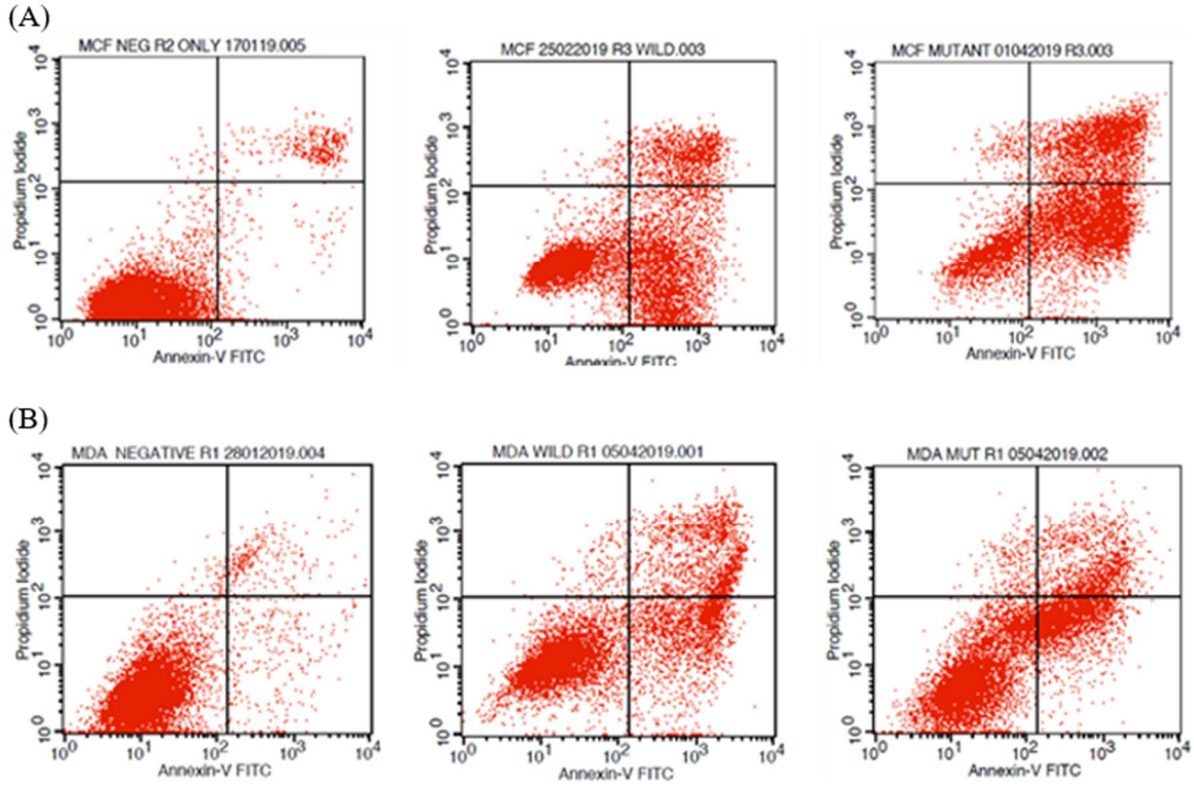

Figure 5. Flow cytometric Annexin V-FITC/PI apoptosis analysis of (A) MCF-7 cells and (B) MDA-MB-231 cells after 24-hr treatment with wild-type and mutant astaxanthin extracts at IC_{50}. Left: untreated control; Centre: wild-type astaxanthin extract; Right: mutant astaxanthin extract

Table 2. Apoptotic rates of MCF-7 and MDA-MB-231 cells after 24-hr treatment with wild-type and mutant astaxanthin extracts at IC_{50}. Results are means of 3 independent experiments and are expressed as mean ± SD. ** $p < 0.01$; * $p < 0.05$ compared to untreated control

Cell line	**MCF-7**			**MDA-MB-231**		
Treatment	**Untreated**	**Wild-type extract**	**Mutant extract**	**Untreated**	**Wild-type extract**	**Mutant extract**
Viable cells (%)	92.25±0.83	52.45±10.00*	20.51±1.85**	94.42±0.24	64.58±1.00*	64.49±3.95*
Necrosis (%)	0.18±0.10	1.26±0.90	0.96±0.74	0.81±0.27	2.44± 1.07	2.94± 1.04
Early apoptosis (%)	3.83±1.08	34.47±3.43**	46.97±0.72**	2.72±0.40	17.58±1.84*	17.96±5.48*
Late apoptosis (%)	3.71±0.70	11.81±6.55*	31.56±2.08**	2.05±0.38	15.40±0.18*	14.60±2.32*

