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Extraction Yield and Biological Activity of Phycobiliproteins from Porphyridium Purpureum Using Atmospheric Cold Plasma Discharge and Jet Systems

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Extraction yield and biological activity of phycobiliproteins from *Porphyridium purpureum* using atmospheric cold plasma discharge and jet systems

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ABSTRACT

Phycobiliproteins (PBPs) extracted from *Porphyridium purpureum* (*P.p*) have bioactive properties and are widely used as ingredients in nutraceutical and food applications. This study investigated the use of two cold plasma systems, namely cold plasma discharge system (CPDS) and cold plasma jet system (CPJS), for the aqueous extraction of PBPs from *P. p*. Three types of PBPs, namely phycoerythrin (PE), phycocyanin (PC) and allophycocyanin (APC) were identified in the crude extracts obtained. The highest PBPs extraction yield (11.31 ± 1.02 mg/g DW *P. p)* was obtained from CPDS treated samples at 25 kV using N_2 for 9 min. CPDS treatments were also shown to be more effective than CPJS treatments in increasing antioxidant activities of the PBPs crude extracts obtained. PBPs crude extracts obtained using CPDS (25 kV; 6 min; N_2) had the highest DPPH (69.44 ± 0.10%) and FRAP (207.34 ± 12.96 μmol/L) antioxidant activities observed. PBPs obtained from samples treated with CPDS (25 kV; 9 min; N_2) exhibited the highest cytotoxicity potential in Caco-2 human colorectal adenocarcinoma cell lines. This study demonstrates that cold plasma treatments increase the extraction yield of PBPs obtained from *P. p* and also enhance antioxidant and cytotoxic properties of the PBP crude extracts. However, an increase in treatment time beyond 6 min for both plasma systems was shown to reduce the level of antioxidant activity in PBPs.

1. Introduction

Microalgae contain an abundance of bioactive compounds including proteins, lipids, carbohydrates, pigments, and secondary metabolites, which are used in many food formulations, cosmetics, nutraceutical, and pharmaceutical applications (Catalani et al., 2016; Salami, Kordi, Bolouri, Delangiz, & Asgari Lajayer, 2021). *Porphyridium purpureum (P. p)* is a red microalgae species, widely known for its red colour due to the presence of phycoerythrin, a red protein-pigment complex from the family of light-harvesting phycobiliproteins (PBPs) (Cecere & Perrone, 1994; Sun, Wang, Gong, & Chen, 2004). PBPs are hydrophilic compounds comprising open-chain tetrapyrrole prosthetic groups, which are covalently attached to cysteine residues of protein by thioether bonds. PBPs are classified into three basic categories based on their colour, namely phycoerythrins (PEs), phycocyanins (PCs), and allophycocyanins (APCs) which can be detected at 480–580 nm, 600–640 nm and 620–669 nm respectively. These pigmented proteins possess high biological activities including antioxidant and cytotoxic properties (Blagosklonny, 2008). In addition, PBPs are used in a wide range of cosmetic, food and nutraceutical applications as a natural colorant

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(Stadnichuk & Tropin, 2017; Viskari & Colyer, 2003; C. Zhao et al., 2017).

Convention extraction of PBPs involves soaking, shaking, and stirring of microalgae biomass in an aqueous phase overnight, followed by centrifugation and recovery using techniques such as ultrafiltration, precipitation, and chromatography (Mittal, Sharma, & Raghavarao, 2019). However, factors such as high viscosity and anionic cell walls limit the extraction of PBP molecules. Mechanical grinding and osmotic shock are conventional methods that are commonly used to improve the efficiency of BPB extraction. However they require long processing times (Ganeva, Galutzov, & Teissié, 2003) and do not effectively disrupt the microalgae biomass resulting in low extraction yields. Consequently, much recent research has focused on exploring extraction strategies that can achieve greater cell disruption of microalgae biomass, leading to higher extraction rates and improved biological qualities (Mittal et al., 2019).

The application of innovative extraction strategies including the use of ultrasound (Ardiles et al., 2020; Sarkarat, Mohamadnia, & Tavakoli, 2022), microwave (Huschek, Rawel, Schweikert, Henkel-Oberländer, & Sagu, 2022), pulsed electric field (Martínez, Delso, Álvarez, & Raso, 2019) and high-pressure processing (Bueno et al., 2020) has been shown to improve the extraction yield of PBPs from microalgae cells in recent research studies. However, it is important to consider the limitations associated with these techniques including scalability challenges when implementing these techniques for large-scale extraction processes. Additionally, microwave extraction may lead to potential sample loss due to evaporation, and there is a risk of sample heating and thermal degradation of heat-sensitive compounds (Huschek et al., 2022). Furthermore, optimizing pressure levels and extraction time is necessary to avoid excessive extraction of undesirable compounds when employing high-pressure processing (Bueno et al., 2020).

Therefore, considering these limitations, an alternative technology, namely cold plasma, was investigated in this study to assess its capabilities for the extraction of PBPs. Cold plasma offers several advantages over other techniques, with minimal drawbacks, making it a promising option for efficient PBPs extraction.

Plasma is termed as cold plasma when it is created by applying a high voltage or electromagnetic fields to a gas or gas mixture under atmospheric pressure below 40 °C. This energy input ionizes the gas, creating a plasma state. Plasma contains a variety of reactive species, including ions, electrons, radicals, and excited molecules. These generated reactive species in the plasma can interact with the surface of the target material or the biomolecules of interest. They can initiate chemical reactions, break molecular bonds, and induce physical or chemical modifications on the surface or within the bioactive compounds (Hoffmann, Berganza, & Zhang, 2013; Pankaj & Keener, 2017).

Generally, cold plasma is used for the modification and functionalization of polymers due to its ability to modulate surface physicochemical properties. It can also disrupt the cell walls of microorganisms or plant cells, releasing the intracellular components. This disruption can enhance the accessibility of bioactive compounds for extraction, improve extraction yields, and facilitate the release of compounds trapped within cell structures (Fatyeyeva et al., 2014; Van Deynse, Morent, & De Geyter, 2016, pp. 506–516). Due to the non-thermal and energy efficient characteristics of cold plasma, it is increasingly employed in the field of food processing. It has been studied for the extraction of bioactive compounds including phenolic compounds from grape and tomato pomace (Bao, Reddivari, & Huang, 2020a; 2020b), anthocyanins from pericarp of colour rice (Poomanee, Wattananapakasem, Panjan, & Kiattisin, 2021) and taxanes from Japanese yew (Z. Zhao et al., 2023). Bursać Kovačević et al. (Kovačević et al., 2016) reported that the total anthocyanin content in pomegranate juice was enhanced by 21–35% after plasma treatment, and Won et al. (Won, Lee, & Min, 2017) demonstrated that plasma treatments improved the antioxidant activity of mandarin peel.

A number of different cold plasma treatment systems have been developed including dielectric barrier discharge, atmospheric pressure plasma jet, microwave plasma, inductively coupled plasma, and surface dielectric barrier discharge systems. The selection of a specific plasma system configuration depends on the application requirements, desired plasma characteristics, and the nature of the target material or surface to be treated.

