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Anticancer activity, oxidative stress

ANTIOXIDANT RICH, ISOLATION AND PARTIAL PURIFICATION OF ZEAXANTHIN FROM *Brassica oleracea* INDUCING IN BREAST CANCER CELL LINE

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ABSTRACT

Zeaxanthin is a member of the xanthophyll family of carotenoids, or plant-produced pigments. They are just somewhat dissimilar to one another in terms of structural similarity. Kale, savoy cabbage, spinach, broccoli, peas, parsley, corn, and egg yolks are the main sources of these carotenoids. Zeaxanthin should be taken daily in doses of 2 mg. Adults need between 1–2 mg of zeaxanthin per day on average. Because humans are unable to make or consume certain substances, they are essential for the health of certain body organs (such as the heart, intestines, skin, and eyes). Including a lot of fruits, vegetables, and dark leafy greens in our diets may aid in maintaining the health of our bodies. Carotenoids, such as zeaxanthin, are essential for scavenging free radicals and preventing oxidative stress, which helps to protect cells. They operate as defence mechanisms for the organism. Zeaxanthin has demonstrated potential in complementing cardiac and breast cancer therapy. This article discusses research on the role of zeaxanthin, namely its absorption and metabolic processes in humans, and explores the importance of eating foods high in xanthophyll.

Keywords: breast cancer, oxidative stress, zeaxanthin, xanthophyll, carotenoids.

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AIMS AND BACKGROUND

Carotenoids, natural pigments found in fruits and vegetables, are associated with a reduced risk of certain cancers[1]. Chemoprevention, a strategy for cancer management, aims to prevent DNA mutational damage using various compounds[2]. Carotenoids are lipid-soluble pigments with distinct types, affecting their properties. They are absorbed through the intestinal tract, influenced by factors like dietary fat and bile[3]. Carotenoids travel in the body through plasma lipoproteins[4]. Chemoprevention studies seek substances in food that can inhibit cancer tumor growth, countering the effects of reactive oxygen species produced by oxygen, which can lead to aging and diseases like cancer[5].

Absorption of zeaxanthin is influenced by the fat content, dietary matrix, and processing techniques. Zeaxanthin is transferred more readily from some foods, such as oranges, kiwis, grapefruits, and sweet potatoes, while it is absorbed less readily from foods like spinach and broccoli[6]. The extractability of bioactive compounds from *Brassica oleracea* (broccoli and cauliflower) is greatly impacted by the techniques of cooking. Specific carotenoids and phenolic components were found in significant concentrations while boiling broccoli inflorescences and steaming cauliflower, respectively[7].

Breast cancer research frequently makes use of breast cancer cell lines. Although they consistently produce components relevant to tumors, they are prone to mutation throughout time. Checking the heterogeneity of these cell lines is essential to confirm their similarity to real carcinomas. Due to various approaches and nomenclatures, it can be difficult to define subgroups in cell lines and malignancies. The study looked at 92 breast cancer cell lines, divided them into five groups based on their molecular features, and described their genetic and epigenetic characteristics. Ten cell lines lacked a distinct subtype[8].

EXPERIMENTAL

MATERIALS

Brassica oleracea and methanol extraction were used in the study. Chemical analysis was performed using lab apparatus such as thin-layer chromatography and column chromatography sets. Antioxidant capability was assessed using a UV spectrophotometer and a DPPH test. Under an inverted microscope, biological experiments used the trypsinised MDA-MB-231 cell line and an MTT test. Fetal bovine serum and Eagle's minimal essential media were used in an incubator to facilitate cell culture activities. Microscope analysis was supported by extra glassware and a fluorescence microscope.

METHODOLOGY

Preparation of methanolic extract of Brassica oleracea. 5 kg of *Brassica oleracea* had to be finely chopped and dried in order to begin the extraction procedure. For ten

days, this dried plant material was immersed in 80% methanol while being stirred occasionally. Following extraction, the extract was evaporated under low pressure while the methanol was filtered[9].

Phytochemical screening. The extract was subjected to phytochemical screening in order to discover alkaloids. Following a hydrochloric acid mixture, the filtrate was put through a series of tests. Wagner's reagent was used in his test, and a reddish-brown precipitate was the sign of a successful result[10]. Dragendorff's reagent was used in the test, and in the presence of alkaloids, it produced a yellow precipitate. Numerous chemical components were screened for, including steroids, flavonoids, terpenoids, alkaloids, proteins, carbohydrates, glycosides, and phenolic compounds[11].

