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Extraction, purification, and bioevaluation of phycoerythrin from *Halymenia refugium* against pathogenic bacteria and A549 lung cancer cell line

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Lung cancer is still among the most frequent cancer-induced deaths, and the traditional and modern treatment options usually lead to severe side effects and lack effectiveness. In this case, the bioactive properties of phycoerythrin (PE), which was extracted in the red alga *Halymenia refugium*, were evaluated. It was extracted using freeze-thaw method, and subsequently purified via ion-exchange chromatography. Extraction technique yielded 5.36 mg of the pigment / g of the alga. The qualitative analyses revealed the presence of flavonoids (6.91 mg/g) and phenolics (2.29 mg GAE/g). The antioxidant properties of PE exhibited good scavenging of DPPH (91.2%), ABTS (81.5%), and phosphomolybdate (86.15%). PE was able to inhibit α-amylase by 88.30% (IC_{50} = 30.81 µg/mL) and β-glucosidase by 91.06% (IC_{50} = 36.34 µg/mL) and defined its anti-diabetic property. anti-inflammatory activity was successfully investigated and confirmed through total protein denaturation (94.09% inhibition, IC_{50} = 44.62 µg/mL) and egg albumin denaturation (85.57% inhibition, IC_{50} = 47.31 µg/mL) assays. Phycoerythrin checked the growth of A549cells to 63.95 % at a specified concentration of 75mM.A good inhibition was observed in bacterial antagonism against S. aureus and B. subtilis. These findings suggested that phycoerythrin from *H. refugium* exhibited strong antioxidant, antidiabetic, anti-inflammatory, antimicrobial, and cytotoxic potential, supporting its development as a natural multifunctional therapeutic agent.

Keywords: Anticancer, Antibacterial, Antioxidant, *Halymenia refugium*, Phycoerythrin

Lung cancer is currently a major leading cause of cancer-related deaths worldwide, with the non-small cell lung cancer (NSCLC) accounting for approximately 85% of diagnosed cases. The treatment outcome in patients with advanced-stage NSCLC stays unfavourable because of drug resistance acquisition and high recurrence together with treatment side effects despite recent technological and therapeutic advances that brought molecularly targeted agents and immune checkpoint inhibitors[1,2]. Because current clinical problems remain ongoing it becomes crucial to identify more secure treatment options which combine multiple targets in addition to natural origin compounds that create minimal adverse effects. Marine macroalgae have emerged as promising reservoirs of pharmacologically active compounds. Red algae (Rhodophyta) are one of them and have been recorded to produce a range of bioactive metabolites, such as phycobiliproteins, sulfated polysaccharides, and phenolic compounds [3,4]. One water soluble pigment protein complex, phycocerythrin (PE), which is mainly present inred algae has drawn considerable research interest due to its antioxidant, anti-inflammatory and anticancer properties [5]. It also has a therapeutic potential that is enhanced by its structural stability, water solubility, and good bioavailability.

The recent research has increased the interest in PE because it can reduce oxidative stress and metabolic disorders[6]. As an example, PE modified with bromelain showed an enhanced thermal and oxidative stability which makes it eligible to bioactive formulations[6]. Peptides derived from Rhodomonas sp. PE inhibited protein denaturation and reduced inflammatory cytokine production, indicating strong anti-inflammatory potential[7]. PE together with related phycobiliproteins from *Gracilaria* edulis and Porphyridium cruentum species were proven to block α-amylase and β-glucosidase carbohydrate-hydrolyzing enzymes for anti-diabetic healthcare potential[8]. However, although these investigations provide evidence of a strong foundation for the pharmacological utility of PE, they are limited in scope and specificity. The research field lacks sufficient studies that assess PE's cytotoxic properties

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Suppl. data available on respective page of NOPR

and therapeutic characteristics against A549 cells. Research on PE as an anticancer agent mainly examines its effects on cancer models that involve the colon, breast or hepatocellular carcinoma[9] yet fails to investigate pulmonary malignancies. The tropical red macroalgae, *H. refugium*, lacks substantial characterization information. Investigation on isolation, characterization and property check of PE from *H. refugium* had not been performed till date. Thus, the present study examined PE by systematically extracting and characterizing it from *H. refugium*, followed by a comprehensive *In vitro* evaluation of antioxidant, antimicrobial, antidiabetic, anti-inflammatory, and anticancer properties, benchmarked against respective conventional therapeutic agents. This work represented the first detailed pharmacological investigation of *H. refugium* PE and established its potential as a candidate for therapeutic development in biomedical applications.

