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SUMMARY

Research background. Breast cancer is one of the most common cancers and remains a major cause of morbidity and mortality among women worldwide. In developed nations, breast cancer as a multifactorial disease is a major health concern, and its incidence is constantly rising in low and middle-income countries. Numerous studies have demonstrated that phytochemicals such as carotenoids inhibit breast cancer growth and induce apoptosis. We recently enhanced the aqueous solubility of capsanthin by encapsulating in diosgenin polyethylene glycol succinate, a novel non-ionic surfactant. Thus, this study aims to evaluate the cytotoxicity of aqueous soluble capsanthin-loaded micelles in MDA-MB-231 cells in vitro through MTT assay.

Experimental approach. In the current study, capsanthin, a hydrophobic carotenoid, is extracted from the fruits of Capsicum annuum. Capsanthin-loaded diosgenin polyethylene glycol succinate-1000 (Cap-DPGS-1000) micelles were prepared from capsanthin extract (CAP) and diosgenin polyethylene glycol succinate 1000 (DPGS-1000) using the solid dispersion method. The capsanthin extract and Cap-DPGS-1000 were characterized by UV–visible spectroscopy, high-performance liquid chromatography (HPLC), Fourier-transform infrared spectroscopy (FT-IR), X-ray diffraction (XRD), particle size distribution, polydispersity, and scanning electron microscopy (SEM). The cytotoxicity of CAP and Cap-DPGS-1000 on a human triple-negative
breast cancer cell line (MDA-MB-231) was performed to check the cell viability, proliferation, and cytotoxicity effects.

Results and conclusions. The aqueous solubility of encapsulated Cap-DPGS-1000 is greatly enhanced and leads to an increased scope for localized drug delivery, a better delivery option for treating residual cancer tumors. The encapsulated capsanthin showed a sustained release in simulated intestinal fluid (pH=6.8). Our research proposes a sustained drug delivery system that ensures effective and controlled release to the affected site. The characterization data revealed no change in structure and functional groups in the encapsulated capsanthin. The \( IC_{50} \) value of the Cap-DPGS-1000 micelles against MDA-MB-231 breast cancer cells was \((3.10\pm1.09) \ \mu \text{g/mL}\), which is much lower than capsanthin extract \((81.06\pm1.51) \ \mu \text{g/mL}\). Capsanthin extract and capsanthin-loaded Cap-DPGS-1000 are promising drug candidates to induce apoptosis and increase reactive oxygen species (ROS) in cancer cells.

Novelty and scientific contribution. The result shows the cytotoxic effect of capsanthin and capsanthin-loaded micelles on MDA-MB-231 cell line for the first time. Capsanthin from \textit{Capsicum annuum} showed remarkable cytotoxic effect on the triple negative MDA-MB-231 cell line.

Keywords: capsanthin; breast cancer; triple negative breast cancer cell line; micelles; water solubility; bioavailability

INTRODUCTION

Cancer, abnormal cell growth, can affect any organ or tissue of the body, and it is a multi-stage process that causes biological damages as a result of biochemical and molecular changes at the cellular level. The mortality rates depend upon prevention, early detection, and effective treatment \((1)\). An estimated 9.6 million cancer-related deaths occurred in 2018 \((2)\), as per the World Cancer Report \((WHO)\). Worldwide, breast cancer is still the principal cause of cancer mortality in women due to its destructive nature, metastasis, and resistance to chemotherapeutic agents \((3)\). Many studies have shown that the human diet plays a crucial role in preventing specific lifestyle diseases like cancer. Specifically, the Mediterranean diet, which consists of a large amount of fruit and vegetables, reduces the incidence of cancer \((4)\).

Dietary carotenoids are potential antioxidants and offer numerous health benefits to the body. Specifically, they prevent certain cancer and age-related macular diseases. The most studied and reported carotenoids are lutein, zeaxanthin, and lycopene \((5)\). The red chili pepper
(Capsicum species) is unique for its diversified carotenoid profile and composition (6). The intense red color of chili is due to the presence of keto-carotenoids, which are capsanthin, capsorubin, and capsanthin-5,6-epoxide, and of which capsanthin is the major carotenoid (7). Capsanthin, a novel carotenoid, gained considerable research attention in the past owing to its usability as a natural food colorant. However, its high antioxidative and anti-carcinogenic activities and potential use as a drug have been revealed recently (8). Studies have shown that capsanthin, a highly potent carotenoid has a significant anti-cancer effect and prevents and protects from the risk of cancer (9).