Cell Cycle Analysis

Dysregulation of the cell cycle associated with abnormal expression of the cell cycle-related proteins is an important mechanism in breast cancer pathogenesis. Cell cycle analysis elaborates on a potential mechanism for cancer cell inhibition. Figure 6 shows the stages of cell cycle arrest in MCF-7 and MDA-MB-231 cells after treatment with the wild-type and mutant astaxanthin extracts at IC_{50} for 24 hr. These data are presented in the format of histogram of cell cycle distribution in Figure 7.

Maejo Int. J. Sci. Technol. **2023**, *17*(01), 24-39

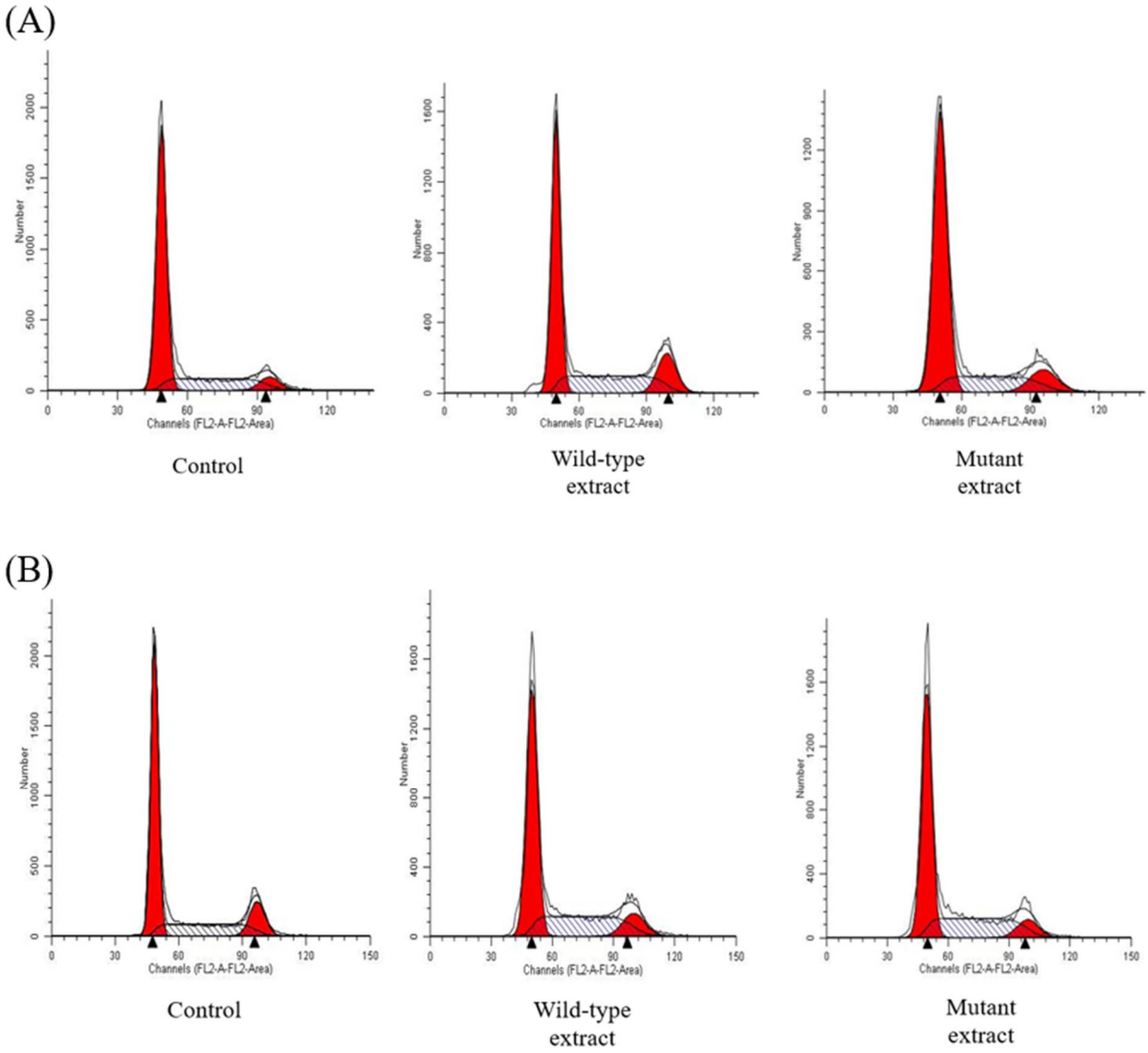

Figure 6. Stages of cell cycle arrest in (A) MCF-7 cells and (B) MDA-MB-231 cells after treatment with wild-type and mutant astaxanthin extracts at IC_{50} for 24 hr

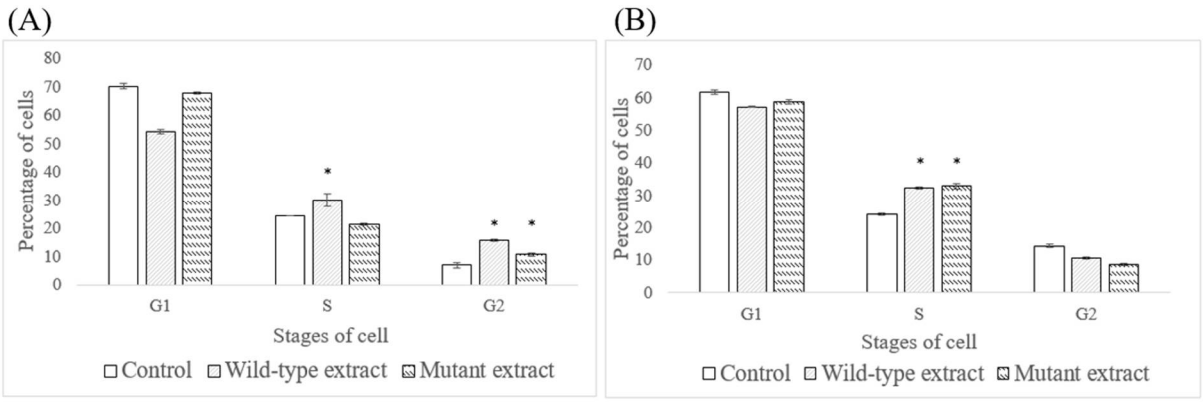

Figure 7. Cell cycle analysis of (A) MCF-7 cells and (B) MDA-MB-231 cells after treatment with wild-type and mutant astaxanthin extracts at IC_{50} for 24 hr. Values are mean ± SD (n=3). * $p < 0.05$ (considered significant as compared to control)

Maejo Int. J. Sci. Technol. **2023**, *17*(01), 24-39

Evidently, there are changes in the percentage of cells in treated groups compared to untreated control in both MCF-7 and MDA-MB-231 cells. However, both types of cells show different susceptibilities to the effects of astaxanthin extracts. For MCF-7 cells, treatment with wild-type and mutant astaxanthin extracts leads to a significant