Thin layer chromatography. Thin layer chromatography (TLC) plates were prepared by mixing an adsorbent like silica gel with a small amount of inert binder and water. The mixture was then spread out on a glass or aluminium foil surface and allowed to dry. The adsorbent layer's thickness varied depending on the use; for analysis, it was usually between 0.1–0.25 mm and between 0.5–2.0 mm (Ref. 12). There are several ways to prepare TLC plates: capillary spotters, TLC spreaders, and pouring. Following preparation, they are oven-dried and activated. The migration of components across the adsorbent layer during solvent movement provides the basis for the identification and separation of components within mixtures using TLC, a commonly used technique.

Column chromatography. Plant extract phytochemicals were separated via column chromatography, which used a silica gel-filled column filled with various eluents. TLC evaluates the components. TLC profiles of comparable fractions were merged[13]. The plant material was first extracted with ethyl acetate, evaporated, and then subjected to silica gel column chromatography. Based on TLC, fractions were categorised and their phytochemical content is examined[14].

Antioxidative assay. Reactive oxygen species and free radicals are known to cause aging and a host of diseases, and antioxidants are essential in the fight against them. The DPHH assay is a widely used technique to evaluate antioxidant activity. It uses DPPH, a stable free radical. It provides information on the overall antioxidant capacity by measuring the decrease of DPPH by antioxidants. When comparing antioxidant activity, the IC_{50} value, which stands for the concentration required to scavenge 50% of DPPH radicals, is utilised. This simple test, which is frequently carried out using a UV-Vis. spectrophotometer, provides a rapid method of assessing the antioxidant capacity and efficacy of novel medications[15].

Molecular docking. Using AutoDock 4.2, the structures of Beta-Sitosterol and Zeaxanthin were prepared before being molecularly docked against the breast cancer target, Progesterone receptor (PR). The technique covered the procedures for acquiring and getting ready the ligands and 3D structures, energy minimisation, and molecular docking simulation[16].

Cytotoxicity test. The Cytotoxicity test entails subculturing, or passing cells to form new cultures as a result of nutrient deficiency or overcrowding in the culture medium. For dis-aggregation, phosphate buffered saline (PBS) that has been defrosted and trypsin medium was utilised. The next step was to seed cells on a 96-well plate, where the cell suspension was spread and various wells got a control, a test film, or varied quercetin drug concentrations[17]. To determine cytotoxicity, the cells were cultured for 24 and 48 h at 37°C. This process guarantees controlled environments for evaluating cell viability and medication reactions[18].

Apoptosis assay by dual staining. Using fluorescent dyes like acridine orange (for cell cycle study) and propidium iodide (for identifying cell death), apoptosis was studied since it is important for eliminating undesirable cells. Rhodamine-123 dye was also used to look into changes in mitochondrial membrane potential, a crucial stage in apoptosis[19]. The research demonstrates that Pa-AuNPs can cause apoptosis by affecting the potential of the mitochondrial membrane.

RESULTS AND DISCUSSION

COLLECTION OF SAMPLES

Brassica oleracea plants, commonly found in various parts of India, particularly in moist soil conditions were collected. These plants are often found throughout India, particularly in moist soil conditions. Using a crude extraction method, 5 kg of *Brassica oleracea* were washed, chopped into small pieces, and then dried. After that, the dried broccoli was immersed in 80% methanol for 10 days while being stirred occasionally. The solvent was filtered through cotton and filter paper (Whatman No 1) to yield the methanol extract, which was then evaporated at 40–45°C under reduced pressure in a rotary evaporator. Figure 1 shows a sample of dried broccoli.

Fig. 1. Dried broccoli

PHYTOCHEMICAL SCREENING

Test tube methods were used to conduct a preliminary phytochemical analysis on samples of broccoli as shown in Fig. 2. These tests are noted to reveal the presence of saponins, phenolic compounds, reducing sugars, alkaloids, carbohydrates, glycosides, and glycosides in the sample.