Materials and Methods

Extraction of phycoerythrin from macroalga

Fresh wet alga, *H. refugium*, was collected from Rameswaram (Mandapam; Lat: 9º16'32.56" N, long: 79º07'5.3" E), Tamil Nadu, India. The algae collected was immediately was rinsed thoroughly with tap water, followed by multiple washes with distilled water water, which was essential in the removal of any surface material and potential chemical that may disrupt the following analysis[10]. The solvents were then added to wet macroalgae in 1:10 ratio (sample to distilled water, with 0.9 percent saline and 0.1 M PBS buffer, in a beaker). To enhance cell disruption and promote pigment release, the mixture underwent three cycles of freeze-thaw reactions[11]. After these cycles, the mixture was incubated overnight at 4°C to allow sufficient extraction of phycoerythrin[5,10]. After incubation, the sample was centrifuged at 6000 rpm in 10 min to segregate the supernatant in which PE was present, and the solid debris. A fresh 1:10 (sample to distilled water) sample was prepared, including 0.9% saline and 0.1M PBS buffer and sonicated in a water bath sonicator with 1440 pulses[12]. After sonication, the sample was centrifuged at 6000 rpm over 10 min to separate the PE containing supernatant and the solid debris to proceed with the analysis of the clear supernatant. The other preparation procedure entailed the addition of the sample to the solvent at a ratio of 1: 10 (sample and distilled water, including 0.9% saline and 0.1M PBS buffer). A 5-second on/off sonication cycle at 80% amplitude was used to probe sonicate the mixture during 5 min in order to enhance extraction efficiency[10]. After sonication, the sample was centrifuged again to separate the supernatant from the solid residues. The supernatant was collected for further analysis after filtering out any remaining particulates[11] (Suppl. Fig. S1).

Purification of PE

The crude extract that contained PE underwent ammonium sulfate precipitation with constant stirring; a 60% saturated solution of ammonium sulfate was gradually introduced to the extract[12,13]. The mixture was maintained at 4°C for 12 h to facilitate protein precipitation (Suppl. Fig. S2). Following incubation and subsequently centrifuged at 8000 rpm for 15 min at 4°C to collect the protein pellet, then the resulting pellet was suspended in 0.01 M phosphate buffer and dialysis was performed against the same buffer to remove residual salts[14]. After dialyzing PE, it was subjected to Q Sepharose column chromatographic purification. Q Sepharose column chromatography as performed at 0.5 mL/min and the fractions were eluted based on the colour variations[13]. The different fractions of collected sample were pooled together and stored at 4°C for further analysis.

Electrophoresis assay of Purified PE

SDS-PAGE was carried out with a 15 percent separating gel and a 5 percent stacking gel, according to a preceding described procedure with some slight variations[12]. Electrophoresis was started at 70 V and then voltage increased to 85 V until the run was completed. In the case of native-PAGE, a 10% separating gel was employed as well as a 5 percent stacking gel, in accordance with a prior experiment[14]. The same trend of voltages and time period, as the above-mentioned, were followed. After electrophoresis, the gels were stained with 0.1% (*w/v*) Coomassie Brilliant Blue R-250. The gel images were captured and analyzed using the ChemiDoc XRS+ imaging system (Bio-Rad, Berkeley, CA, USA).

Total protein content determination

The Bradford test was used to analyze the total protein concentration, by using bovine serum albumin (BSA) as the reference[15]. A 24-well microtiter plate was addeed with 100 µL of each extract and 160 µL of Bradford reagent. After incubation, a spectrophotometer was used to detect absorbance at 565 nm. PE concentration was calculated by extrapolating using a standard calibration curve (Suppl. Fig. S3).

Estimation of purity and Yield percentage of PE

PE concentration was quantified using a UV–Vis absorption spectrophotometer that followed the dichromatic equations established by Sampath-Wiley and Neefus[16]. The quantification of PE concentrations was done by implementing equations 1 and 2. The PE purity index was calculated by dividing the absorbance value at 565 nm by the absorbance value at 280 nm which is shown in Equation (1)[10,16].