Several plasma systems configurations have been reported for the extraction of bioactive compounds using cold plasma. These include arc discharge systems (Nutrizio, Maltar-Strmečki, Chemat, Duić, & Jambrak, 2021), dielectric systems (Jin, Zhou, Zhou, Ouyang, & Wu, 2021) and jet systems (Pogorzelska-Nowicka et al., 2021). However, the extraction of bioactive compounds using cold plasma techniques is complex. The reactive nature of plasma can lead to unwanted reactions or degradation of compounds, resulting in the loss of target bioactive compounds or the formation of undesired by-products. The effectiveness and degradation of compounds during cold plasma extraction depend on various parameters, including gas composition, discharge power, treatment time, and the distance between the plasma source and the sample (Jin et al., 2021). Further studies are required to determine the optimal combination of these parameters for maximum extraction yield of bioactive compounds.

There are no reported studies on the utilization of cold plasma treatments to improve the extraction of PBPs from *P. p.* The present study aims to address this research gap by investigating the extraction of PBPs from *P. p* samples using atmospheric cold plasma discharge and jet systems. Additionally, the antioxidant activity and cytotoxic effects on Caco-2 human colorectal adenocarcinoma cell lines were evaluated for the crude extracts of PBPs obtained.

2. Materials and methods

2.1. Microalgae and culture conditions

Porphyridium purpureum (P.p) was cultured at Swansea University, Wales under controlled environmental conditions over several weeks using a range of working volumes from 250 mL up to 80 L. Cultured stains in 80 L bags were subsequently transferred to an 800 L photobioreactor (PBR) located in an outdoor greenhouse environment. The temperature of the greenhouse was maintained in a range of 19–21 °C and pH was kept constant at 7.5. Vaporized CO_2 was injected into the PBR at a rate of 0.6 L/min and turbulence was created using a centrifugal pump. Illumination was provided using natural light with 18:6 cycles of light: dark. Samples of 1000 mL of culture were taken from the PBR and centrifuged (Beckman Coulter Centrifuge, Avanti J-20XP, JLA rotor) for 10 min at 4000 rpm, followed by separation of supernatant and pellets to harvest the red algae cells. The recovered pellets were freeze-dried using a freeze drier (BUCHI Lyovapor™ L-300). The freeze-dried *P. p* powder was vacuum-sealed and stored at −20 °C prior to extraction studies.

2.2. Extraction of phycobiliproteins from Porphyridium purpureum

Freeze-dried red microalgae samples were rehydrated in distilled water at a dilution ratio of 1:20 *w/v* for recovery of phycobiliproteins (PBPs). The resultant solution was incubated at 22 °C for 30 min at 160 rpm using a shaker (Thermo Fisher Scientific MAXQ6000, Thermo Fisher Scientific, Life Technology Ltd., London, UK). The incubated samples were then subjected to two cold plasma and control treatments as illustrated in Fig. 1. All extraction treatments were carried out in duplicate.

Two cold plasma systems were investigated for extraction of PBP, namely a cold plasma discharge system (CPDS) and a cold plasma jet system (CPJS). Based on preliminary experiments using CPDS and CPJS, optimized experimental parameters of frequency, voltage, pulse width, time, supply gas, and mass to solvent ratio were selected for sample treatments.

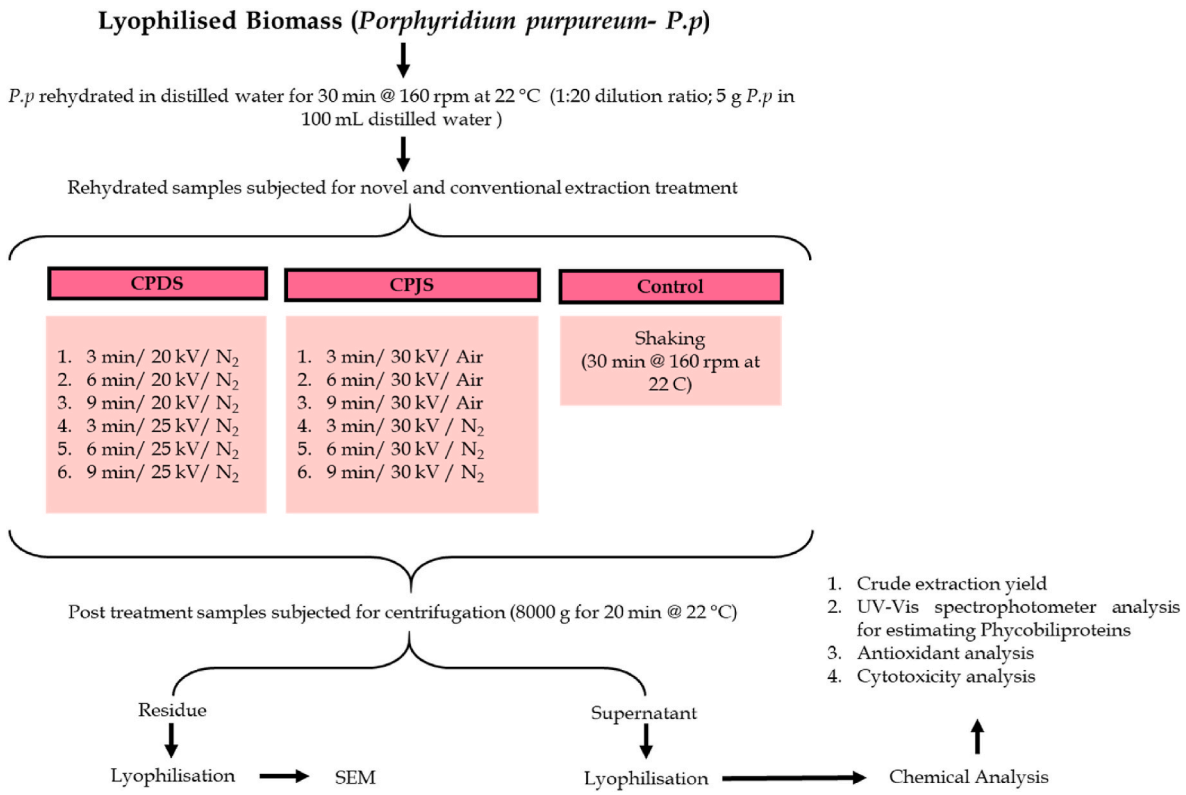

Fig. 1. Schematic workflow of the extraction protocols employed to obtain phycobiliproteins from *Porphyridium purpureum (P.p)* (CPDS- Cold Plasma Discharge System; CPJS-Cold Plasma Jet System).

The CPDS system (Model 'IMP-SSPG-1200', Impel Group, Croatia) shown in Fig. 2a had a maximum adjustable current of 30 mA and maximum voltage of 25 kV. The rehydrated mixture of algae biomass and distilled water was transferred for plasma treatment to a 100 mL beaker-shaped reactor (Fig. 2a). A needle was inserted at the bottom of the reactor to allow N_2 gas to pass through at a flow rate of 5 L/min during treatment. Experimental parameters were fixed as follows, frequency of 100 Hz, distance between electrodes was 15 mm, voltage of 20 kV and 25 kV, and treatment times of 3, 6 and 9 min. A total of six extracts were prepared in duplicate and all experiments were carried out at room temperature.

The CPJS system (National Centre for Plasma Science and Technology, Dublin City University, Ireland) shown in Fig. 2b had a variable high voltage power supply unit which was adjusted to 30 kV at 20 kHz, and was operated using both N_2 and air at a flow rate of 11 L/min. Treatment times of 3, 6 and 9 min were investigated for both gases. Algae biomass samples (50 mL) rehydrated with distilled water were positioned below the plasma probe in sterile glass containers. The diameter of the plasma jet was 30 mm and the distance between the jet and the sample during treatment was set at 5 cm. A total of six extracts were prepared in duplicate and all experiments were carried out at room temperature.