percentage of cells in the G2/M phase (15.7% and 10.7% respectively, $p < 0.05$) compared to untreated control (6.9%). However, the cell cycle arrest at G0/G1 and S phases is different for the wild-type and mutant extracts in MCF-7 cells. For wild-type extract, the number of S phase cells is significantly higher relative to control (30% vs 23%, $p < 0.05$) and the percentage of G0/G1-phase cells is significantly reduced to 54%. MCF-7 cells treated with wild-type astaxanthin extract tend to stop replicating at the S phase and G2/M phase as the percentage of the cells increases, but for the mutant extract the increase is shown only in the G2/M phase. With the mutant extract, the numbers of the G0/G1- and S-phase cells are slightly reduced compared to control and the cell cycle arrest is mainly observed at the G2/M phase. This indicates that the normal cell cycle progression has been compromised, resulting in a cell cycle arrest in the G2/M process. These data are consistent with previous studies by Hormozi et al. [21], which showed that treatment with astaxanthin led to a significant accumulation of cells in the G2/M phase compared to control. Similar findings were reported demonstrating a G2/M arrest induction in two different colon cancer cell lines (HCT116 and HT29) [22] and in mice hepatoma cells [23] due to the treatment with astaxanthin. These data suggest that astaxanthin might trigger a mitotic arrest in the G2/M phase in the MCF-7 cell line.