Purity ratio: $PE=A_{565}/A_{280}$

Total protein content:A_{280}

Quantitative Phytochemical profile

Determination of total phenolic content (TPC)

The total phenolic content of PEextract was measured using Folin-Ciocalteau reagent method, with gallic acid as the standard. A 0.5 mL aliquot of the sample was mixed with 0.5 mL of Folin–Ciocalteu reagent and incubated for 5 min[17]. Following this, 2 mL of 7.5% sodium carbonate solution was introduced into the mixture. The reaction mixture was kept in the dark for 15 min, subsequently, absorbance was recorded at 765 nm using a spectrophotometer. Results were expressed as micrograms of gallic acid equivalents (µg GAE) per gram of sample.

Determination of total flavonoid content (TFC)

The aluminium chloride technique was employed to determine the total flavonoid content of PE, using quercetin as the standard. To a volume of 1 mL sample, 0.5 mL of 5% sodium nitrite was added and kept for 5 min. Then, 0.5 mL of 10% aluminium chloride was added and incubated for 6 min[17]. Finally, 2 mL of 4% sodium hydroxide was introduced to the mixture. Absorbance was recorded at 510 nm using a UV-Vis spectrophotometer. Results were expressed as micrograms of quercetin equivalents (µg QE) per gram of sample.

Quantification of free radical scavenging potential

DPPH Assay

The free radical scavenging activity of PE was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay with slight modifications from the method described by Chen Y *et al.* 2014[17]. To prepare the DPPH stock solution, 0.0024 g of DPPH was dissolved in 100 mL of methanol. The standard ascorbic acid solution was prepared at a stock concentration of 1 mg/mL Different concentrations of ascorbic acid were used to generate a standard curve. A 1 mL aliquot of the DPPH solution was mixed with 1 mL of each ascorbic acid concentration and incubated in the dark at ambient temperature for 30 min[18,19,21]. Absorbance was then measured at 520 nm using a UV–VIS spectrophotometer. A similar procedure was carried out for the PE samples. Distilled water was used as the blank.

$$\% \text{ of inhibition} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

ABTS Assay

PE's antioxidant capacity was determinedby the ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) radical cation decolorization test which followed modified experimental conditions. The reduction of $ABTS^{+}$• radicals by antioxidants leads to lower readings at 734 nm according to this assessment method[20]. The ABTS radical cation ($ABTS^{+}$•) required a solution mixture of 7 mM of ABTS in deionized water with an equal amount of 2.45 mM potassium persulfate. After incubate in dark at room temperature for 12 to 16 h the solution became ready for use with completely formed $ABTS^{+}$• radicals. Assessment of the working solution required ethanol or deionized water to dilute it until the absorbance reached 0.7 ± 0.02 at 734 nm wavelength. This assay combined 10 µL of test sample with 190 µL of the $ABTS^{+}$• working solution in 96-well microplates. The reaction mixture remained in dark conditions at room temperature for 6 to 10 min. The examination of absorbance occurred at 734 nm with either a UV-Vis Spectrophotometer or a microplate Reader. The percentage of scavenging activity is assessed by the following equation.

$$\% \text{ of inhibition} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

Phosphomolybdate Assay

PE's antioxidant property was also determined using the phosphomolybdate assay, where molybdenum (Mo^{6+}) gets reduced to molybdenum (Mo^{5+}). A mixture was prepared by combining of 1 mL phosphomolybdate (containing 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) with 0.1 mL of the sample[17]. The reaction tube containing the mixture incubated at 95°C for a period of 90 min. After incubation, the absorbance of the reaction solution was measured at 765 nm using a spectrophotometer. The antioxidant activity assessment occurred through absorbance measurements which showed increased reducing potential as absorbance value increased.

$$\% \text{ of inhibition} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

***In vitro* anti-diabetic assay of PE**

α-amylase inhibitory assay

The inhibitory activity of PE towards α-amylase was determined based on a modified DNSA protocol[22], in the presence of acarbose as a reference standard inhibitor. First, 250 µL of PE was combined with 250 µL of alpha-amylase solution (0.5 mg/mL) in the phosphate buffer solution (pH 6.9) and kept at 25°C for 10 min. Subsequently, a 1% buffered starch solution was added, followed by further incubation for 10 min. The reaction was terminated by adding 500 µL of 3,5-dinitrosalicylic acid (DNSA) and heating for five min. The prepared samples were diluted with 5 mL of distilled water after cooling, and absorbance was read at 540 nm using a UV-Vis spectrophotometer, and acarbose was used as the positive control.