A control extraction treatment was also carried out based on a method previously described by Noore et al. (Noore et al., 2022) with minor modifications. Rehydrated algae biomass samples in distilled water were subjected to shaking at 160 rpm for 30 min at 22 °C. Duplicate samples were prepared for all treatments.

All treated samples were centrifuged at 10,000 g for 20 min at 4 °C to separate the supernatant and pellets. Supernatant samples were analysed using ultraviolet–visible spectroscopy to quantify the level of extracted PBPs in crude aqueous extracts. Both supernatant and pellets were freeze-dried (FD 80 model, Cuddon Engineering, Blenheim Marlborough, New Zealand) and stored at −20 °C prior to further analyses.

2.3. UV–Vis spectroscopy of aqueous extracts

Absorption of PBPs in aqueous extracts of *P. p* was measured using an ultraviolet–visible (UV–Vis) spectrophotometer (Epoch™ 2, Biotek, VT, USA) over a wavelength range of 250–700 nm. Aqueous samples of 1.5 mL were used for spectral measurement to quantify the concentration of PEs, PCs, and APCs in the crude extracts using Equations (1)–(3) (Dumay & Morançais, 2016, pp. 275–318; Kursar, van der Meer, & Alberte, 1983) below. All spectroscopic measurements were carried out in triplicate.

$$PE\ (\text{mg}/\text{mL}) = 155.8 \times A_{498nm} - 40 \times A_{614nm} - 10.5 \times A_{651nm} \quad (1)$$

$$PC\ (\text{mg}/\text{mL}) = 151.1 \times A_{614nm} - 99.1 \times A_{651nm} \quad (2)$$

$$APC\ (\text{mg}/\text{mL}) = 181.3 \times A_{651nm} - 22.3 \times A_{614nm} \quad (3)$$

2.4. Extraction yield

Crude aqueous soluble PBP extract yields were calculated as outlined by Park et al. (Park, Kim, Lee, Lim, & Hwang, 2019):

$$\text{Yield (PBPs mg / g of DW } P.p) = (W_1 \times W_2/W_0) \quad (4)$$

Where,

W_0 is the initial mass of freeze-dried *P. p* (g, DW) sample treated,
W_1 is the mass of PBPs in aqueous soluble extracts based on UV–Vis spectroscopic analysis (PBPs mg/mL of aqueous extract of *P. p*).
W_2 is the total volume (mL) of the aqueous extracts of PBPs from *P. p* post treatment.

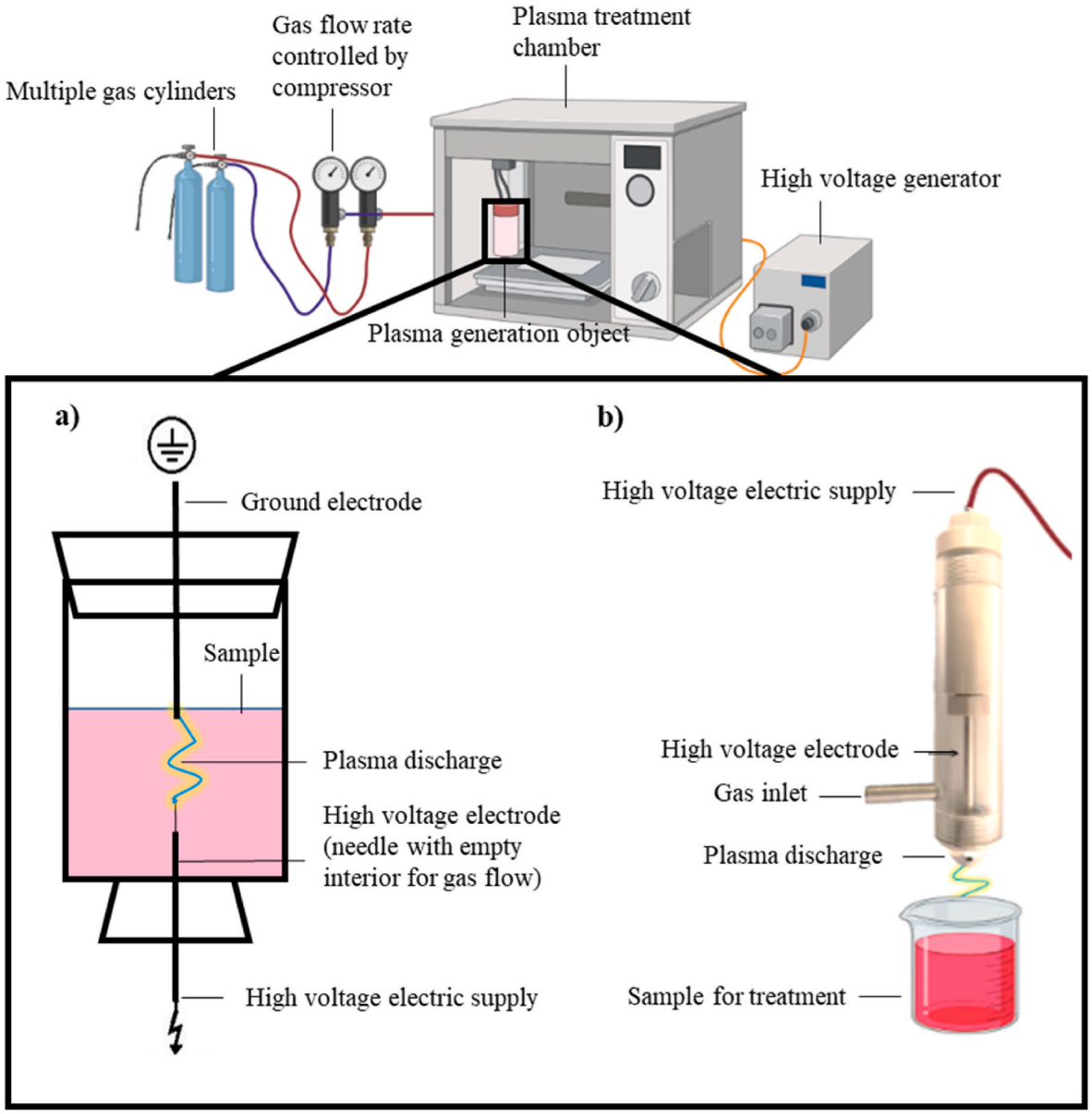

Fig. 2. Schematic set-up of atmospheric a) cold plasma discharge system (CPDS); b) cold plasma jet system (CPJS).

2.5. Antioxidant activities

2.5.1. DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) assay

DPPH activity was measured according to the method described by Madhubalaji et al. (Madhubalaji, Mudaliar, Chauhan, & Sarada, 2021). Briefly, freeze-dried extracts of *P. p* samples were diluted to 1 mg/mL in 0.1 M citrate phosphate buffer containing 0.3% Triton X-100. Further, 10 μL of a 2 mM methanolic DPPH solution was added to each well in a UV plate, and incubated at 22 °C in the dark for 30 min to allow the reaction to take place. Post incubation reaction, the absorbance at 517 nm of the UV plate was measured using a Varioskan Lux multi-plate reader (Thermo Scientific). The scavenging activity (%) was calculated using Equation (5). All analysis was carried out in duplicate with three independent absorbance readings per sample.

$$\text{DPPH Radical scavenging activity } (\%) = \frac{y_0 - y_1}{y_0} \times 100 \quad (5)$$

Where, y_0 is absorbance of blank and y_1 is absorbance of sample.