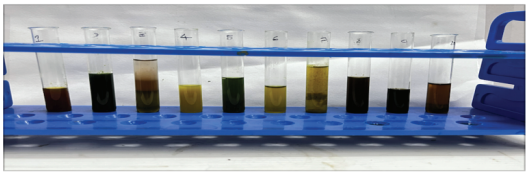

Fig. 2. Phytochemical screening

Table 1. Results of phytochemical analysis

Phytochemical tested	Ethanol	Ethyl acetate	Petroleum ether	Hexane
Alkaloid	+	+	+	+
Carbohydrates	+	+	+	+
Glycosides	+	+	+	+
Saponins	–	–	–	+
Protein	+	+	+	–
Amino acids	–	–	–	–
Phenol	+	+	+	–
Flavonoid	+	+	+	+
Terpenoid	+	+	+	–
Steroid	+	+	–	+

Table 1 indicates several phytochemicals were examined in ethanol, ethyl acetate, petroleum ether, and hexane, among other solvents. The outcomes showed whether certain phytochemicals were present or not. Alcohol, ethyl acetate, and petroleum ether extracts contained alkaloids, carbohydrates, glycosides, protein, phenol, flavonoids, terpenoids, and steroids. However, amino acids were not found in any of the studied solvents, while saponins were only found in the hexane extract. The hexane extract did not contain any protein, either.

THIN LAYER CHROMATOGRAPHY

The maximum retention factor was seen in the TLC of crude *Brassica oleracea* extract when it was examined under UV light and ethyl acetate (Figs 3–5). Ethyl acetate and methanol were used as the mobile phase. Ethyl acetate, with a retention factor of 0.834 cm, came next. The least retention factor was 0.238 cm for methanol. The solvent system with ethyl acetate:methanol was then used in column chromatography since the crude ethyl acetate extract displayed the highest retention value when compared to all other solvent extracts are displayed in Table 2.

Table 2. Retention factor values of crude extracts of *Brassica oleracea*

No	Solvent system	Distance of solute (cm)	Distance of solvent (cm)	R_f(distance solute/ distance solvent) (cm)
1	Ethyl acetate	2.2	5.5	0.834
2	Methanol	1.3	5.5	0.238

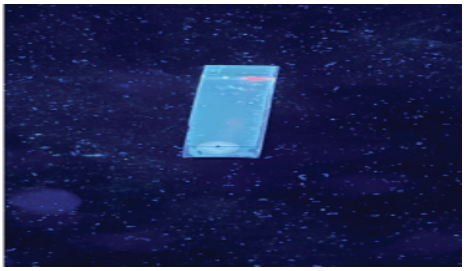

Fig. 3. Chromatogram of crude extract ethyl acetate in UV-light

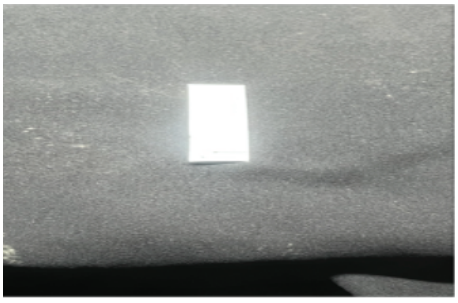

Fig. 4. Chromatogram of crude extract ethyl acetate in normal light

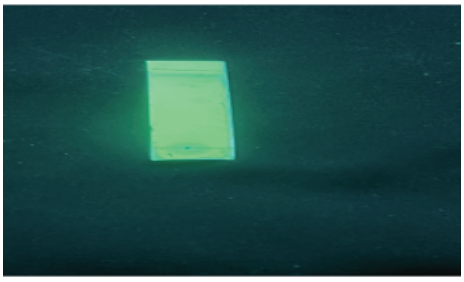

Fig. 5. Chromatogram of crude extract ethyl acetate in visible light

COLUMN CHROMATOGRAPHY

The Elite-5MS packed fused silica column (5% biphenyl, 95% dimethylpolysiloxane, 30 m, 0.25 mm ID, 250 m df) employed in the analysis was housed in a Clarus 680 GC. Helium served as the carrier gas during the separation process, with a steady flow rate of 1 ml per min as shown in Fig. 6. For the chromatographic run, the injector temperature was set at 260°C. When the one-liter extract sample was placed inside

the apparatus (Fig. 7), the oven's temperature was as follows: Following two min at 60°C, 300°C was attained at a pace of 10°C per min, and 300°C was held for six min. The mass detector was operated in an electron impact ionisation mode at 70 electron volts (eV), with 0.1-second scan intervals, a 240°C transfer line and ion source temperature, and a 0.2-second scan period. Compounds with masses between 40 and 600 Da were examined. These solvent conditions were used with a scan range of 50 to 600 Da, a bulk delay of 2.00 min, and source and transfer temperatures of 240°C.