Lack of aqueous solubility of phytochemicals is always a challenge in drug development. Although these phytochemicals have shown remarkable efficacy in cell line studies, they have shown limited efficacy in various human clinical studies. The limited oral bioavailability and rapid elimination, or combination of both, eventually result in poor therapeutic benefits (10). Issues of poor oral bioavailability of cancer chemo preventives have limited the usage of phytochemicals in cancer prevention and treatment.

A novel drug delivery system that modifies the pharmacokinetics of existing active pharma ingredients (drug substances), such as liposomes, niosomes, nanoparticles, cyclodextrin-complexes, could be used to enhance the aqueous solubility and bioavailability of chemo preventive agents. Developing a new drug delivery system is essential for targeted delivery and enhancing maximum absorption for the approved and newly investigated drug substances (11). Capsanthin micelle was prepared using diosgenin polyethylene glycol succinate (DPGS-1000) and characterized by various techniques. A non-ionic, amphiphilic DPGS-1000 possesses a hydrophobic head and hydrophilic tail with a hydrophile-lipophile balance (HLB) value of 14.2. Various spectroscopic techniques characterized the synthesized Cap-DPGS-1000.

The present study aims to compare the possible effects of novel keto-carotenoid capsanthin (hydrophobic) and capsanthin-loaded Cap-DPGS-1000 (hydrophilic) on cytotoxic and cell apoptosis effects on human triple-negative breast cancer cell line (MDA-MB-231).

MATERIALS AND METHODS

Chemicals

Diosgenin polyethylene glycol succinate-1000 (DPGS-1000) was provided by Phytosol India Pvt Ltd. Bangalore, India. MDA-MB-231, the triple-negative breast cancer cells, were purchased from the NCCS (National Centre for Cell Science, Pune, India). The capsanthin
reference standard was procured from Sigma-Aldrich (Sigma-Aldrich, Co, LLC, St. Louis, USA). Dulbecco’s modified eagle medium (Merck Lifesciences, Bangalore, India), acridine orange (Merck Lifesciences, Bangalore, India), ethidium bromide (Merck Lifesciences, Bangalore, India), propidium iodide (Merck Lifesciences, Bangalore, India), Höechst 33342 (Merck Lifesciences, Bangalore, India), rhodamine 123 (Merck Lifesciences, Bangalore, India) and dichlorodihydrofluorescein diacetate (Merck Lifesciences, Bangalore, India) MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Merck Lifesciences, Bangalore, India), FBS (fetal bovine serum (Merck Lifesciences, Bangalore, India), PBS (phosphate buffer saline (Merck Lifesciences, Bangalore, India)) was procured from Merck Lifesciences, Bangalore, India.

**Extraction of capsanthin**

The dried Capsicum annuum fruits were extracted with n-hexane at a temperature ranging from 40 to 60 °C (12). The n-hexane layer was filtered through a 0.5 μm filter cloth and concentrated under a vacuum (400 mm of Hg).

The oleoresin obtained was further enriched by supercritical fluid extraction (40–60 °C and 25–50 mPa). The enriched extract was saponified using 50% alcoholic potassium hydroxide at a temperature of 80–85 °C for 2 h. The alcohol was removed under vacuum, and the product was washed with hot water until neutral pH. Ethyl acetate was used to extract the saponified capsanthin, and aqueous soluble impurities were removed by washing with water. Anhydrous sodium sulfate was used to dry the ethyl acetate and concentrated under a vacuum to obtain capsanthin extract (13).