2.5.2. Ferric reducing antioxidant power assay (FRAP)

FRAP activity was measured as outlined by Owusu-Apenten et al. (Spiegel et al., 2020). Briefly, freeze-dried extracts of *P. p* were diluted to 1 mg/mL concentration in distilled water in triplicate. Further, Trolox at concentrations (50–500 μmol/L) were prepared to create a standard curve. Working reagents of FRAP were also prepared using 300 mM sodium acetate buffer at pH 3.6; 20 mM Ferric Chloride Hexahydrate; 10 mM Ferric 2,4,6-Tripyridyl-s-Triazine (TPTZ) in 40 mM HCl in a 10:1:1 ratio respectively, followed by incubation for 5 min at 37 °C. Test samples (50 μL Samples + 100 μl FRAP reagent) and blank samples (50 μL H_2O + 100 μL FRAP reagent) were prepared in a UV plate and absorbance at 593 nm was measured using a Varioskan Lux multi-plate reader (Thermo Scientific) post incubation for 30 min at 37 °C in dark. All analysis was carried out in duplicate with three independent absorbance readings per sample.

2.6. In-vitro cytotoxicity assay on human colorectal adenocarcinoma cells

2.6.1. Cell culture

Human colorectal adenocarcinoma (Caco-2), (ECACC 86010202)

cells were obtained from an ATCC European distributor (LGC Standards, UK). The absence of mycoplasma was checked using a MycoAlert PLUS Mycoplasma detection kit (Lonza, UK). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) - high glucose (Sigma, Ireland) supplemented with 10% fetal bovine serum (FBS) (Sigma, Ireland) and 1% penicillin-streptomycin solution (Sigma, Ireland) in TC flask T 75, standard for adherent cells (Sarstedt, Ireland). Cells were maintained in a humidified incubator containing 5% CO_2 atmosphere at 37 °C. Cells were routinely sub cultured when 80% confluence was reached using 0.25% w/v Trypsin-EDTA solution (Sigma). Cells were then seeded at a density of 2.5×10^3 cells/well (6 days treatment) (100 μL culture medium per well), in triplicate in 96-well plates (Sarstedt, Ireland). Plates were incubated overnight at 37 °C with 5% CO_2 to allow proper adherence. Existing media was removed from each well and cells were treated with 200 μg/mL of each PBPs fractions, serially diluted from 200 μg/mL to 1.5625 μg/mL 20% dimethyl sulfoxide (DMSO) was used as a positive control while culture medium was used as a negative control.

2.6.2. Cell viability assay

Cell viability was analysed using Alamar Blue™ cell viability reagent (Thermo Fisher Scientific) according to the method of Wanigasekara et al., (Wanigasekara, Barcia, Cullen, Tiwari, & Curtin, 2022). After treatment and subsequent incubation at 37 °C in 5% CO_2, the cells were rinsed once with phosphate buffered saline (Sigma), and incubated for 3 h at 37 °C with a 10% Alamar Blue™ solution and 90% DMEM-high glucose solution. Fluorescence was measured using an excitation wavelength of 530 nm and an emission wavelength of 590 nm with a Varioskan Lux multi-plate reader (Thermo Scientific). All experiments consisted of three independent tests and three replicates per sample.

2.6.3. Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) (Model: Regulus 8230, Hitachi Ltd., Tokyo, Japan) was carried out using the protocol of Murtey and Ramasamy (Murtey & Ramasamy, 2016, pp. 161–185) on pellets which were freeze-dried and stored at −20 °C. Sample preparation for SEM involved the following steps:

(a) Fixation of protein: briefly, freeze dried cold plasma treated microalgae cell were subjected to fixation in 2.5% glutaraldehyde using 0.1 M phosphate buffer (pH 7.4) at 4 °C for 3h. After 3h, samples were rinsed three times in phosphate buffer followed by deionized water for 15 min each;
(b) Post-fixation of lipids: prefixed samples were treated with 0.1% osmium tetroxide using 0.1 M phosphate buffer (pH 7.4) for 1h and then rinsed three times with phosphate buffer followed by deionized water for 15 min each;
(c) Dehydration: fixed samples were dehydrated in series of alcohol with ascending concentration (i.e., 30, 50, 70, 80, 90 and 100% ethanol) for 15 min per concentration;
(d) Critical drying using nitrogen gas: post dehydration, samples were completed dried using nitrogen gas to remove left over moisture from the samples.

Samples were placed on double-sided carbon tape, mounted on an aluminium stub, and placed in a vacuum chamber for gold coating prior to scanning electron microscopy with a 50.0 μm measuring scale at 1000x magnification level.

2.7. Statistical analysis

All the experiments were carried out in duplicate unless otherwise stated. Prism (version 9.1.0, GraphPad Prism Software, Dotmatrics, California, USA) was used to carry out curve fitting and statistical analysis on extraction yield, and antioxidant activities including DPPH, and FRAP. Dose-response curves were created using nonlinear curve fitting. Two-way ANOVA analyses were performed using Tukey's multiple comparison tests.

3. Results and discussion

3.1. Phycoboliprotein extraction yield

Extraction yields of phycobiliproteins (PBPs) recovered from *Porphyridium purpureum (P.p)* using two cold plasma systems, namely cold plasma discharge system (CPDS) and cold plasma jet system (CPJS) were measured.

3.1.1. Cold plasma discharge system (CPDS)

The highest extraction yield (8.42 ± 2.20 mg/g DW *P. p*) of PBPs at 20 kV using CPDS was quantified in samples treated for 9 min compared to yields at 3 min, 6 min and control of 4.75 ± 0.60, 6.58 ± 0.45 and 3.90 ± 0.22 mg/g DW *P. p* respectively (Fig. 3a). The extraction yields of PBPs increased at 25 kV to 4.81 ± 0.86, 6.40 ± 0.92 and 11.33 ± 2.77 mg/g DW *P. p.* for 3, 6 and 9 min treatment times respectively (Fig. 3b).

Three types of PBPs were quantified in the crude extracts obtained using UV–Vis spectroscopy namely phycoerythrins (PEs), phycocyanins (PCs) and allophycocyanins (APCs). PEs extraction was significantly ($p < 0.01$) higher (3.79 ± 0.86 mg/g DW *P. p)* for samples treated for 9 min at 20 kV using N_2 compared to control (1.61 ± 0.08 mg/g DW *P. p*) (Fig. 3a). At the higher voltage of 25 kV (Fig. 3b), increased PEs yield were observed for longer treatment times. For a treatment time of 9 min, a greater than 2 fold increase in PEs yield to 4.82 ± 1.25 mg/g DW *P. p* was observed compared to control. Due to longer treatment times and higher voltage, more reactive species were generated along with a stronger plasma discharge (Režek Jambrak et al., 2021) which reacted with microalgae cells leading to enhanced extraction yields of PBPs. However, a longer treatment time and the presence of higher levels of reactive species resulted in a decrease in the antioxidant activity of PBPs after 6 min (Fig. 5). These results suggest that increasing the treatment time beyond 6 min creates high levels of reactive species which leads to higher oxidation of PBP antioxidant compounds." was added in the revised manuscript.