For MDA-MB-231 cells, a similar trend is observed for both wild-type and mutant extract treatments (Table 2). The number of the S-phase cells significantly increases relative to control (32% vs 24%) for both extract treatments and the numbers of cells in G0/G1 and G2/M phases are slightly lowered compared to control. The accumulation of cells in the S phase is accompanied by a corresponding decrease in the percentage of cells in G0/G1 and G2/M phases. Increment of the cell population shows that astaxanthin extracts have successfully intercepted the cell cycle in the S phase and prevented it from entering the proliferative phase. The findings indicate that cell cycle arrest in the S phase may be the major mechanism for the observed cell growth inhibition in MDA-MB-231 cell line by the wild-type and mutant astaxanthin extracts.

Intracellular ROS Analysis

ROS are important regulators of apoptosis and proliferation and are involved in anti-cancer mechanisms. While minimal increment of ROS may promote cell proliferation and differentiation, excessive ROS levels cause oxidative damage and induction of apoptosis. According to recent research, ROS generation plays a crucial role in pro-apoptotic activities of a variety of anti-cancer agents [24]. ROS is found in control samples as it occurs naturally in the cell and its level in the control samples is set as 100%. As shown in Figure 8, ROS accumulates approximately 2- to 2.3-fold compared to the untreated control in MCF-7 and MDA-MB-231 cells after treatment with the wild-type and mutant astaxanthin extracts at IC_{50}. The astaxanthin extract treatment did not attenuate the ROS production in the cells as there was significant increase in ROS percentage in both the cell lines. This suggests that the astaxanthin extracts may mediate their anti-cancer activity through ROS elevation and enhanced oxidative stress, leading to apoptosis stimulation. Atalay et al. [16] showed that the effects of astaxanthin treatment at 5, 10 and 15 μg/mL on ROS accumulation were not significantly different from DMSO control after 24 hr incubation. However, at 30 μg/mL treatment, ROS production increased 2.5-fold compared to DMSO control in MCF-7 cells.

Maejo Int. J. Sci. Technol. **2023**, *17*(01), 24-39

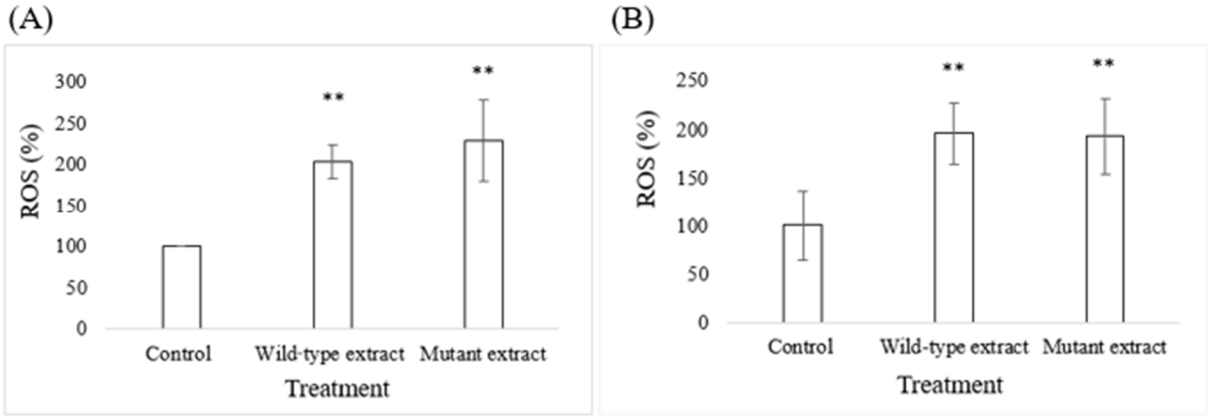

Figure 8. Effects of wild-type and mutant astaxanthin extracts on intracellular ROS level in (A) MCF-7 cells and (B) MDA-MB-231 cells. Data are expressed as mean ± SD (n=3).
** p< .01 vs control group