**Preparation and characterization of Cap-DPGS-1000**

The Cap-DPGS-1000 was prepared by the hot-melt solid dispersion method (14). First, a three-neck round-bottom flask fitted with an overhead stirrer was kept in an oil bath and heated to 120–130 °C. 50 g of DPGS-1000 was added to the round-bottom flask, and 50 g of capsanthin extract was added slowly under stirring. In the mixture, the molar ratio of DPGS-1000 to capsanthin extract was equal to 1:1. After adding the capsanthin extract, the stirring was continued, and the same temperature was maintained for two more hours for complete dissolution. Cooled and 500 mL of distilled water was added, filtered, and concentrated under a vacuum.
UV–visible (UV-Vis) spectroscopy characterization and HPLC quantification of Cap-DPGS-1000 is done as per the method described in Paprika extract, FAO JECFA monograph (15). The UV-Vis spectrum was recorded on a spectrophotometer (model 1900i, Shimadzu Corporation, Kyoto, Japan) by dissolving the samples in acetone. The HPLC quantification was conducted with the Shimadzu i- Series Plus HPLC (model: LC-2030C, Shimadzu Corporation, Kyoto, Japan) using binary gradient elution. USP L1 reversed-phase column (Sunfire, Waters Corporation, Milford, USA) with a dimension of 250x4.6 mm and particle size of 5 µm were used. The capsanthin reference standard from Sigma Aldrich was used for the identification and quantification. Acetone (solvent A) and water (solvent B) were used as mobile phases. The gradient program described in the WHO monograph was followed (15). The capsanthin reference standard (0.01 mg/mL) and capsanthin extract (0.5 mg/mL) were dissolved in acetone and Cap-DPGS-1000 (1 mg/mL) was dissolved in water. The solutions were filtered through a 0.45 µm filter before injection. The total flow rate was maintained at 1.2 mL/min, and the detection wavelength was set at 474 nm. Capsanthin in the capsanthin extract and Cap-DPGS-1000 were identified based on the retention time of the capsanthin reference standard.

Particle size analysis

The mean particle diameter of Cap-DPGS-1000 and capsanthin extract was measured by phase analysis light scattering performed on particle size analyzer (model: ZetaPALS Particle Sizing Software Version 5.23, Brookhaven Instruments, Holtsville, USA).

The samples were dispersed in deionized and distilled water followed by sonication for 5 minutes at room temperature. A scanning electron micrograph (SEM) of the aqueous dispersion was recorded on a scanning electron microscope (model: EVO 18, Car Zeiss, White Plains, USA) by spreading the micelle dispersion over a carbon tape and drying it under a nitrogen stream.

Fourier-transform infrared (FT-IR) analysis

Attenuated total reflectance Fourier-transform infrared (FT-IR) spectra of capsanthin extract and Cap-DPGS-1000 were analyzed to confirm any change in the structure and functional groups after capsanthin loading using an FT-IR spectrophotometer (model: Nicolet iS5, Thermo Fisher Scientific, Waltham, USA). The wavelength range was set at 400–4,000 ㎛/cm⁻¹.
The crystallographic nature of capsanthin extract and Cap-DPGS-1000 was evaluated by X-ray diffraction (XRD). Using a pro diffractometer (model: X-pert, Philips, Farnborough, United Kingdom), the test items were scanned at a voltage of 40 kW and 30 mA. The scanning rate was 3 °C per minute, and the angle was maintained at 2 θ°.

**In vitro drug release studies**

The cumulative drug release of Cap-DPGS-1000 was studied on a calibrated dissolution apparatus (model: DS 8000, Labindia Analytical Instruments Pvt. Ltd., Thane, India) with paddles at 100 rpm; the bath temperature was set at (37±0.5) °C with a dissolution volume of 900 mL. A drug release experiment was conducted using 500 mg of Cap-DPGs-1000 in each bowl. The dissolution was conducted for the first 2 h in simulated gastric fluid (pH=1.2 buffer) followed by 6 h in simulated intestinal fluid (pH=6.8 buffer). The pH=1.2 buffer was prepared by mixing 250 mL of 0.2 M potassium chloride solution with 425 mL of 0.2 M HCl and diluted to 1000 mL with water. The phosphate buffer of pH=6.8 was prepared by mixing 200 mL of 0.2 M potassium phosphate monobasic with 89.6 mL of 0.2 M sodium hydroxide, diluted to 1000 mL with water. At predetermined intervals, 5 mL of dissolution media were removed for quantification and replaced with the same volumes of similar media (16). The release profile quantification of capsanthin was determined by the WHO HPLC method (15). All measurements were performed in triplicate.

**Cell culture** MDA-MB-231 triple-negative breast cancer cells were purchased from the National Centre for Cell Science, Pune, India. The cells were cultured in DMEM (Dulbecco’s modified eagle medium) supplemented with 10% V/V fetal bovine serum (FBS) and 1 % m/V antibiotics (penicillin/streptomycin) at 37 °C in a humidified atmosphere with 5 % CO₂ and 95 % oxygen (17).