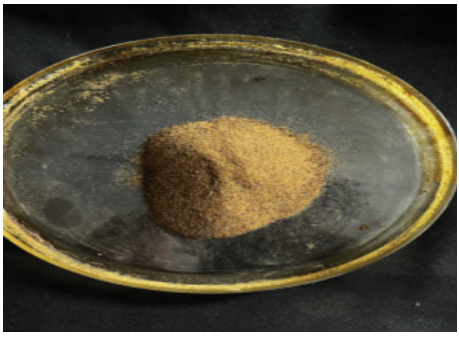

Fig. 6. Dried sample for column chromatography

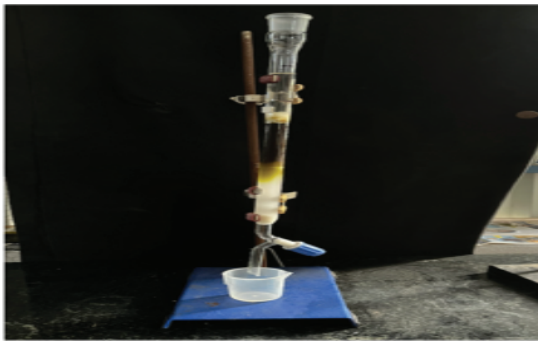

Fig. 7. Column chromatography

SPECTROSCOPIC CHARACTERISATION

Utilising a UV-Vis. spectoscopy, in the 200–800 nm range, spectrometry analysis was carried out on the fractions of *Brassica oleracea*. Plotting of the graph was placed between 200 and 800 nm of wavelength (Fig. 8).

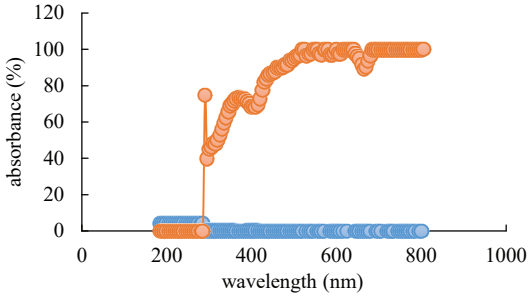

Fig. 8. UV-Vis. spectrum of ethyl acetate extract

FTIR ANALYSES

The BRUKER FT-IR spectrometer was used to examine the purified chemical's vibration patterns. In the FTIR spectra, characteristic bands appeared in the frequency range of 4000 to 500 cm^{-1}. Compared to the functional group region's three peaks, the finger print region observed four peaks (Fig. 9). The alkyl stretching at 3491 cm^{-1} (OH), 2925 cm^{-1} 1, and 2855 cm^{-1} (C–H) was where the FTIR's functional group area peaked. In the finger print region of the spectrum, peaks at 1680 cm^{-1} (CC) cyclic bending, 1469 cm^{-1} (C–H) cyclic bending, and 1052 cm^{-1} (C–O) stretching were discernible.

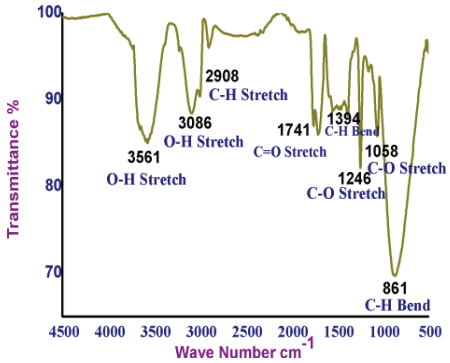

Fig. 9. FTIR analysis of chemical constituents in ethyl acetate extract

CYTOTOXICITY ACTIVITY

Table 3 displays the absorbance values and corresponding cell viability percentages for different concentrations (µg/ml) of a substance in two sets. The average absorbance of the control group is 0.5805, which indicates 100% cell viability. The absorbance values fall as the substance's concentration rises from 20 to 100 µg/ml, indicating decreased cell viability. For example, the average absorbance at 20 µg/ml is 0.5745, which indicates about 98.97% cell vitality; at 100 µg/ml, the average absorbance is 0.5125, which indicates 88.29% cell viability.

Table 3. Cytotoxic effect

Concentrations (µg/ml)	Absorbance		Average	Cell viability (%)
	I	II		
Control	0.579	0.582	0.5805	100
20	0.573	0.576	0.5745	98.96640827
40	0.566	0.571	0.5685	97.93281654
60	0.559	0.558	0.5585	96.21016365
80	0.543	0.549	0.5460	94.05684755
100	0.509	0.516	0.5125	88.28596038

Figures 10–15 depict different concentrations (20, 40, 60, 80 and 100 µg/ml, respectively) treatments of Vero cells.