$$\% \text{ of inhibition} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

β-Glucosidase inhibitory assay

A modified technique based on previously established methods was used to assess PE's β-glucosidase inhibitory activity utilizing p-nitrophenyl-β-D-glucopyranoside (pNPG) as the substrate[22]. The assay mixture was comprised of 50 µL of the test solution, containing PE or a standard inhibitor, blended with 50 µL of a β-glucosidase enzyme solution at afinal concentration of 1 U/mL, which was prepared in 0.1 M phosphate buffer at a pH of 6.8. This solution maintained at 37 °C for 10 min before the experiment began. The reaction was initiated by adding 50 µL of a 5 mM p-nitrophenyl-β-D-glucopyranoside (pNPG) solution, which was dissolved in the phosphate buffer. The reaction mixture was then incubated at 37 °C for thirty min. To terminate the reaction, 100 µL of a 0.2 M sodium carbonate (Na_2CO_3) solution was added. The release of p-nitrophenol, indicated by the fprmation of a yellow colour, was recorded at 405 nm using a UV-Vis spectrophotometer.

$$\% \text{ of inhibition} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

Anti-inflammatory activity of PE

Protein denaturation Assay

PE was prepared at varying concentrations ranging from 20 to 100 µg/mL into a 100 µL solution of 1% (*w/v*) bovine serum albumin (BSA) in 800 µL of phosphate-buffered saline (PBS, pH 64) to formulate the reaction mixture[23]. The mixture was maintained at 37°C for 20 min to facilitate the reaction with BSA. Subsequently, the mixture was subjected to heating at 70°C for an additional 5 min , resulting in protein denaturation[24]. The turbidity of the reaction mixture was measured at 660 nm using a UV-Vis spectrophotometer after the temperature was normalized. Diclofenac sodium served as the standard drug for assessing anti-inflammatory activity, while dimethyl sulfoxide (DMSO) was employed as a control[24].

$$\% \text{ of inhibition} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

Egg albumin denaturation assay

The egg albumin denaturation assay was performed to assess PE's anti-inflammatory properties, incorporating minor modifications to the standard procedure[25]. The reaction mixture comprised 0.2 mL of fresh egg albumin and 2.8 mL of phosphate-buffered saline (PBS, pH 6.4), along with 2 mL of PE solution at different concentrations (*e.g.*, 20, 40, 60, 80, 100 µg/mL). The mixture was maintained at 37 °C (15 min) before being heated to 70 °C for 5 min. After the heating process, the mixture was allowed to cool, and the turbidity of the test solutions was evaluated at a wavelength of 660 nm using a UV–Vis spectrophotometer[24]. A control solution without PE was maintained for comparison. Additionally, diclofenac sodium as a reference anti-inflammatory agent under the same experimental conditions.

$$\% \text{ of inhibition} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

Characterization of PE

UV-Vis spectrophotometry

A UV–Vis absorption spectrophotometer was used to analyze PE (PE) in order to examine its spectral qualities and determine its pure state[25]. An absorption spectrum measurement of PE spanned from 200 to 800 nm for peak identification assessment.

Circular dichorism (CD)

PE was analyzed by applied photophysics spectrum. The CD spectrum was recorded over the wavelength ranging between 190 and 260nm. A quartz cuvette with an optical path length of 0.1cm and a bandwidth of 1 nm were utilized. Subsequently, the obtained spectra were analyzed to determine the protein's secondary structure content using the K2D3 online tool.

FTIR spectroscopy

The Fourier Transform Infrared Spectroscopy (FTIR) measured functional groups that connected to protein structure in PE. The FTIR spectra acquisition for the analysis was carried out within the range from 4000 to 400 cm^{-1} by using an attenuated total reflectance (ATR) accessory at resolution of 4 cm^{-1}[1,27].

Anti-microbial activity

Agar well diffusion method

Bactericidal potential of PE isolated from *H. refugium* was evaluated by using the agar well diffusion technique against three bacterial strains, *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus*. The bacterial suspensions were freshly prepared by subculturing the bacterial strains in sterile nutrient broth and incubating them for 24 h at 37 ± 2°C. For the assay, sterile nutrient agar plates were set up, and each bacterial culture was evenly spread on the agar using a swabbing method[27]. Wells of 4 mm diameter were cut in the agar by a sterile gel puncher, and various concentrations of PE (200, 400, 600, 800, and 1000 µg/mL) were placed into the wells. The positive control used was a 15 µg/mL ampicillin solution, with sterile distilled water as the negative control. The plates were maintained at 37 ± 2°C for 24 h, after which time the inhibition zones (in cm) were measured.

