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RESEARCH ARTICLE

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In vitro cytotoxic activity of microalgal extracts loaded nano–micro particles produced via electrospraying and microemulsion methods

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Abstract
Reactive oxygen species can bind protein, DNA, lipids, and carbohydrates and thus cause an oxidation reaction that induces various syndromes such as cardiovascular diseases, degenerative disease, and cancer types in the human body. Bioactive compounds, such as PUFA, EPA, DHA, and carotenoids in algae, have a chain ring and protect the tissue from chemical damage and reverse the symptoms of some diseases. Algal bioactives also have various biological properties such as anticoagulants, antiviral, antiangiogenic, antitumor, anti-inflammatory, antioxidant, antiproliferative, and immune modulation properties. This study aimed to show in vitro cytotoxic activity effect of *Chlorella protothecoides* and *Nannochloropsis oculata* microalgal extracts loaded nano–microparticles on A-172 (*Homo sapiens* brain glioblastoma) and HCT-116 (*H. sapiens* colon colorectal carcinoma) cell lines because of the increasing importance of algal biotechnology. MTT viability tests were performed on HUVEC, A172, and HCT 116 cells with particles obtained at optimum process parameters. The cell viability rates of encapsulated particles were also compared with pure algae extracts. Microalgal extracts loaded nano–micro particles showed very promising results for cytotoxic effect on cancer cells.

KEYWORDS
anticancer effect, electrospraying, microalgae, microemulsion, nanotechnology

1 | INTRODUCTION

Marine ecosystems have a wide range of living organisms compared to terrestrial ecosystems that provide countless resources for human nutrition and health. Marine species contain diverse multifaceted groups with habitats in all ocean ecosystems, and their habitats range from intergenerational to deep-sea environments. Algae are photosynthetic organisms classified as micro and macro; and green, blue-green, brown, and red algae. Algae, storing valuable metabolites within the cell, can be used in different areas such as food support and health preservatives, animal feed, soil structure improving properties, natural food coloring, cosmetic industry and they can be also used in wastewater treatment with their metal bonding capabilities.[1]

Algae are used directly in the variety of foods, medicines and other industrial products as well as in the production of certain substances of these products. Algae produce their own nutrients through photosynthesis, but also produce energy for their survival.

Nowadays, the importance of algal biotechnology is rapidly developing and increasing. Since the first studies, different species have been discovered and algae have become the focal point of new researches in a short time with the evaluation of materials obtained from these species.[2,3] Macroalgae, microalgae, bacteria, and cyanobacteria produce secondary metabolites as adaptation to their environment. These secondary metabolites include biologically active compounds such as polyunsaturated fatty acids (PUFAs), sterols, proteins, polysaccharides, antioxidants, and pigments. Bioactive components, which can be considered as marine-based pharmaceutical and nutritional products, can be derived from a large number of sources, including marine plants, microorganisms and sponges, all of which contain their own biomolecules. Natural products from marine living creatures have been used as a promising bioactive molecule source for preventing various diseases over the centuries. Therefore, marine organisms are one of the most important sources of food that have the potential to treat various diseases. These bioactive molecules and secondary metabolites have antibiotic, antiparasitic, antiviral, anti-inflammatory, antifibrotic, and anticancer effects. Compounds produced from these organisms are generally effective as therapeutic agents for infectious and non-infectious diseases with a high specificity for target molecules. These components derived from marine-derived products have excellent potential for use as functional foodstuffs and pharmaceutical products, as they have advantageous physiological effects along with health benefits such as anticancer or anti-inflammatory effects. In recent years, researches on the use of marine-derived components in pharmaceutical and nutraceutical fields have been carried out. In this context, especially in the field of bioengineering, raw materials sourced from marine sources have been widely used.[4-6]

Chlorella protothecoides and *Nannochloropsis oculata* were utilized as microalgae species in this study. *C. protothecoides* is a microalgae that can grow photototropically or heterotrophically under different culture conditions. Heterotrophic growth of *C. protothecoides* in culture medium with added organic compounds as acetate, glucose, or carbon source results in high biomass and high lipid content in cells.[7] Its high lipid content has made this microalgae remarkable especially in biotechnological and biofuel applications, and it has also become an important source of many products such as aquaculture feed, human food supplements, and pharmaceuticals.[8,9] *N. oculata* is a marine microalga of great biotechnological interest, mainly due to its large production of lipids containing PUFAs. It has various pigments such as zeaxanthin, beta-carotene, chlorophyll-a, violaxanthin, vaucheraxanthin, and high oil content for several industrial applications such as biotechnological product, fish feed, and biodiesel production.[10]