**In vitro cytotoxicity**

MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed to assess the cytotoxicity of capsanthin extract and Cap-DPGS-1000 against human triple-negative cell breast cancer cells. MDA-MB-231 cells were seeded at a density of 10⁴ cells per well in 96-well plates and incubated overnight. The next day the cells were treated with different concentrations of capsanthin extract (0.5, 1, 2, 4, 6, 8, 10, 25, 50, 75, and 100 μg/mL) and Cap-DPGS-1000 (0.5, 1, 2, 4, 6, and 8 μg/mL) for 24 h. After completing the incubation time, 20 μL of freshly prepared MTT (5 mg/mL of PBS) was added to each well, followed by incubation in a CO₂
incubator (Thermo Fisher Scientific, Waltham, USA) for 3 h. The medium was removed thoroughly, and 200 μL of DMSO was added to each well to dissolve the formazan crystals. Finally, the absorbance of the 96-well plates was read at 590 nm using a microplate reader (Bio-Rad Laboratories, Hercules, USA).

The relative cell viability percentage after treatment with capsanthin extract and Cap-DPGS-1000 was normalized with control. Using the following equation, the cell viability was calculated:

\[
\text{Percentage of cell viability} = \frac{A \text{ value of treated samples}}{A \text{ value of control samples}} \times 100 / 1
\]

**Morphological staining**

The morphology and membrane permeability of capsanthin extract and Cap-DPGS-1000 treated MDA-MB-231 cells were analyzed using the acridine orange/ethidium bromide (AO-EB) dual staining method (18). In brief, MDA-MB-231 cells (10^5 cells per well) were grown in six-well plates and incubated with IC50 of concentration of capsanthin extract and Cap-DPGS-1000 for 24 h.

The media was removed after incubation, and the cells were washed with PBS and stained with a 2 μL mixture of AO/EtBr (100 μg/mL) for 5 minutes. The cells were visualized under a fluorescence microscope (model: EX310, ACCU-SCOPE, Commack, USA) at 20x magnifications.

**Nuclear staining—Hoechst 33342 and propidium iodide (PI)**

The cell-permeant nuclear stain Hoechst 33342 (19) and propidium iodide (PI) (20) were used to identify the apoptotic cells.

MDA-MB-231 cells (10^5 cells per well) were cultured and treated with IC50 concentration of capsanthin extract and Cap-DPGS-1000 for 24 h. The media was removed, and sufficient Hoechst 33342 stain (10 mg/mL stock) and propidium iodide (PI) (10 g/mL stock) were added separately to cover the cells. After incubation for 10–12 minutes in the dark, the media were removed, washed with PBS twice, and visualized under a fluorescence microscope (model: EX310, ACCU-SCOPE, Commack, USA) at 20x magnification.
Mitochondrial membrane potential

Rhodamine 123 (RHO) staining was used to evaluate mitochondrial membrane potential. The fluorescent dye RHO binds to metabolically active mitochondria. MDA-MB-231 cells were grown and treated with IC\textsubscript{50} value of capsanthin extract and Cap-DPGS-1000 for 24 h. The cells (10\textsuperscript{5} cells per well) were washed with PBS, stained with rhodamine 123 stain for 1 h in the dark at 37 °C, and visualized under a fluorescence microscope (model: EX310, ACCU-SCOPE, Commack, USA) at 20x magnification.

Generation of reactive oxygen species (ROS)

The intracellular reactive oxygen species (ROS; free radicals) accumulation within MDA-MB-231 cells was analyzed by 2',7'dichlorodihydrofluorescein diacetate (DCF–DA) staining. For this, 10\textsuperscript{5} cells per well were seeded into six-well plates (grown overnight) and incubated with IC\textsubscript{50} value of capsanthin extract and Cap-DPGS-1000 for 24 h. The medium was removed, washed with PBS, and stained with 100 μL of DCF-DA (50 μM) for 25–20 minutes in the dark at room temperature. Fluorescence was detected using a fluorescence microscope (model: EX310, ACCU-SCOPE, Commack, USA) at 20x magnifications.

Statistical analysis

Data normality was determined with the student’s t-test using GraphPad Prism V7.04 software (GraphPad Software, Inc., San Diego, USA) (21). Values are given as mean ± standard deviation (SD) obtained from three independent experiments. Statistical comparison between the treated and control groups was carried out by one-way analysis of variance (ANOVA), and p < 0.05 was considered to indicate a statistically significant difference.

RESULTS AND DISCUSSION

In women, breast cancer is the most common cancer, followed by skin cancer, and the second leading cause of death (22). Each year, approximately one million women join the breast cancer battle (23).