Interestingly, ROS has been shown to destroy cancer cells selectively. Hileman et al. [25] proved that ROS preferentially destroys human leukemia cells while causing no cytotoxicity in normal lymphocytes. To counteract the toxic effects of ROS, cancer cells have evolved to increase their antioxidant capacity to maintain a redox balance; hence cancer cells are more susceptible to changes in ROS levels [26]. Furthermore, certain carotenoids have been shown to have pro-oxidant effects on cancer cells with the generation of free radicals. Kim et al. [24] reported the inhibition of leukemia cell growth by fucoxanthin and attributed it to ROS production mediated by fucoxanthin that leads to apoptosis. This suggests that carotenoids act either as antioxidants or as pro-oxidants, depending on their environment. At low concentrations, they inhibit ROS production and are ineffective in modifying cell growth while at high concentrations, they increase the formation of free radicals and inhibit cell growth by inducing apoptosis. The ability of carotenoids to modulate the intracellular redox status may be one explanation for their dual functions in cancer prevention.

Wound Healing Assay

Invasion and migration are two critical processes in the development of cancer. Malignant cancer cells move away from the primary tumour and destroy the surrounding extracellular matrix to invade and migrate to other parts of the body via the lymphatic system, bloodstream or by direct extension [27]. Since metastasis is the leading cause of death in cancer patients, prohibiting migration will have a substantial positive effect on survival rates. As observed in Figure 9A, the untreated MDF-7 and MDA-MB-231cells nearly heal after 24 hr of incubation. For MCF-7 cells treated with either the wild-type or mutant astaxanthin extract, the wound areas are not significantly reduced after 24 hr. Some minor cell migration is observed for the wild-type extract treatment (Figure 9B). The wound gaps of MDA-MB-231 cells treated with the wild-type and mutant astaxanthin extracts are slowly reduced in a time-dependent manner and the gaps do not heal totally after 24 hr (Figure 9C).

Figure 10 shows that the untreated cells grow normally when cultured in control media settings and nearly heal after 24 hr. Upon treatment with the wild-type or mutant astaxanthin extract, the migration distances of MCF-7 and MDA-MB-231 cells decrease and the wound gaps remain open after 24 hr, indicating that the astaxanthin extracts reduce breast cancer cell migration. The mutant extract shows a slightly better inhibition on both cell lines as the migration rate is

Maejo Int. J. Sci. Technol. **2023**, *17*(01), 24-39

slower and the gap distance is larger compared to the wild-type extract. Both astaxanthin extracts give a slightly better inhibition of MCF-7 cells as slower migration rate and bigger wound gap are observed compared to the highly metastatic MDA-MB-231 cells. Le et al. [28] reported that astaxanthin inhibited the migration of MDA-MB-231 TNBC and MCF-7 ER+ breast cancer cells. Nevertheless, no substantial changes in migration, cell number, or apoptotic gene expression of the regular breast epithelial cell line MCF-10A were observed following astaxanthin treatment. The fact that astaxanthin has no impact on general breast cells is crucial since normal cells can still migrate and proliferate in response to astaxanthin cancer treatment. The normal cells will continue to work normally and grow into voids left by dying tumour cells throughout the healing process.