To protect valuable effects of algal bioactives, encapsulation of these bioactives has been promising importance in recent years. Electrospinning technology is one of the new methods for this purpose. The process of obtaining the product from the polymer solution with the help of the electric field is divided into two according to the structure of the product being produced. When electrospinning is used, the ultra-thin continuous fibers are produced; while the spherical particle is produced if the electrospraying technology is used. The purpose of this technology is to encapsulate pharmaceuticals, enzymes, dyes, flavors, and so forth, in the active core containing the protective polymer to protect them from the external environment by means of a shell. The shells of these nanoparticles can consist of carbohydrate polymers, herbaceous gums, or synthetic polymers. Micro and nanocapsules are applied in areas such as pharmaceutical, cosmetics, and food industry.[11-13] In electrospraying process, electrostatic forces are used to produce electrically charged jets from viscoelastic polymer solutions; these solutions are converted to particles by evaporation of the solvent.[14,15] The size of particles prepared by emulsions are generally in micro-order. This magnitude in electrospraying is suitable for improving the effect of particle size on cell uptake.[16]

This study aimed to show in vitro cytotoxic activity effect of *C. protothecoides* and *N. oculata* microalgal extracts loaded nano–microparticles on A-172 (*Homo sapiens* brain glioblastoma) and HCT-116 (*H. sapiens* colon colorectal carcinoma) cell lines.

2 | MATERIALS AND METHODS

2.1 | Materials

Alginic acid sodium salt from brown algae (Sigma-Aldrich, UK), calcium chloride (Merck, Germany), Tween 20 (Sigma-Aldrich, UK), poly vinyl alcohol (Merck, Germany), Sudan Red G (Sigma-Aldrich, UK), chitosan (Sigma-Aldrich, UK), ethanol, acetic acid (90%), and methanol (Merck, Germany) were purchased. Bioactives from *C. protothecoides* and *N. oculata* as the core material were obtained in the Algal Biotechnology Laboratory at Bioengineering Department of Yildiz Technical University.[17] In the cell experiments, HUVEC, A172, and HCT116 cells were obtained from Department of Pharmaceutical Technology in the Faculty of Pharmacy of Istanbul University. Of note, 10% fetal bovine serum supplemented with DMEM was from Biochrom AG, Berlin, Germany.

2.2 | Electrospraying method

A laboratory-scale Nano Fiber Electrospinning device (Unit model no: HO-NFES-0434) of Holmarc Opto Mechatronics Pvt. Ltd. was used to obtain nanoparticles. The injected polymer solution–algae oil mixture was attached to the pump section and the electrode was attached to the other end of the metal syringe tip. In this way, an electric field of attraction was created with oppositely charged electrodes. Experiments were initiated primarily at the 15 cm collector plate and a rate of 0.5 mL/hr. Droplet-forming, and nanoparticle-like structures were obtained at 3 mA of current and 12 kV of voltage by the formation of the Taylor cone.[16]

2.2.1 | Synthesis of PCH particles

PVA: Chitosan solution (PCH) was prepared that PCH in a mass of 80:20 (wt/wt) was completely dissolved in acetic acid (25% of vol/vol) to obtain PVA-Chitosan solution with 2% (wt/vol) at 75°C with 1.5 hr of stirring on a magnetic stirrer.[18-20] Then 1% (wt/vol) of algae oil and 1% (wt/vol) of Tween 20 were added and mixed for a further 15 min to obtain a completely homogeneous creamy solution for PCH1 (with *N. oculata* extract) and PCH2 (with *C. protothecoides* extract) particles production via electrospraying.