Pathogenesis involves several etiological factors, including age, family history, unbalanced diet, and lack of physical activity, and endocrine factors, such as hormonal imbalance (24). In a multicellular organism, programmed cell death is essential for normal growth, known as apoptosis, but this process may be blocked in cancer cells. The uncontrolled cell division and
accumulation of abnormal cells are due to induction to apoptosis (25). Therefore, apoptosis plays a significant role in cancer treatment (26).

Exploring complementary and alternative breast cancer treatment options is inevitable due to the irreversible and undesirable effects associated with chemotherapy (27,28). Secondary metabolites from plants have been extensively researched for their biological effect, especially inducing apoptosis in breast cancer (29,30). Naturally available phytochemicals play an essential role in treating several pathologies, among them cancer (31). Epidemiological studies and experimental results suggested that carotenoids can be used as dietary anti-cancer agents. The free radical quenching and neutralizing ability of carotenoids can exert antiproliferative and antitumor activities (32). Carotenoids reduce inflammation that can stimulate breast cancer tumor growth (33). When combined, carotenoids can be used to suppress breast cancer cell growth. Capsanthin significantly induced apoptosis and reduced cell viability in MCF-7 cells dose-dependently (34). Capsanthin causes oxidative stress and decreased mitochondrial membrane potential by reducing glutathione and catalase in MCF-7 cells.

**Preparation and characterization of capsanthin-loaded micelles**

Capsanthin was successfully loaded into diosgenin polyethylene glycol succinate by solid dispersion technique. The content of capsanthin in capsanthin extract and Cap-DPGS-1000 was > 50 m/m and > 20 m/m, respectively. Further, UV Vis spectrum and HPLC characterization confirmed the presence of capsanthin in Cap-DPGS-1000. The UV spectrum and HPLC chromatograms are shown in Fig. S1 and Fig. S2, respectively. The change in the structure and functional groups were further investigated by FTIR after encapsulation of capsanthin. The FT-IR spectra of capsanthin extract exhibit -CH₃ bending band at 1365 cm⁻¹, stretching band at 1634 cm⁻¹, typical for conjugated ketone (-C=0), and a broad -OH stretching band at 3436 cm⁻¹ (35). The IR spectra for Cap-DPGS-1000 are concordant with capsanthin extract. The FT-IR spectra may suggest no change in structure or functional group due to encapsulation of capsanthin in Cap-DPGS-1000, and the FT-IR spectra are shown in Fig. S3. The crystalline nature of capsanthin extract and Cap-DPGS-1000 was further analyzed by XRD (Fig. S4). The crystalline structure of capsanthin in capsanthin extract was observed as distinct peaks in the range of 15° # 2θ° # 30°. However, in capsanthin-loaded micelle Cap-DPGS-1000, no such crystalline peaks were observed. The disappearance of characteristic crystalline peaks in the Cap-DPGS-1000 demonstrated that capsanthin molecules exist as amorphous or disordered
crystalline forms. DPGS-1000 could protect the capsanthin molecules from X-ray diffraction, causing the undetectable crystalline structure. Thus, the encapsulation of capsanthin in DPGS-1000 was further confirmed by the XRD pattern. The solubility of a material is higher when it is in the amorphous form than the more stable crystalline form because of the higher Gibbs free energy. Most recently, amorphous solid dispersion is one of the most significant strategies to enhance the bioavailability of poorly aqueous soluble drugs (36). The higher solubility and more significant biological effect of Cap-DPGS-1000 compared to capsanthin extract due to lesser crystalline nature.