Previously cold plasma treatment significantly ($p < 0.05$) improved the extraction yield of polyphenols such as vanillin, 4-hydroxybenzaldehyde, *p*-coumaric, ferulic acid, sinapic acid, and chlorogenic acid from rice and corn bran by three fold (Mehta, Yadav, Chaturvedi, Shivhare, & Yadav, 2022). In another study (Rezaei, Ghobadian, Ebadi, & Ghomi, 2020), plasma treatment was demonstrated to enhance the level of oil extraction from flax seed. Samples treated with plasma at 18 kV for 16 min, showed significantly higher extraction yield (31.5%) compared to untreated samples (24.5%). Additionally, the level of protein extracted was significantly ($p < 0.05$) higher (39.5%) in cold plasma treated samples compared to untreated samples (26.4%) (Rezaei et al., 2020).

3.1.2. Cold plasma jet system (CPJS)

For samples treated with CPJS using N_2, a yield of 5.03 ± 0.68 mg/g DW *P. p* of PBPs was obtained at 30 kV and 9 min treatment time, compared to yields of 3.41 ± 0.31, 3.65 ± 0.19 and 3.90 ± 0.22 mg/g DW *P. p* for 3 min, 6 min, and control treatments respectively (Fig. 4a). Reduced PBP yields of 2.29 ± 0.64; 2.71 ± 0.37; 4.19 ± 1.87 mg/g DW *P. p.* were observed for samples treated with CPJS using air for treatment times of 3, 6 and 9 min respectively (Fig. 4b). The working gas used and the treatment duration employed for CPJS treatments influences the reactive nitrogen species generated which react with microalgae cells and influence the extraction yields obtained (Charoux et al., 2020).

The extraction yields of PEs, PCs and APCs obtained in the crude extracts are shown in Fig. 4. The highest extraction yield of PEs (2.12 ± 0.30 mg/g of DW *P. p*) was observed in samples treated for 9 min at 30 kV using N_2, compared to yields of 1.43 ± 0.13, 1.52 ± 0.07 and 1.61 ± 0.08 mg/g DW *P. p* for treatments of 3 min, 6 min and control respectively. However, no significant differences were identified in PC and APC extraction yields between all CPJS treatments and control (Fig. 4a).

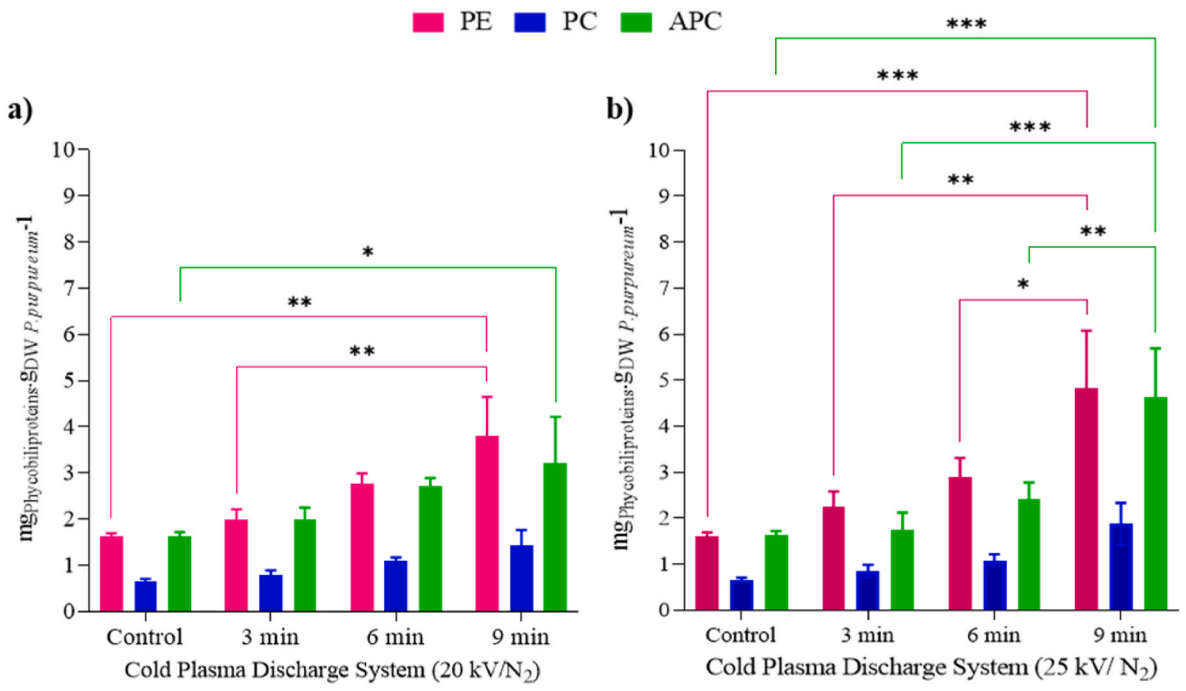

Fig. 3. Extraction yield (PBPs mg/g DW*P. p*) of phycobiliproteins (PBPs) from *Porphyridium purpureum* (*P.p*) treated with cold plasma discharge system; (a) 20 kV/N_2; b) 25 kV/N_2 for treatment times of 3, 6, and 9 min and control. Results are expressed as mean ± standard deviation; *p < 0.05; **p < 0.01; ***p < 0.001.

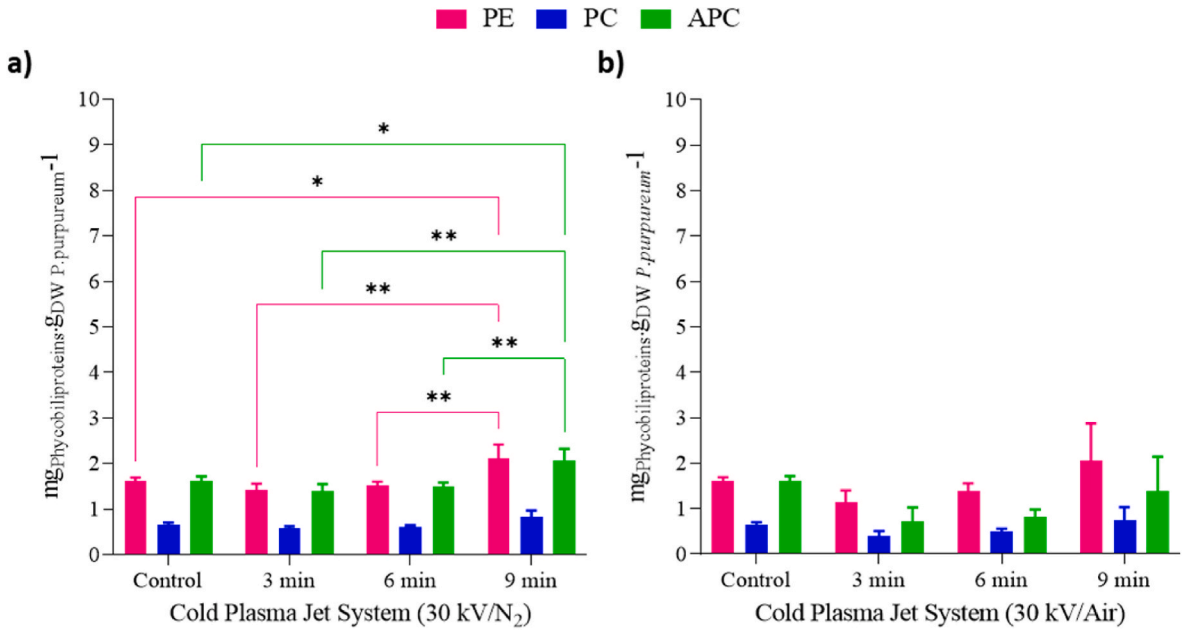

Fig. 4. Extraction yield (PBPs mg/g DW *P. p*) of phycobiliprotein (PBPs) from *Porphyridium purpureum (P.p)* treated with cold plasma jet system; a) 30 kV/N_2; b) 30 kV/Air for treatment times of 3, 6, and 9 min and control. Results are expressed as mean ± standard deviation; *p < 0.05; **p < 0.01.