2.2.2 | Synthesis of PNA particles

PVA: NaAlg solution (PNA) was prepared to obtain the particles (with *C. protothecoides* extract) via electrospraying technique. The concentrations of 7% and 8% (wt/vol) PVA solution were prepared in distilled water at a ratio of NaAlg with 2% (wt/vol) and mixed to obtain PVA-NaAlg solutions of 80:20 (vol/vol) for PNA1 and PNA2 particles production, respectively.[21]

2.3 | Microemulsion method

The alginate solution was obtained by dissolving sodium alginate in deionized water. In the following step, an emulsifier containing microalgae oil was included. This mixture, which was put within the mechanical stirrer, was emulsified and mixed by mechanical mixing (C-Mag hs 7, IKA) at 3000 rpm until the creamy appearance. At that point, the calcium chloride was included quickly and the blend was blended. Sodium alginate in different concentrations, microalgae oil, and emulsifier blend were created utilizing 200 W and 24 kHz ultrasonic wave created by ultrasonic gadget (Alex machine ultrasonic cleaner). The sonication handle was performed at indicated times of 5 and 15 min. At last, to get the microemulsion, a foreordained sum of calcium chloride arrangement was included to deliver nanocapsules. In this process, the fundamental guideline is that the Ca^{+} ions bond within the alginate chains to make the grid structure and trap the center fabric within the arrangement into the egg.

2.3.1 | Synthesis of NPAL particles

During the microemulsion process, sodium alginate as a wall material, calcium chloride ($CaCl_2$) as a gelling agent, Tween 20 as an emulsifier and alginate as a core material were sed.[22] The capsules of NPAL1 (obtained at 5 min of process time) and NPAL2 (obtained at 15 min of process time) were prepared by using emulsified sodium alginate solution as aqueous polymer phase, *C. protothecoides* microalgae oil as the oil phase and Tween 20 as emulsifier.

2.4 | Entrapment and loading efficiencies of PCH, PNA, and NPAL particles

The amount of oil in the particles was measured according to the OD value, and the percentage of the encapsulated oil was calculated by using a standard curve. For each production: 0.2 mg of the particles was weighed and 4 mL of chloroform was added. Chloroformed samples were incubated in a water bath at 40°C for 5–6 hr, then centrifuged at 4200 rpm and supernatant fractions were measured by a UV Spectrophotometer at 501 nm. The EE and LE of the PCH, PNA, and NPAL nano–microparticles were calculated using the following equations[23]:

$$\text{Loading efficiency (LE)} = \frac{\text{Total amount of algae oil} - \text{Amount of free algae oil}}{\text{Weight of nanoparticle}} \times 100 \tag{1}$$

$$\text{Entrapment efficiency (EE)} = \frac{\text{Total amount of algae oil} - \text{Amount of free algae oil}}{\text{Initial amount of algae oil used for the drug encapsulation}} \times 100 \tag{2}$$

2.5 | Particle size and functional groups of PCH, PNA, and NPAL particles

Particle diameters were measured by using a zetasizer device (Malvern Zetasizer Nano ZS instrument). A quartz cuvette was used during the measurements. FT-IR device (Thermo Scientific, Nicolet 6700) was used to identify the functional groups of nanocapsules of microalgal oil encapsulated with sodium alginate and to determine whether the encapsulation process was performed and the absorbance values of the functional groups contained in the samples in the range of 600–4,000 cm^{-1}.

2.6 | Release profile of algal extracts

To evaluate the release profile of hydrophobic algal extract included bioactive compounds, Sudan Red G, a water-soluble dye, was used as a hydrophobic material. The release medium was ready a pair of (wt/vol) Tween 20 in double-distilled water. Assays to be utilized in unleash studies comprised 5 mL release medium with 25 mg of particles for every sample, and these were stirred at 100 rpm in an exceedingly flask at 25°C. At selected time intervals, the discharge medium was collected and centrifuged at a speed of 4,200 rpm for 10 min. Then, a pair of milliliter of supernatant was removed for UV measurements. The concentration of dye within the supernatant was determined by using UV (UV-2600 photometer, Shimadzu, Japan) absorption at a wavelength of 501 nm.[24]

2.7 | MTT assay: Cytocompatibility of nano–microparticles

Human umbilical vein endothelial cells (HUVECs) and human glioblastoma cells (A172) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), human colon carcinoma cell line HCT116 were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum (Panbiotech, Germany) in an incubator maintained at 37°C and 5% CO_2.

For MTT assay, HUVECs, human glioblastoma cells (A172), and human colorectal cancer cells (HCT-116) were seeded onto a 24-well plate with a density of 37,500 cells per well and grown for 24 hr. After the incubation, 17.5, 35, and 70 μM PCH and PNA particles and 12.5, 25, and 50 μM NPAL particles were added to the wells and incubated for 48 hr.