Investigation of physicochemical properties of capsanthin-loaded micelles

Particle size is an important factor for insoluble drugs (37) and hydrophobic phytochemicals. Smaller particles have a large surface area, higher dissolution, and faster absorption than the bigger particles. The dynamic light scattering (DLS) technique was used to examine the particle size distribution and zeta potential of capsanthin-loaded micelles. The average particle size of micelles was (508±3.2) d/nm, almost 80 times lower than the capsanthin extract’s average particle size of (31993±5.2) d/nm. Particle size population and their distributions in a sample can be defined by polydispersity index (PDI). The value of PDI ranges from 0.0 (uniform particle size) and 1.0 (multiple particle size population). Usually, the acceptable value for a micelle is 0.2 and below. From the PDI value for Cap-DPGS-1000, which is (0.261±1.1), we can conclude that the particle size population is almost uniform, but for the free capsanthin, the PDI is 0.616±0.9, which is a characteristic of multiple particle size groups (38). The zeta potential of micelles is one of the most critical factors when nanoparticles enter the bloodstream. The positive charge of micelles leads to an increase in the removal of nanoparticles by the reticuloendothelial system and increases adsorption by non-specific proteins (39,40). The negative zeta potential of the capsanthin-loaded micelles was found to be (-38.54±0.3) mV indicates greater stability of the particles in solution. The data are shown in Table 1. The size and morphology of the Cap-DPGS-1000 were confirmed by SEM imaging. The exact concentration of capsanthin and Cap-DPGS was dispersed in water, and SEM readings were recorded (Fig. 1). Capsanthin-loaded micelle shows that the particles have an average particle size of (50–100) d/nm, and it is well within the optimal range below 200 d/nm. There is a notable decrease in the size between capsanthin extract and Cap-DPGS-1000, probably due to DPGS-1000 preventing aggregation formation. Particle size reduction enhances drug wettability
and bioavailability significantly. Lipophilic drugs suffer from slow dissolution, incomplete release, and poor bioavailability. The poor solubility and bioavailability of lipophilic capsanthin can be enhanced by reducing the particle size by converting it into capsanthin-loaded micelles.

**Aqueous solubility and drug release**

Aqueous solubility is an essential parameter for drug absorption, desired concentration in the systemic circulation, bioavailability, and a desired biological response (41). The amphiphilic Cap-DPGS-1000 could form self-assembled micelles in aqueous solutions. Capsanthin remains as a hydrophobic core, while DPGS-1000 could form a hydrophilic outer shell of the micelles. Dissolution of Cap-DPGS-1000 was performed in simulated gastric fluid (pH=1.2 buffer) and found very few capsanthin was released, as shown in Fig. S5. The Cap-DPGS-1000 particles were intact even after 120 minutes, and the acid-resistant property ensured that very low capsanthin (13.19 %) was released in simulated gastric pH. The release profiles of capsanthin in Cap-DPGS-1000 for 240 minutes in simulated intestinal fluid (pH=6.8) are shown in Fig. S5. From this profile, it was found that the capsanthin was gradually released over the period, and the release percentage at 240 minutes was 88.82 %. Cap-DPGS-1000 did not show any rapid burst, probably due to capsanthin being strongly bound or adsorbed in Cap-DPGS-1000 (42). Based on the above results, the formulation method affects the dissolution profile, and capsanthin loaded by the solid dispersion method can influence rapid burst and delayed-release characteristics (43).

**In vitro cytotoxicity and apoptosis**

Programmed cell death (apoptosis) is crucial for normal cell development and turnover, and the apoptosis pathway is deregulated in cancer. Triple-negative breast cancer (TNBC) is defined by the lack of estrogen and progesterone receptor expression and HER2 (human epidermal growth factor receptor 2) overexpression/amplification and represents 10-15% of all breast cancer patients (44). Carotenoids may exhibit cytotoxic activity in various cancer cell types (45). Lutein, the extensively studied carotenoid, inhibits a variety of human breast cancer cells, including the BT-474 (ER/PR+HER2+), MDA-MB-453 (triple-negative), and MDA-MB-231 (triple-negative) cell lines. Further, lutein significantly reduced colony numbers in MCF-7 and MDA-MB-468 cells (46). Lycopene, zeaxanthin, and capsanthin showed the highest total apoptosis in the MDA-MB-231 breast cancer cell line, according to Molnár et al. (47).
In this present MTT assays on the MDA-MB-231 cancer cell line, the study showed that the percentages of viable cells in the groups treated with different concentrations of capsanthin and capsanthin-loaded micelles were significantly reduced in a dose-dependent manner, and the reduction was statistically significant compared with the control group (p < 0.05). The IC$_{50}$ at 24 h for capsanthin and capsanthin loaded micelles were 81.06±1.51 and 3.10±1.09 μg/mL, respectively. The data are shown in Fig. 2. Our study validates the previous findings of the cytotoxic activity of capsanthin, but capsanthin-loaded micelles against MDA-MB-231 were not studied earlier and reported. The conspicuous morphological changes such as growth inhibition, distorted cell shape, and cytoplasmic condensation were observed by inverted phase-contrast microscope in capsanthin extract (Fig. 3b) and Cap-DPGS-1000 treated-cells (Fig. 3c), compared to the untreated control (Fig. 3a). The cells in the control group were live and found to be uniform in shape. The morphological alterations induced by capsanthin extract and Cap-DPGS-1000 were observed using acridine orange-ethidium bromide (AO-EB) dual staining (nucleic acid binding stains). Under a fluorescence microscope, untreated cells appeared uniform shaped, light green colored, and without distortion. The morphology of treated cells was entirely different from untreated. The early process of apoptosis, such as nuclei condensation and cell shrinkage (48), were observed in treated cells. The treated cells were smaller due to the dense cytoplasm and more tightly packed organelles. The AO-EB staining of control, IC$_{50}$-treated cells, and change in the morphology are shown in Figs. 3d, 3e and 3f.