Also no significant differences in PE, PC and APC yields were observed between CPJS treatments using air for all treatment times compared to control (Fig. 4b).

A previous study on plasma treatments for preservation of bioactive compounds in Spirulina powder reported that cold plasma discharge treatments using N_2 resulted in higher extraction yields of chlorophyll *a*, carotenoid and PBPs compared to air. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are formed during air plasma treatment, while RNS are mostly formed during nitrogen plasma treatments. The observed changes in extraction yields are associated with the presence of reactive oxygen species (ROS) or a combination of reactive nitrogen species (RNS) (Beyrer, Pina-Perez, Martinet, & Andlauer, 2020). Zhang et al. (Zhang et al., 2019), investigated plasma treatment of red chili peppers and reported that the extraction rate of pigment contents was improved after plasma treatment for exposure times up to 30 s. However, longer treatment times were shown to cause pigment loss and reduced yield, leading to the degradation of bioactive compounds.

Overall, the observed variation in extraction yield between CPDS and CPJS can be attributed to the reactive species formed and plasma chemistry. The direct exposure of the sample to the corona discharge in

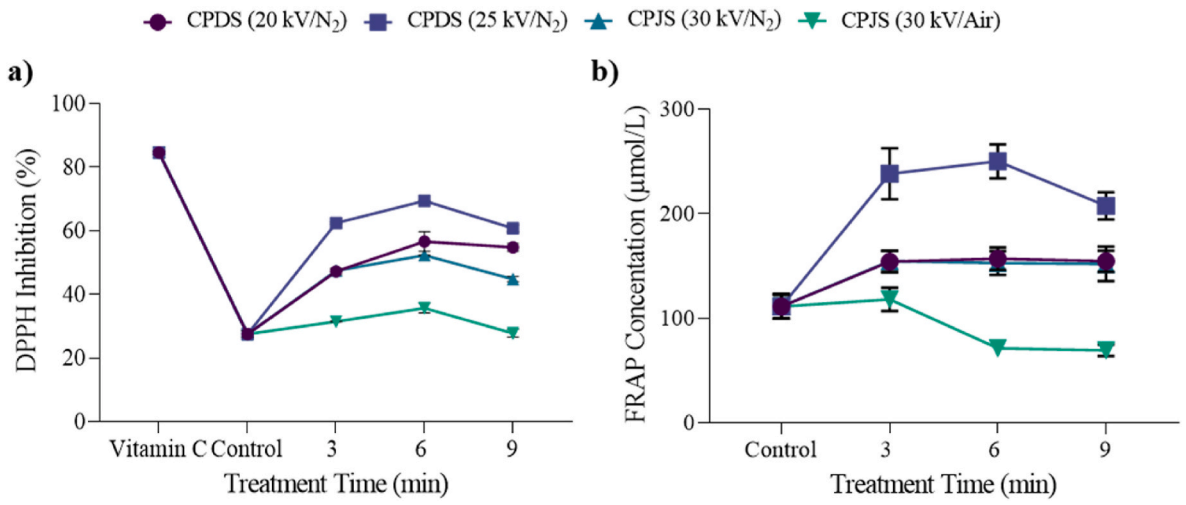

Fig. 5. Antioxidant activities of crude phycobiliprotein (PBP) extracts obtained from *Porphyridium purpureum* using cold plasma discharge system (CPDS); cold plasma jet system (CPJS) at 20 and 25 kV for treatment times of 3, 6, and 9 min using N_2 and air. a) 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay; b) Ferric Reducing Antioxidant Power Assay (FRAP). Results are expressed as mean ± standard deviation and statistically analysed using two-way ANOVA with Tukey's post-test.

CPDS allows for more effective interaction and thus higher extraction yield, while the spatial gap in CPJS may hinder the efficiency of the extraction process.

3.2. Antioxidant activities

The antioxidant activities of PBP extracts measured using DPPH and FRAP assays are shown in Fig. 5. The highest level of DPPH activity