Then, 50 μL of MTT (Biofroxx, Germany) solution at the concentration of 2 mg/mL was added into each well and incubated for 3 hr at 37°C. The medium was discarded and 200 μL/well of DMSO (Biofroxx, Germany) was added for 30 min. The optical density (OD) of the wells was determined at 570 nm using microplate reader (Thermoscientific Multiskan Ex). Experiment was performed in triplicate to validate the data. in vitro experiments were done with an "n" value of 4. All the results were analyzed using Student's *t* test as the statistical method and *p* value of .05 was considered to be significant.

2.8 | GC chromatograms of microalgae oil

Gas chromatography device was used to determine the content of microalgae oil. The analyzes were performed on a YL Instruments 6100 GC gas chromatography device. The gas chromatography device contains a flame ionization detector and a 30 m × 0.32 mm × 0.25

IDm ZB-FFAP column. The column temperature program starts at 50°C and reaches 175°C at 15°C/min, then at 230°C and 5°C/min. The injector temperature was 230°C and the flow rate was set at 1.8 mL/min. The detector temperature was kept at 280°C. Hydrogen gas was used as the carrier gas. Methyl heptadeconate (C17: 0) was used as internal standard and the samples were made ready for gas chromatography by mixing with methyl heptadeconate and n-heptane.

3 | RESULTS AND DISCUSSION

3.1 | Particle size analysis

The size of the nanocapsules affects their quality and stability, so the smaller particles, the more stable they are. The nanocapsules produced at a concentration of PNA2 are more stable than the nanocapsules produced at a concentration of PNA1. The reason is that the increased PVA concentration is more affected by the electric field strengths by increasing the conductivity and thus more stable structure.[15] PCH polymer blend particles were obtained in larger size than PNA particles because of moleculer weight of chitosan.[19] For NPAL particles, when particle size results measured by zetasizer device are examined, it is observed that particle size increases with increasing alginate concentration.

The particle size distribution of PCH, PNA, and NPAL particles was confirmed using Zetasizer equipment. The characterization results confirmed the particle sizes as 132.3 ± 15 nm and 123.9 ± 10 nm for PNA1 and for PNA2 particles; 325.2 ± 40 nm and 334.5 ± 40 nm for PCH1 and PCH2 particles; 756.8 ± 60 nm and 762.4 ± 65 nm for NPAL1 and NPAL2 particles, respectively.

3.2 | FT-IR analysis

FT-IR spectral analysis was performed to show the interaction between the algal extracts and the particles, and to characterize molecular bonds and functional groups. The chemical structure and changes in the structure of particles were determined using Fourier Transform Infrared Spectroscopy (Thermo Scientific, Nicolet 6700). The adsorbance values of the functional groups in the range of 600–4,000 cm^{-1} were determined.

FT-IR spectrum of microalgae oil-loaded nanoparticles (PNA1, PNA2, PCH1, NAPL1) were given in Figure 1. The overlap in peaks