Minimal inhibitory / minimal bactericidal concentration

To further validate the antimicrobial activity detected in agar well diffusion test, other tests like Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) are needed to give more detailed information regarding the antimicrobial activity of PE. MIC detects the minimum concentration that can inhibit bacterial growth, while MBC detects the minimum concentration that can kill bacteria[27]. A stock solution of PE (1 mg/mL) was diluted serially using nutrient broth to obtain a final concentration of around 0.9 µg/mL. To a microtiter plate, 10 µL of bacterial inoculum was introduced in each well, and the plate was incubated at 37 ± 2°C for 24 h. To count the MBC, 10 µL from every well was plated onto solid nutrient agar plates, which were incubated at 37 ± 2°C for the additional 24 h. The MBC was taken as the least concentration of PE that completely suppressed bacterial colony growth.

***In vitro* animal cell line study**

Cell culturing

The lung cancer cell line, A549, was used to assess the anti-cancer activity of PE[28]. Cells were keptin Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin-streptomycin antibiotics in culture plates. They were then incubated in a humidified incubator with 5% CO_2 at 37°C for 24 h[29].

Cytotoxicity assay

The MTT assay determined the anti-proliferative effect of PE on A549 cells. Cells, at a density of 2000 cells per well, were seeded in 96-well micro titerplates. They were treated with varoius concentrations of PE for 48 h[30,31]. Cell viability was performed using MTT assay according to the manufacturer's protocol (Sigma-Aldrich). The determination of cell viability was assessed by detecting the formation of formazan product and its absorbance was measured at 520 nm using a microplate reader (BioTek, SynergyH1, USA)[24].

Result and Discussion

Quantification of PE

PE extraction from wet *H. refugium* biomass proved effective when multiple extraction methods and solvent systems were combined according to this research investigation. The freeze-thaw method with 0.1 mM phosphate-buffered solution provided optimal extraction of PE at maximum concentration (Fig. 1). The use of phosphate-buffered saline solutions (pH 6–7.5) proved effective for protein structure stability and enhanced extractability while reducing protein aggregation according to the earlier findings[32]. PBS exhibits known buffering properties that help maintain an optimal pH range for protecting protein structures from denaturation while supporting pigment retention and slowing down enzymatic activity toward sensitive phycobiliproteins including

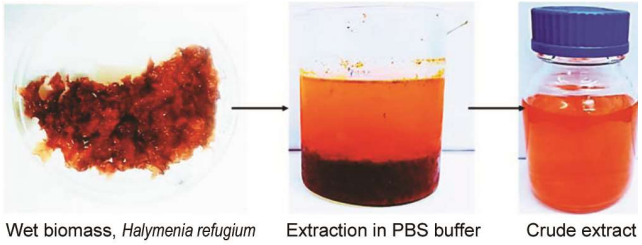

Fig. 1 — Crude extraction of PE. The wet biomass of *H. refugium* was subjected to extraction in phosphate buffer solution to subsequently yield a crude extract

PE[33]. The freeze-thaw technique outperformed other extraction methods due to the formation of intracellular ice crystals, which effectively disrupted cell walls without inducing mechanical or thermal stress. This approach preserved both the structural and functional integrity of PE, enabling further bioactivity assessment. Repeated freeze-thaw cycles, when paired with PBS stabilization, maintained pigment integrity and minimized denaturation, thus supporting continued pharmacological investigation.

Protein quantification of the PBS-extracted samples revealed a total protein yield ranging from 4.69 to 5.36 mg/g of algal biomass, with a corresponding purified PE concentration of 6.23 mg/mL. Compared to saline and distilled water, PBS consistently yielded greater amounts of PE, a trend also reported in red algae species such as *Gracilaria* and *Porphyra*[10]. PE purification from *H. refugium* was verified through SDS-PAGE analysis (Fig. 2A). The crude extract displayed several protein bands together with a significant band at ~20 kDa which matched the α-subunit of PE[34]. Efficient protein binding occurred together with effective purification of impurities because the target protein showed minimal detection during flow-through and wash fraction analysis. The 18-20 kDa strong protein band showed maximum presence in Elution 1 and decreased in concentration in successive elutions. The chromatographic procedure