The occurrence of apoptosis was further confirmed by DNA binding dyes Höechst 33342, which detect chromatin condensation, a hallmark of apoptotic cell death (49), and propidium iodide (PI) stains were used to confirm AO-EB staining results. These nuclei binding dyes stains all the cells without discriminating their viability (50). The nuclei of capsanthin extract and Cap-DPGS-1000-treated cells showed a brighter blue fluorescence than the untreated cells. These morphological changes due to the nuclei of treated cells were fragmented and shrank compared to the untreated control group. The Höechst 33342 control, IC$_{50}$-treated cells capsanthin extract, and Cap-DPGS-1000 are shown in Figs. 3g, 3h and 3i.

Furthermore, the apoptosis and morphological changes in the capsanthin extract and Cap-DPGS-1000-treated cells were confirmed by red PI staining, which can stain necrotic and late apoptotic cells. Distinguishable changes like condensed nuclei and apoptotic bodies were observed in the treated cells compared to control. The fluorescence image of PI-stained control, IC$_{50}$-treated capsanthin extract, and Cap-DPGS-1000 are shown in Figs. 3j, 3k and 3l.
Mitochondrial membrane potential

Mitochondrial membrane potential for capsanthin extract and Cap-DPGS-1000 was evaluated by rhodamine 123 (RHO) staining. MDA-MB-231 cells were treated with IC$_{50}$ value of test items for 24 h, stained with RHO and examined under a fluorescence microscope. The control cells with high membrane potential showed green fluorescence (Fig. 4a). The decrease in fluorescence in treated cell lines indicates the decrease in the mitochondrial membrane potential, as shown in Figs. 4b and 4c.

Evaluation of intracellular reactive oxygen species (ROS).

It is well known that ROS generation was usually associated with cell apoptosis (51). Generation and accumulation of ROS can elicit apoptosis and inhibit the cell cycle aggravating oxidative stress in cancer cells (52). To compare the level of ROS generation in MDA-MB-231 cells with or without capsanthin extract and Cap-DPGS-1000, the fluorescent dye, DCF-DA (2′,7′-dichlorofluorescein diacetate) stain was used and examined under a fluorescence microscope. Finally, there was a significant increase in the fluorescence (Figs. 4e and 4f) compared to control (Fig. 4d).

CONCLUSIONS

In this study, we have extracted capsanthin from Capsicum annuum fruit and developed capsanthin-loaded Cap-DPGS-1000 micelles successfully the first time, and the micelles exhibited suitable physicochemical properties. A solid dispersion technique was employed to prepare Cap-DPGS-1000 micelle as a delivery system for the aquaphobic capsanthin. The FTIR and other characterization data confirmed no changes in capsanthin’s structure and functional groups in the capsanthin-loaded micelles. The capsanthin-loaded micelles were nanosized with low polydispersity, indicating the size’s uniformity. In vitro release behavior of capsanthin showed a controlled manner for 240 minutes. Finally, capsanthin extract and capsanthin-loaded micelles were evaluated for their cytotoxic activity on triple-negative breast cancer cells MDA-MB-231. In conclusion, the current study’s results show that capsanthin extract and Cap-DPGS-1000 both showed cytotoxic effects on MDA-MB-231 breast cancer cells. The IC$_{50}$ value of the Cap-DPGS-1000 micelles against MDA-MB-231 breast cancer cells was 3.10 μg/mL, which is much lower than capsanthin extract (IC$_{50}$ 81.06 μg/mL). Capsanthin extract
and capsanthin-loaded Cap-DPGS-1000 are promising drug candidates to induce apoptosis and increase reactive oxygen species (ROS) in cancer cells.