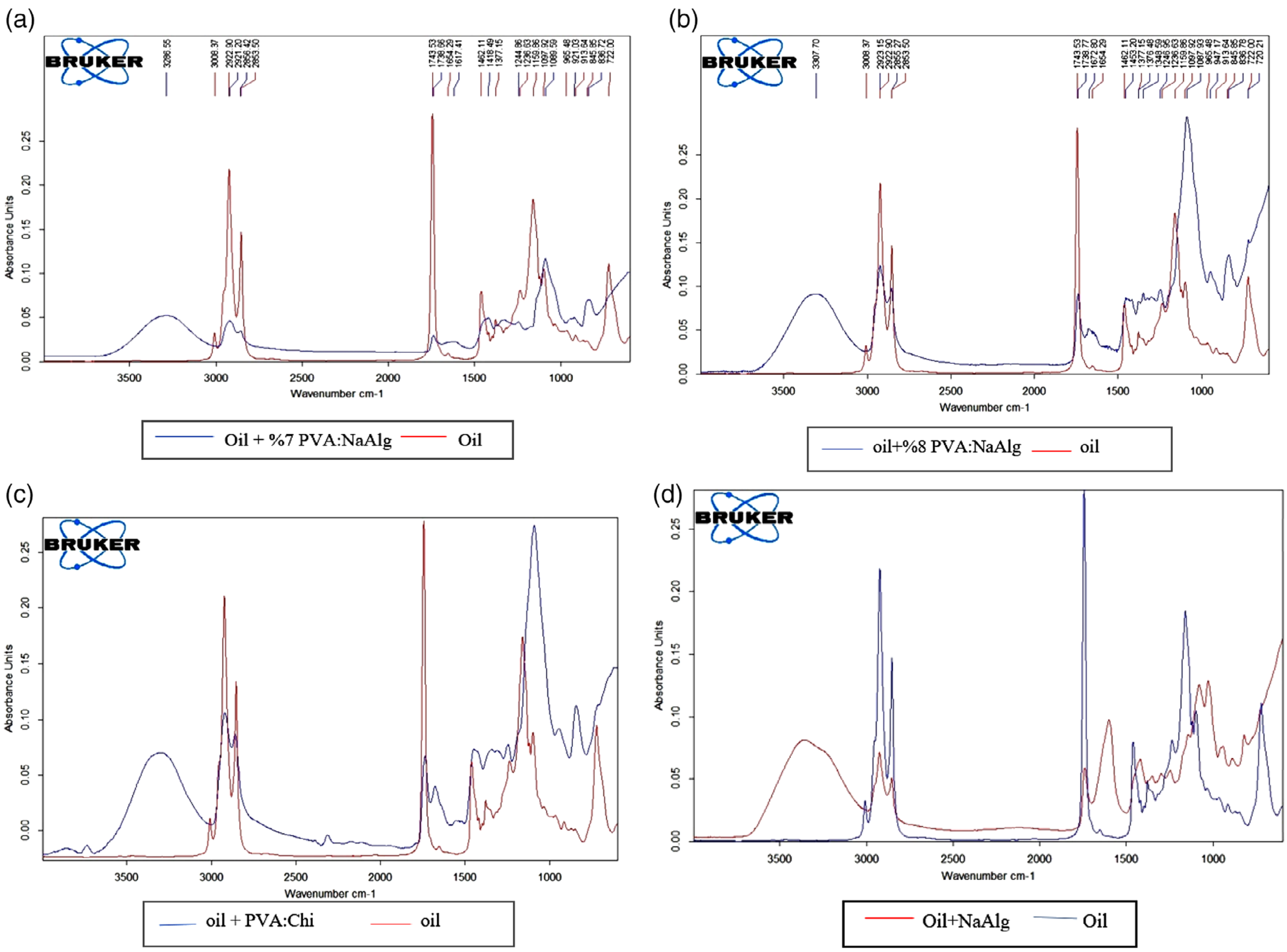

FIGURE 1 FT-IR spectrum of (a) PNA1, (b) PNA2, (c) PC1, and (d) NAPLl

indicates the presence of oil in the all capsules produced.[25] All these data showed that the amount of oil trapped in NAPL1, PCH1, PNA1, and PNA2 particles is highest in PNA2 by comparing with microalgal oil peaks.[26]

3.3 | GC chromatograms of microalgae oil

As shown in Figure 2, *C. protothecoides* oil has the content of palmitic acid (C16: 0), oleic acid (C18: 1), linoleic acid (C18: 2), eicosatrienoic acid (C20: 3), and eicosapentaenoic acid (EPA) (C20: 5) while *N. oculata* oil has palmitic acid (C16:0), palmitoleic acid (C16:1), oleic acid (C18:1), linoleic acid (C18:2), EPA (C20:5). According to the obtained chromatograms, the highest fatty acid content was seen as linoleic acid in *C. protothecoides* and as palmitoleic acid in *N. oculata*. Other fatty acids were found in trace amounts and therefore they could not be evaluated. Both of microlgal oils were found to have bioactive value because of being rich in beneficial PUFAs.[9,27]

3.4 | Entrapment and loading efficiencies

By this method, oil amount in the capsules is measured as the OD value and the formula contains the percentage of oil that can be encapsulated using the standard curve. For each production, 0.2 mg of particle is weighed and 4 mL of chloroform is added. The chloroformed samples were centrifuged at 4200 rpm after waiting for 5–6 hr at 40°C in a water bath to measure the supernatant portions at 501 nm at the UV Spectrophotometer. When the obtained OD values are compared to the standard graph, the results obtained are written in the Equation (1), and the yields are found (Table 1). The highest efficient encapsulation was obtained as 77 ± 5.4% for PNA2 sample. The lowest yield was obtained as 60 ± 3.8% for NPAL2 sample.

3.5 | Algal extract release profile of PCH, PNA, and NPAL particles

To assess the release profile of hydrophobic algal extract compounds included bioactives, the Sudan Red G model, a fat-soluble dye, was used as the hydrophobic material. The measurement results of the light absorbing at the UV spectrophotometer at 501 nm are given in Figure 3. In the first 24 hr, it was observed that the algal extract in the capsule were released. At the same time, this oscillation was not sudden but it was in a controlled manner. This stabilized release level and duration showed that controlled release of the drug could be achieved if a drug was encapsulated.