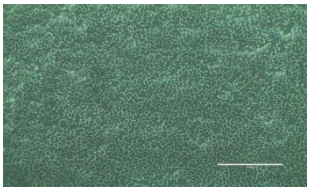

Fig. 10. Control of Vero cells

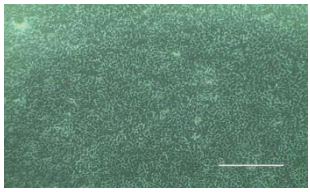

Fig. 11. 20 µg/ml of Vero cells

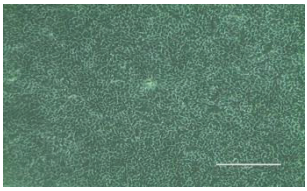

Fig. 12. 40 µg/ml of Vero cells

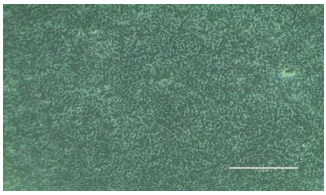

Fig. 13. 60 µg/ml of Vero cells

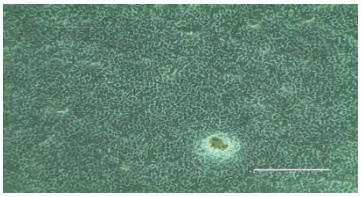

Fig. 14. 80 µg/ml of Vero cells

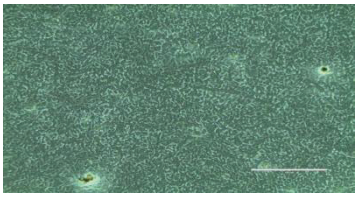

Fig. 15. 100 µg/ml of Vero cells

ANTICANCER ACTIVITY

Using the MTT assay, the biogenic zeaxanthin derived from *Brassica olareacea* was evaluated for its capacity to reduce the proliferation of breast cancer MDA-MB 251 cell lines. As shown in the image, zeaxanthin was tested for its ability to fight cancer at levels ranging from 20 to 100 g/ml. Table 4 shows that cell viability significantly dropped from 90.54 to 21.26% when concentration rose from 20 to 100 µg/ml.

Table 4. Anticancer activity

Concentrations (µg/ml)	Absorbance		Average	Cell viability (%)
	I	II		
Control	0.613	0.624	0.6185	100
20	0.566	0.554	0.56	90.54163298
40	0.471	0.483	0.477	77.12206952
60	0.360	0.351	0.3555	57.4777688
80	0.243	0.241	0.242	39.12691997
100	0.126	0.137	0.1315	21.2611156

Figures 16–21 depict the control (Fig. 16) and different concentrations (20, 40, 60, 80 and 100 µg/ml, respectively) treatments of cancer cells.

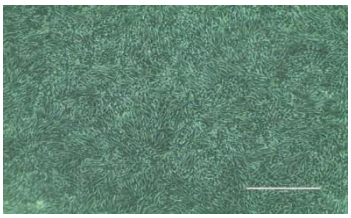

Fig. 16. Control of MDA-MB 251

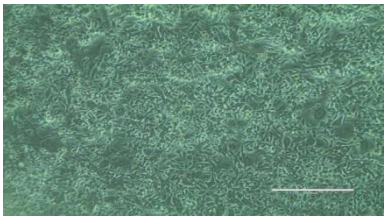

Fig. 17. 20 µg/ml of MDA-MB 251

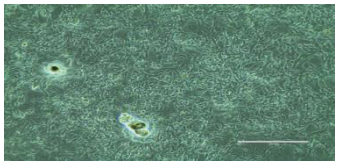

Fig. 18. 40 µg/ml of MDA-MB 251

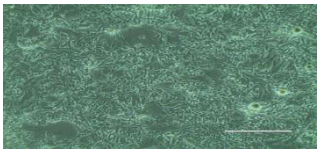

Fig. 19. 60 µg/ml of MDA-MB 251

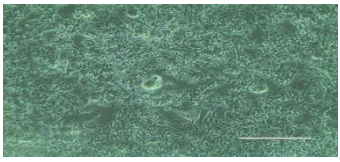

Fig. 20. 80 µg/ml of MDA-MB 251

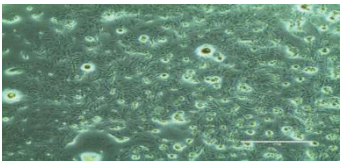

Fig. 21. 100 µg/ml of MDA-MB 251

ANTIOXIDANT ACTIVITY

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) technique was used to evaluate Zeaxanthin's antioxidant potential. Zeaxanthin had considerable antioxidant and free radical scavenging properties, according to the results (Table 5). The greatest antioxidant activity was found at a concentration of 100 µg/ml, with the DPPH values ranging from 23.71 to 39.97% for different concentrations (20 to 100 µg/ml). These results showed a significant improvement in antioxidant performance, which is consistent with the characteristics of zeaxanthin that have been reported from various natural extracts.