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CONFLICT OF INTEREST

Authors have no conflict of interest with this study.

SUPPLEMENTARY MATERIALS

Supplementary materials are available at www.ftb.com.hr.

AUTHORS’ CONTRIBUTION

V. S: Performed the analysis, collected data, analyzed and interpreted data and drafted the article. R.S: Conceptualized and supervised the work, helped with the data analysis and interpretation, provided critical revision of the article along with its editing and final approval of the version to be published.

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Table 1. Average particle size, zeta potential and PDI of capsanthin extract and Cap-DPGs-1000 (capsanthin loaded micelles)

<table>
<thead>
<tr>
<th>y(extract)</th>
<th>Average particle size (d/nm)</th>
<th>Mean zeta potential (m/V)</th>
<th>Poly dispersity index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capsanthin extract</td>
<td>(31993±5.2)</td>
<td>(-33.25±1.8)</td>
<td>(0.616±0.9)</td>
</tr>
<tr>
<td>Cap-DPGS-1000</td>
<td>(508±3.2)</td>
<td>(-38.54±2.7)</td>
<td>(0.261±1.1)</td>
</tr>
</tbody>
</table>

Data are expressed as mean value±standard deviation (N=3)
Fig 1. Scanning electron microscopy (SEM) micrographs a) capsanthin extract b) Cap-DPGS-1000 (Capsanthin loaded micelles). Magnification= 30 K X
Fig. 2. MTT assay. a) capsanthin extract (0.5, 1, 2, 4, 6, 8, 10, 25, 50, 75 and 100 µg/mL) and b) Cap-DPGS-1000 (capsanthin loaded micelles) (0.5, 1, 2, 4, 6, and 8, µg/mL) for 24 h. MTT assay experiment was performed in triplicate and the final values were represented as the mean ± standard deviation. Data represent the mean value±standard deviation (N=3) *p=<0.05, **p=<0.05, and ****p=<0.0001 and ns =not significant were considered statistically significant.
Fig. 3. Morphology of MDA-MB-231 cells after treatment with capsanthin extract (0.5, 1, 2, 4, 6, 8, 10, 25, 50, 75 and 100 μg/mL) and Cap-DPGS-1000 (capsanthin loaded micelles) (0.5, 1, 2, 4, 6, and 8, μg/mL). a) control, b) IC$_{50}$-treated capsanthin extract inverted-phase contrast image, c) IC$_{50}$-treated Cap-DPGs-1000 inverted-phase contrast image. d) control, e) IC$_{50}$-treated capsanthin extract AO-EB staining image, f) IC$_{50}$-treated Cap-DPGS-1000 AO-EB staining image. g) control, h) IC$_{50}$-treated capsanthin extract Hoest-33342 staining image, i) IC$_{50}$-treated Cap-DPGS-1000 Hoest-33342 staining image. j) control, k) IC$_{50}$-treated capsanthin extract Propidium iodide (PI) staining image, l) IC$_{50}$-treated Cap-DPGS-1000 Propidium iodide (PI) staining image.
Fig. 4. Morphology of MDA-MB-231 cells after treatment with capsanthin extract (0.5, 1, 2, 4, 6, 8, 10, 25, 50, 75 and 100 μg/mL) and Cap-DPGS-1000 (capsanthin loaded micelles) (0.5, 1, 2, 4, 6, and 8, μg/mL). a) control and b) IC$_{50}$-treated capsanthin extract Rhodamine staining image, c) IC$_{50}$-treated Cap-DPGS-1000 Rhodamine staining image. d) control, e) IC$_{50}$-treated capsanthin extract 2',7'-dichlorofluorescein diacetate staining image, f) IC$_{50}$-treated Cap-DPGS-1000 2',7'-dichlorofluorescein diacetate staining image
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Fig. S1. UV absorbance spectrum
Fig. S2. HPLC chromatograms

Fig. S3. FTIR spectrum of capsanthin extract and Cap-DPGS-1000 (capsanthin loaded micelles)
Fig. S4. XRD spectrum of a) capsanthin extract and b) Cap-DPGS-1000
Fig. S5. Dissolution conditions. Method=UP type II, paddle=100rpm, dissolution media=900ml.
Release of capsanthin from Cap-DPGS-1000 (Capsanthin loaded micelles) a) in simulated
gastric pH=1.2 buffer b) simulated intestine pH=6.8 buffer. Data represent the mean
values±standard deviation (N=3)