3.6 | In vitro cytocompatibility of particles toward HUVEC, A172, and HCT116 cells

In this study, the cytotoxic effects of PCH1, PCH2, PNA1, PNA2 particles produced by electrospraying method; and NPAL1, NPAL2 particles produced by microemulsion method, and directly *C. protothecoides* and *N. oculata* microalgae extracts without encapsulation on three different cell lines were determined by MTT test (Figure 4).

For cytotoxicity tests, *C. protothecoides* and *N. oculata* microalgae extracts were used as 70, 35, and 17,5 μg/mL for particles produced by electrospraying, 50, 25, and 12.5 μg/mL for particles produced by microemulsion; 100, 50, and 25 μg/mL for microalgal extracts without encapsulation.

Cell viability rates obtained after the application of the samples on the cell cultures, the OD values obtained by the MTT test, the ratio to the control group optical densitometer values (%) were expressed as cell viability, and the results of each group's own control group was accepted as 100%. Mosmann (1983) rated the cytotoxic effect with a scale based on the rate of cell viability after the test material was applied.[28] In this study, the cell viability rates were evaluated based on this classification.

TABLE 1 Entrapment and loading efficiencies of samples

Codes	Size (nm)	Entrapment efficiency (%)	Loading efficiency (%)
PNA1	132.3 ± 15	80 ± 2.6	66 ± 2.1
PNA2	123.9 ± 10	82 ± 3.6	77 ± 5.4
PCH1	325.2 ± 40	79 ± 3.2	72 ± 2.6
PCH2	334.5 ± 40	81 ± 2.8	70 ± 3.2
NPAL1	756.8 ± 60	75 ± 3.2	73 ± 3.1
NPAL2	762.4 ± 65	73 ± 3.6	60 ± 3.8

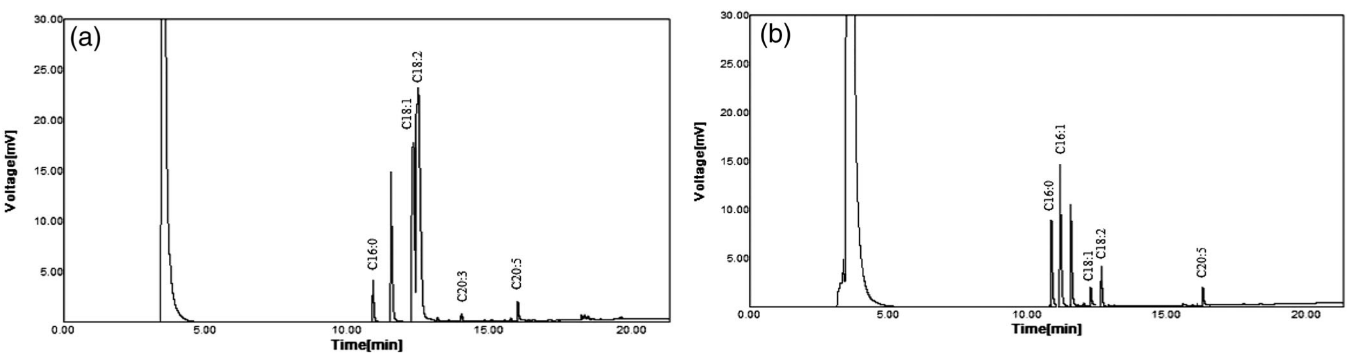

FIGURE 2 Chromatograms of (a) *Chlorella protothecoides* oil and (b) *Nannochloropsis oculata* oil

First, the cytotoxic effect of each nano and microparticle was tested in the HUVEC cell line, which is a noncancerous cell line. Particles and extracts in this cell line showed no cytotoxic effect.

The concentration of 17.5 μg/mL for PCH1, PCH2, and PNA2 particles showed a mild cytotoxic effect while 70 and 35 μg/mL had more cytotoxic effect on two types of cancer lines. PNA1 showed no cytotoxic effect at a concentration of 17.5 μg/mL and more severe cytotoxic effect at concentrations of 35 and 70 μg/mL. Generally, particles produced from PVA: Chitosan polymer blend had much more cytotoxic effect on cancer lines compared to particles produced from PVA: NaAlg polymer blend. And, there was no much difference between 35 and 70 μg/mL concentrations effect on HCT-116 cell line for all particles. PCH1 particle with *N. oculata* extract is the most anticancer effected particle in all particles. It can be concluded that *N. oculata* microalgae has a little more anticancer effect compared to *C. protothecoides* microalgae.