Table 5. DPPH assay

Concentration (μg/ml)	Radical scavenging activity (%)
20	2.422
40	24.11
60	38.22
80	57.15
100	64.64

MOLECULAR DOCKING STUDIES

Gamma-Sitosterol and Zeaxanthin were docked into the binding pockets of the Progesterone receptor (PR) with PDB ID 4OAR during molecular docking studies using AutoDock 4.2. The top cluster conformations were examined for binding energy and interactions with active site residues, such as hydrogen bonds, hydrophobic interactions, and van der Waals forces. Each complex produced 100 docked conformations. Zeaxanthin and gamma-Sitosterol's top clusters had binding energies of –7.56 and –12.19 kcal/mol, respectively (Table 6).

Table 6. Binding energy and hydrogen bond interactions of gamma-Sitosterol and Zeaxanthin with PR by AutoDock

Complexes	Binding energies (kcal/mol)	Hydrogen bond interactions (D-H…A)	Distances (Å)	Predicted inhibition constants
PR with Gamma-Sitosterol	–12.19	O–H…N (ARG-766)	2.7	1.16 nM
		O–H…O (GLN-725)	2.8	
		O–H…N (GLN-725)	2.9	
PR with Zeaxanthin	–7.56	O–H…O (PRO-696)		624 μM

APOPTOSIS

Studying apoptosis, a crucial step in tissue homeostasis and the emergence of cancer, involved the use of dual staining techniques. In contrast to untreated cells, those that had been exposed to zeaxanthin revealed early symptoms of apoptosis. Propidium iodide staining was used to determine the extent of cell death, and untreated pure NF mat exhibited few necrotic or late apoptotic cells. Treatment with zeaxanthin caused necrosis and morphological alterations. DNA structure was impacted by the increased necrosis and apoptosis caused by nanofiber mats (NF2 and NF3). These results support the anticancer properties of particular nanofiber mats containing zeaxanthin. Figures 22–26 show the treated cells with AO, PI, AO and PI, control of MMD and treated sample of MMD, respectively.

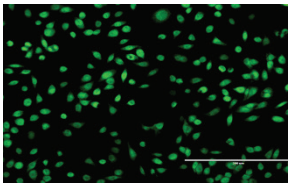

Fig. 22. Treated cell stained with AO

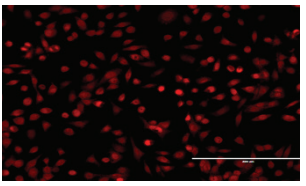

Fig. 23. Treated cell stained with PI

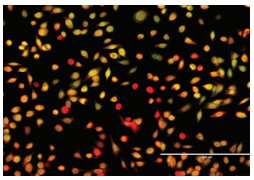

Fig. 24. Treated cell stained with AO and PI

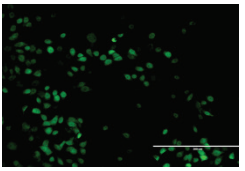

Fig. 25. Control of MMD

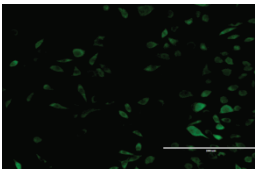

Fig. 26. Treated sample of MMD

CONCLUSIONS

Because of its antioxidant properties, the carotenoid zeaxanthin, which is found in the eyes, protects against eye diseases and promotes heart health. Its potential as a breast cancer anti-cancer therapy is being researched. Zeaxanthin, which is present in a variety of foods including broccoli, leafy greens, and goji berries, combats free radicals and lowers oxidative stress. It affects mood and cognitive function, acting as a protective antioxidant for eye and brain health along with lutein and meso-zeaxanthin. Dietary lipids are necessary for the absorption of zeaxanthin. Although eating fat seems to raise lutein levels, more research is required to determine the exact effect of fat on zeaxanthin bioavailability. Compared to low-fat eating, blood lutein levels were higher in those who consumed high-fat foods.

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