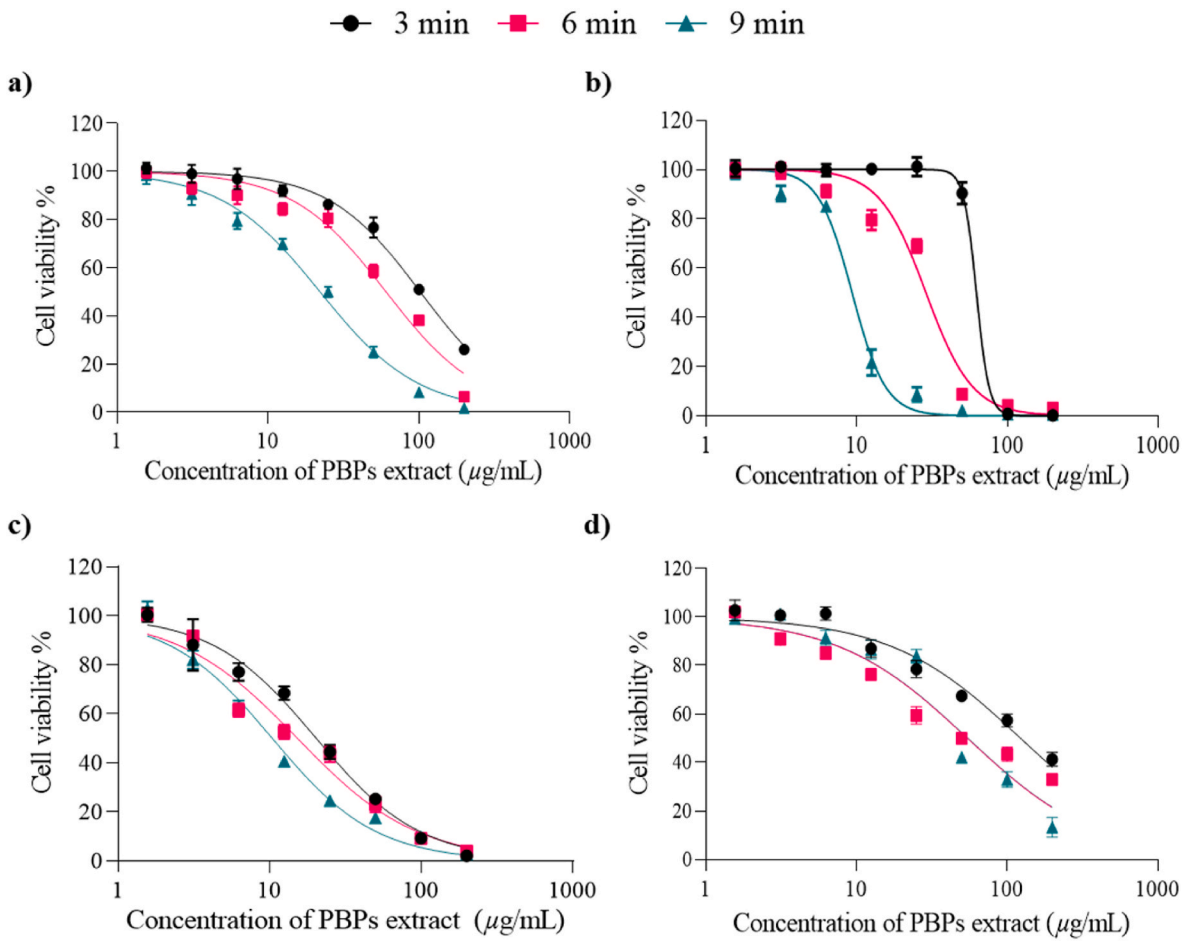

Fig. 6. Cytotoxicity effect of crude phycobiliprotein (PBP) extracts obtained from *Porphyridium purpureum* using cold plasma discharge system (CPDS) and cold plasma jet system (CPJS) for treatment times of 3, 6, and 9 min, on Caco-2 human colorectal adenocarcinoma cell lines: a) CPDS (20 kV/N_2); b) CPDS (25 kV/N_2); c) CPJS (30 kV/N_2); d) CPJS (30 kV/Air). Two-way ANOVA demonstrated that there is a significant difference in viability between the highest and lowest concentration ($p < 0.0001$).

(69.44 ± 0.10%) was measured in CPDS treated samples at 25 kV using N_2 for 6 min, which was significantly ($p < 0.05$) higher than the control (27.53 ± 1.05%) (Fig. 5a). The increased antioxidant activity observed is due to the effects of the reactive species formed during treatment on the extracts. Further increasing the treatment time to 9 min, resulted in a 9% decrease in DPPH activity, indicating that longer plasma treatment durations degrade the antioxidants in the extracts.

Comparatively, samples treated with CPJS using air had significantly ($p < 0.05$) lower antioxidant activities of 31.48 ± 0.12, 35.75 ± 1.56, and 27.75 ± 1.22% for treatment times of 3, 6, and 9 min respectively. This is due to the higher oxidation caused by the reactive oxygen species formed during the plasma treatments using air compared to N_2.

Similar trends were observed for the FRAP assay. The highest antioxidant activity (207.34 ± 12.96 μmol/L) was measured in samples treated with CPDS at 25 kV using N_2 for 9 min, whereas samples treated with CPJS at 25 kV using air for 9 min and control had significantly lower activities of 69.13 ± 5.50 and 11.14 ± 11.58 μmol/L respectively.

Zhang et al. (Zhang et al., 2019) reported that longer plasma treatment times increased antioxidant activity of chili pepper. Another study (Mehta et al., 2022) on cold plasma assisted-extraction of phenolic compounds from rice and rice bran reported that antioxidant activity significantly ($p < 0.05$) increased from 64.96 ± 2.20 to 72.65 ± 1.75 DPPH % after cold plasma treatment at 60 kV for a treatment time of 30 min.

3.3. Cytotoxicity effects of phycobliprotein extracts on Caco-2 human colorectal adenocarcinoma cell lines

The cytotoxicity of PBP extracts in Caco-2 human colorectal adenocarcinoma cell lines was investigated. Half maximal inhibitory concentrations (IC_{50}) of 102.0 μg/mL, 60.56 μg/mL and 22.13 μg/mL were determined for crude PBP extracts obtained from samples treated using CPDS with N_2 gas at 20 kV for 3, 6, and 9 min treatment times respectively (Fig. 6a, Table 1). Lower IC_{50} values of 62.05 μg/mL, 28.70 μg/mL and 9.282 μg/mL were determined for crude PBP extracts obtained from samples treated using CPDS with N_2 gas at 25 kV for 3, 6, and 9 min treatment times respectively (Fig. 6b, Table 1).

Table 1
IC_{50} Values and ranges of crude PBPs extracts obtained from *P.p* using CPDS and CPJS in Caco-2.

Cold Plasma Techniques	Treatment Parameters		IC_{50} (μg/mL)	IC_{50} Range (μg/mL)	Figure
	Voltage/ Carrier gas	Time (min)			
CPDS	20 kV/N_2	3	102.00	97.86 to 106.20	6a
		6	60.56	56.47 to 64.94	6a
		9	22.13	21.09 to 23.21	6a
	25 kV/N_2	3	62.05	57.28 to 67.22	6b
		6	28.70	27.08 to 30.43	6b
		9	9.28	8.95 to 9.62	6b
CPJS	30 kV/N_2	3	21.25	14.39 to 31.39	6c
		6	17.48	12.67 to 24.13	6c
		9	10.90	7.78 to 15.27	6c
	30 kV/Air	3	122.80	113.70 to 132.90	6d
		6	54.50	48.82 to 60.89	6d
		9	54.91	49.22 to 61.31	6d

CPDS: Cold plasma discharge system; CPJS: Cold plasma jet system; IC_{50}: Half maximal inhibitory concentrations.

IC_{50} values of 21.25 μg/mL, 17.48 μg/mL and 10.90 μg/mL were determined for crude PBP extracts obtained from samples treated using CPJS with N_2 gas for 3, 6, and 9 min treatment times respectively (Fig. 6c, Table 1). Higher IC_{50} values of 668.67 μg/mL, 761.6 μg/mL and 357.6 μg/mL were determined for crude PBP extracts obtained from samples treated using CPJS with air for 3, 6, and 9 min treatment times respectively (Fig. 6d, Table 1). These preliminary results demonstrate the growth inhibitory potential of PBPs extracted from plasma treated samples in Caco-2 human colorectal adenocarcinoma cell lines. These results are in line with the extraction results. For CPDS treated samples, both the highest extraction of PBPs and the highest level of Caco-2 cell toxicity (IC_{50} value of 9.28 μg/mL) were observed on samples treated with N_2 gas at 25 kV for 9 min treatment time. Similarly, for CPJS treated samples, both the highest level of PBPs extraction and the highest level of Caco-2 cell toxicity (IC_{50} value of 10.90 μg/mL) were observed in samples treated with N_2 gas at 30 kV for 9 min treatment time.

The cytotoxicity potential of PBPs was previously reported for leukemic (HL60), colon-human (HCT116), glioblastoma-human (SF295) and prostate (PC3) cell lines (Viana Carlos et al., 2021). The highest cytotoxicity effects were demonstrated for leukemic (HL60) cells with an IC_{50} of 112.6 μg/mL. Pekkoh et al. (Pekkoh et al., 2023) investigated the cytotoxicity of PBPs in human colorectal carcinoma (Caco-2) cells and reported an IC_{50} of 4870 μg/mL which is higher than IC_{50} of 9.282 μg/mL determined in this study for a similar cell line (Caco-2).

3.4. Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) images were acquired from untreated and treated (CPDS and CPJS) *P. p* cells to observe the effects of plasma treatments on cell membranes (Fig. 7). The SEM images of untreated *P. p* cells in Fig. 7a are intact and have a single circular ellipsoid shape. The untreated cells have a defined thick cell wall layer which is similar to previously published SEM images of *P. p* cells with a diameter of 6–12 μm (Aizdaicher, Stonik, & Boroda, 2014). Cell wall disruption is evident in *P. p* cells treated with CPDS at 20 and 25 kV for 9 min treatment time using N_2 (Fig. 7b and c). A higher level of cell wall disruption occurred in CPDS samples treated at the higher voltage of 25 kV. Decreased cell wall disruption was evident in samples treated with CPJS at 30 kV for 9 min using N_2 and air (Fig. 7d and e) compared to CPDS treated samples. Cold plasma treatment induced cell membrane damage which facilitated the increased extraction of PBPs compared to untreated cells.