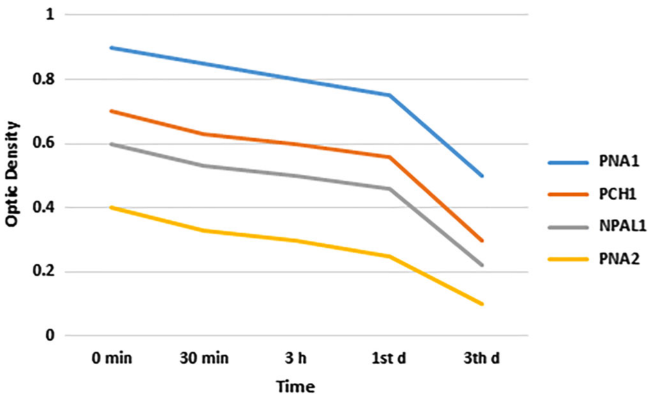

FIGURE 3 The release test results using Sudan Red G hydrophobic dye (30 min, 3 hr, 1 days, 3 days)

NPAL1 particles showed moderate cytotoxic effect at a concentration of 12.5 and 25 μg/mL, and made more cytotoxic effect at a concentration of 50 μg/mL on A-172 cancer line while NPAL2 particles showed severe cytotoxic effects on A-172 cell line at all concentrations of 12.5, 25, and 50 μg/mL. Both of NPAL1 and NPAL2 particles showed similar cytotoxic effect on HCT-116 cancer line. As seen in Figure 4 that *C. protothecoides* microalgae has cytotoxic effect on cancer lines compared to empty capsules (None). As shown in the studies of Tannoury, M.Y. et al. (2017), Sanjeewa, K.K.A. et al. (2016), Atasever-Arslan, B. et al. (2016), Zhu, X. et al. (2012), Borges, O. et al. (2006), algae show cytotoxic effects against various types of cancer, and the nanoparticles increase this cytotoxic effect.[29-33]

The reason why algae show cytotoxic effects on cancer cells is explained in the literature. Microalgae contain various bioactive compounds, minerals, polysaccharides, PUFAs, and vitamins. Therefore, they are potential candidates for possible anticancer activity and as modulators of spesific intracellular signaling pathways, And the algae extracts trigger apoptotic-signaling pathways in cancer cell lines and decrease cell proliferation rate. Various studies have shown that extracts of some algae species lead to death by examining which