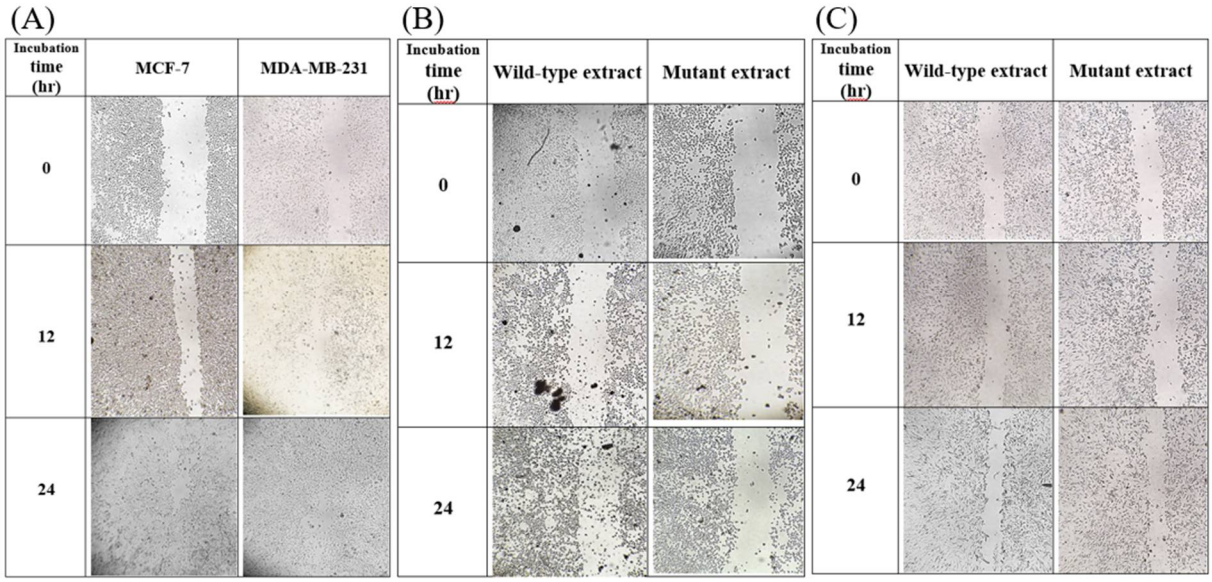

Figure 9. Cell migration (wound healing assay) of (A) untreated control, (B) MCF-7 cells after treatment with wild-type and mutant astaxanthin extracts and (C) MDA-MB-231cells after treatment with wild-type and mutant astaxanthin extracts. Images were recorded at 0, 12 and 24 hr after wounding. Representative images are shown from three independent experiments.

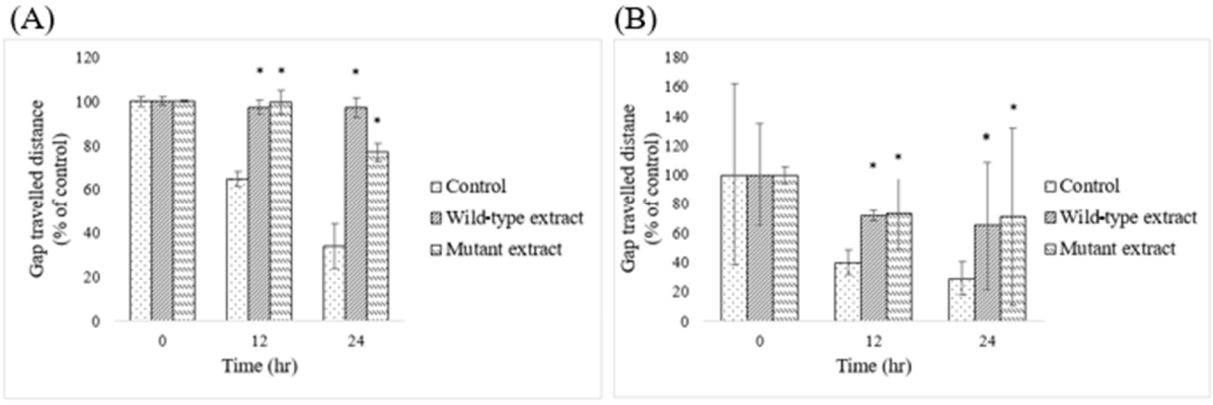

Figure 10. Wound healing of (A) MCF-7 cells and (B) MDA-MB-231 cells at 0, 12 and 24 hr after treatment with wild-type and mutant astaxanthin extracts. Data are expressed as mean ± SD (n=3). * $p < .05$ vs control group

Maejo Int. J. Sci. Technol. **2023**, *17*(01), 24-39

CONCLUSIONS

The wild-type and mutant astaxanthin extracts have been shown to exhibit anti-cancer properties by *in vitro* assays, namely cytotoxicity assay, cell morphological observation, apoptosis assessment, cell cycle analysis, ROS analysis and would healing assay. These studies should provide a wider basis of knowledge on the potential of *X. dendrorhous* astaxanthin extracts, especially the hyperproducing mutant extract, as anti-cancer agent from natural sources. This may lead to novel therapeutic strategies against cancer in future, although more work is required to further reveal the complicated mechanisms involved in the anti-tumorigenesis in a detailed fashion.

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