A previous study (Seol, Kim, Park, & Young Chang, 2017) reported that plasma treatments using helium induced rupturing of cell wall structure in tissues of plant epidermis. Other studies reported that cold plasma treatments also resulted in other surface morphological alterations such as roughness (Grzegorzewski, Rohn, Kroh, Geyer, & Schlüter, 2010), cracking (Huang, Wu, Wu, & Ting, 2019), and scorching (Kodama, Thawatchaipracha, & Sekiguchi, 2014) on the cell surface of different plant matrices. Xu el al (Xu, Garner, Tao, & Keener, 2017). reported that *S. enterica* cells treated with cold plasma at 90 kV using air resulted in an etching effect with irregular surface.

4. Conclusion

This study demonstrates the potential of cold plasma discharge system (CPDS) and cold plasma jet system (CPJS) treatments to enhance the extraction of phycobiliproteins (PBPs) from *Porphyridium purpureum (P. p)* compared to control. CPDS treatments were shown to be more effective than CPJS treatments in increasing extraction yields. Both cold plasma extraction treatments investigated also increased the antioxidant activities and the cytotoxicity effects of the PBP crude extracts obtained compared to control. Crude PBP extracts obtained after CPDS treatments at 25 kV using N_2 had higher extraction yields, antioxidant activities and cytotoxicity effects compared to extracts from CPDS treated samples at

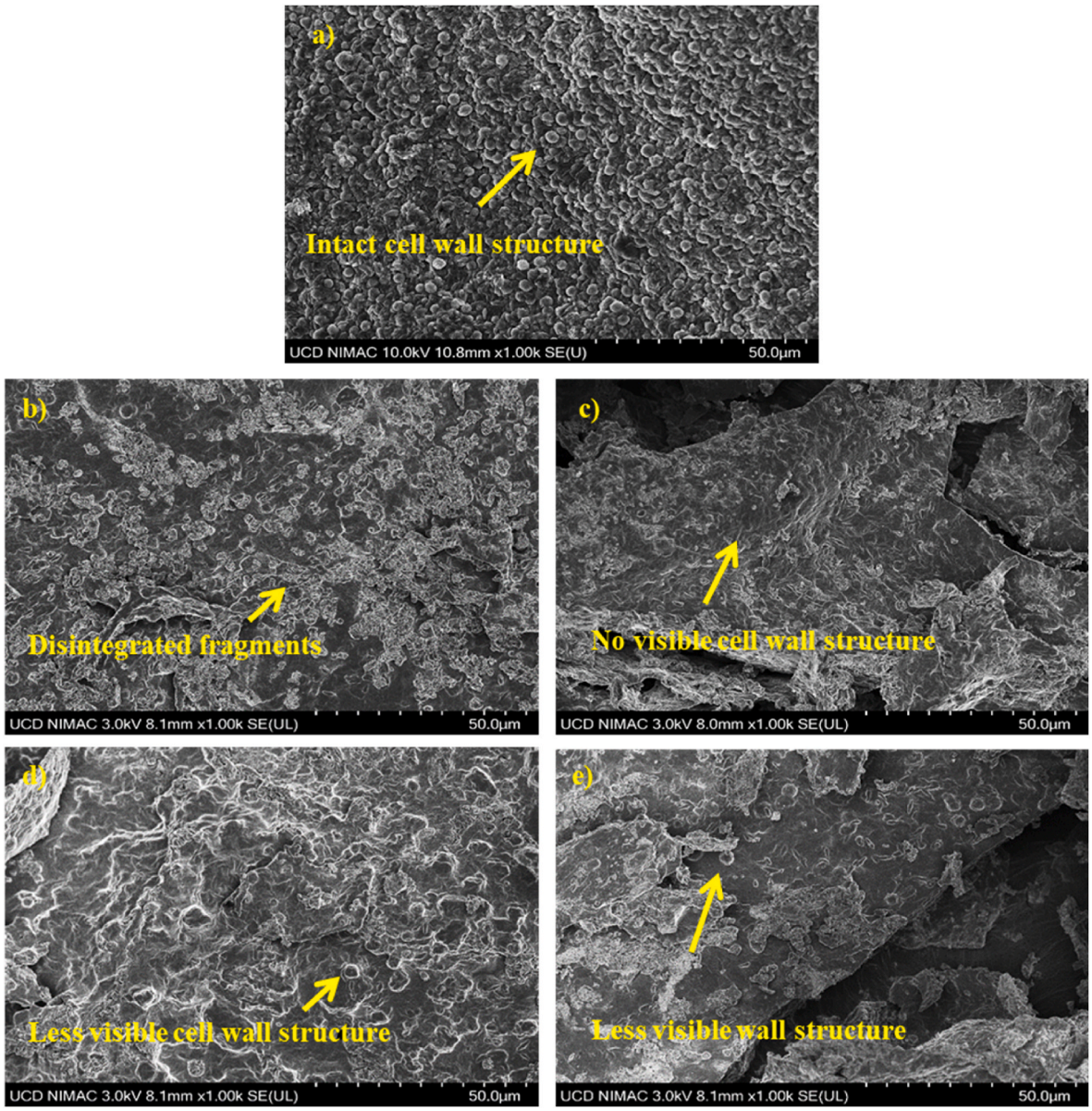

Fig. 7. Scanning electron micrographs of *Porphyridium purpureum (P.p)* cells, after cold plasma treatment at 50 µm scale at 1000x magnification level. a) Untreated *P. p* cells; b) CPDS (20 kV/N_2/9 min); c) CPDS (25 kV/N_2/9 min); d) CPJS (30 kV/N_2/9 min); e) CPJS (30 kV/Air/9 min); cold plasma discharge system (CPDS); cold plasma jet system (CPJS).

20 kV using N_2. While crude PBP extracts obtained after CPJS treatments using N_2 at 30 kV had higher extraction yields, antioxidant activities and cytotoxicity effects compared to extracts from CPJS treated samples using air at 30 kV. Overall, CPDS treatment at 25 kV using N_2 gas for 9 min treatment time resulted in the highest extraction yield and cytotoxicity effects. However, the highest antioxidant activity was observed for CPDS treatment at 25 kV using N_2 gas and a treatment time of 6 min. Additional studies are recommended to further investigate the cytotoxicity effects of PBP crude extracts and address scale up challenges to facilitate the adoption of plasma treatments in commercial applications.

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Informed consent statement

Not applicable.

CRediT authorship contribution statement

Shaba Noore: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, preparation, Visualization, All the authors have read and agreed to the published version of the manuscript. **Brijesh K. Tiwari:** Conceptualization, Methodology, Investigation, Resources, Data curation, Writing – review & editing, Supervision, Project administration, Funding acquisition, All the authors have read and agreed to the published version of the manuscript. **Anet R. Jambrak:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing – review & editing, Supervision, All the authors have read and agreed to the

published version of the manuscript. **Josipa Dukić:** Methodology, Formal analysis, Investigation, Writing – review & editing, All the authors have read and agreed to the published version of the manuscript. **Janith Wanigasekara:** Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, preparation, All the authors have read and agreed to the published version of the manuscript. **James F. Curtin:** Resources, All the authors have read and agreed to the published version of the manuscript. **Claudio Fuentes-Grunewald:** biomass production and harvesting, All the authors have read and agreed to the published version of the manuscript. **Colm O'Donnell:** Conceptualization, Methodology, Investigation, Resources, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition, All the authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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