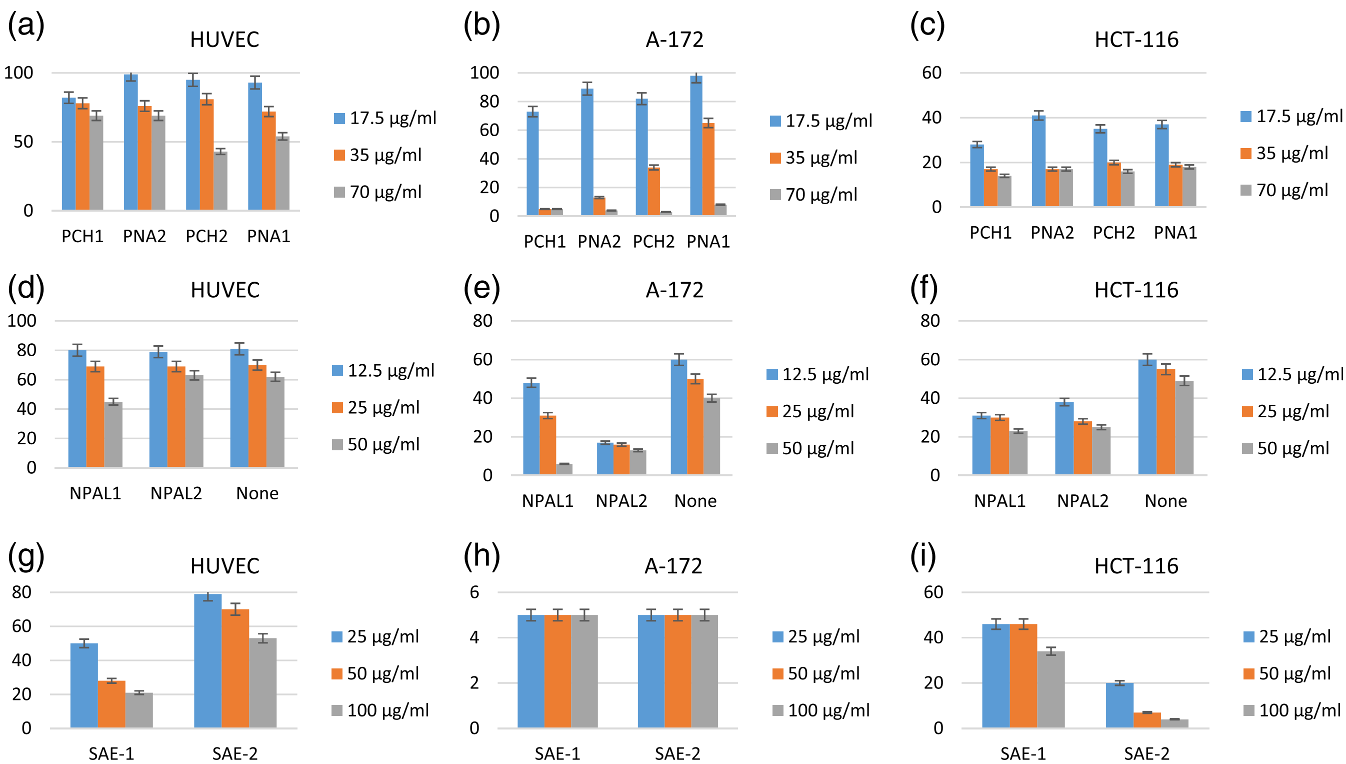

FIGURE 4 MTT test results (viability percentage): Citotoxic effect of electrosprayed nanoparticles on (a) HUVEC cell line, (b) A-172 cell line, (c) HCT-116 cell line; citotoxic effect of microparticles produced via microemulsion method on (d) HUVEC cell line, (e) A-172 cell line, (f) HCT-116 cell line (none: particles without microalgal extract); citotoxic effect of microalgae extracts (SAE1: *Chlorella protothecoides extract*, SAE2: *Nannochloropsis oculata extract*) on (g) HUVEC cell line, (h) A-172 cell line, (i) HCT-116 cell line

signaling pathways in various cancer cells.[31,34] Goh et al. demonstrated that the mode of cell death induced by the extract was mainly apoptosis as confirmed by the AO/PI staining, annexin V-FITC, and TUNEL assay.[35] Van Beelen et al. tested EPA and DHA for their effect on mitochondrial activity and cell proliferation of human colon adenocarcinoma Caco-2 cells. It was reported that, algal oils gave a modest dose-dependent decrease in cell proliferation.[34] Also, fatty acids that contained in *C. protothecoides*; palmitic (16:0), oleic (18:1), linoleic (18:2), eicosatrienoic acid (20:3), and eicosapentaenoic acid (20:5) show high antioxidant activity.[36] Each algae extract contains a wide range of essential oils. Although their effect on cancer cells is known as apoptosis, it is known that antagonistic or synergistic effects occurring between essential oils may alter cytotoxicity. Specifically, the effects of these extracts on intracellular signal nodes and apoptotic pathways need to be examined.

4 | CONCLUSIONS

Two different microalgal extracts were encapsulated, cytotoxic effects of extracts and encapsulated extracts produced by two different methods (electrospraying and microemulsion techniques) were compared. Both microalgal extracts and encapsulated microalgal extracts had cytotoxic effects on two different cancer cell lines and no effect on normal cells. The release test performed using the Sudan Red G, hydrophobic dye, showed controlled release of the compounds within the particles in the first 24 hr. The maximum loading efficiency of the particles was found to be 77%. The cytotoxicity of these nano–microparticles and two algae extracts on A-172, HCT-116 cancer cells, and HUVEC cells were determined by MTT test. It was observed that particles and extracts did not have cytotoxic effects on healthy cells but only cytotoxic effects on cancerous cells. This cytotoxicity study has shown that microalgal extracts have cytotoxic effects on cancer cells and do not lose their cytotoxic effects after encapsulation. Future experiments with encapsulated particles of different algal species and with different concentrations of their extracts are required to obtain advance scientific knowledge.

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CONFLICT OF INTEREST

The authors confirm that there is no conflict of interest.

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