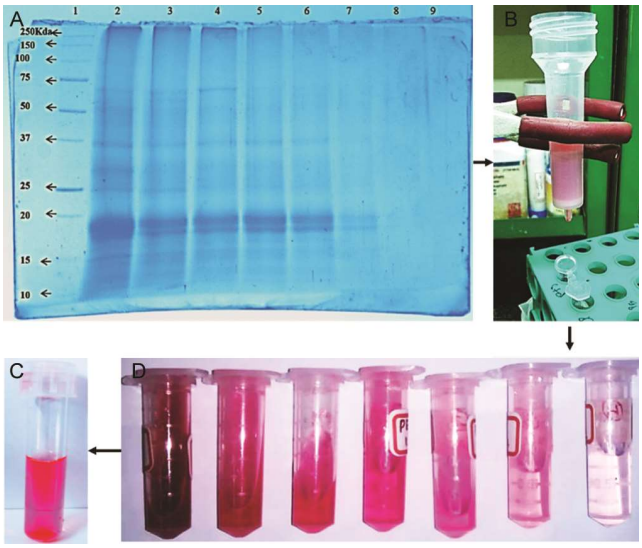

Fig. 2 — Isolation of PE. (A) SDS-PAGE revealed the presence of PE with a molecular weight of 20 kDa (lane 1: protein marker, lane 2: crude extract, lane 3: flow-through, lane 4: PBS wash, lane 5: eluent 1, lane 6: eluent 2, lane 7: eluent 3, lane: eluent 4 and lane 9: NaOH wash; (B) chromatography chromatography – Q Sepharose column with the crude extract; (C) various eluents or fractions collected upon chromatographic run; and (D) purified PE

demonstrates standard protein desorption behaviour which occurs through stepwise method in affinity or ion-exchange purification[35]. The results from the final NaOH wash showed that all the target protein successfully returned to the collection tube (Fig. 2B-C and Suppl. Fig. S4). Recent research revealed that the purified PE from *Kappaphycus* and *Porphyra* species showed a distinct ~22 kDa protein band[33,34]. The established purification method showed high selectivity while preserving most of the PE substance (Fig. 2D) with good recovery rates thus sustaining protein integrity and activity for future research[36].

Phytochemical analysis of PE

Red algae and cyanobacteria contain the light-gathering pigment PE which scientists continue to understand because it demonstrates varied biological functions. The PE extracted from *H. refugium* showed strong phenolic and flavonoid content levels (TPC reached 71.8 mg GAE/g while TFC reached 63.5 mg QE/g) that exceeded earlier measurement results (TPC at 2.29 ± 0.01 mg GAE/g and TFC at 6.91 ± 0.09 mg/g) by significant margins[17,37]. The exceptional levels of bioactive PE indicated that *H. refugium* could be an exceptionally strong natural source of these compounds. This pigment benefits from its elevated phenolic and flavonoid compounds which enhance antioxidant potential and enable antibacterial and anticancer properties through the regulation of oxidative stress while simultaneously breaking microbial and cancer cell membranes and interrupting nucleic acids and intracellular signal transmission pathways[17].

Characterization of PE

UV-Visible spectrophotometry

A UV-Visible spectral evaluation of the *H. refugium* extract was performed to identify and measure pure PE concentrations based on its strong visible light absorption property. The maximum absorption occurred at 480 nm (OD_{480} 0.72) due to the protein components primarily identifying peptide bonds in PE. The extract contains PEalso exhibitedmoderate absorption levels at wavelengths 539 nm (OD_{539} 0.81) and 560 nm (OD_{560} 1.24) as illustrated in (Fig. 3A). The results corroborated the earlier reports on PE[26].

Circular dichroism

Circular Dichroism (CD) spectrum of PE was recorded in the far-UV region (200-260 nm) to analyze its secondary structure. The CD spectrum,

shown in (Fig. 3B), reveals patterns indicative of ordered secondary structures. Analysis of the obtained spectrum using the K2D3 online tool determined the following composition: - α-helix content: 7.06% - β-strand content: 18.79%. The CD spectrum showed negative ellipticity at 210-220 nm, indicating a β-strand-rich structure which was consistent with a previous report in which crude precipitated PE showed negative ellipticity from 201 nm[27]. The low α-helix content aligned with previous studies highlighting β-sheet dominance, which contributed to the protein's stability and functionality. An increase in ellipticity beyond 230 nm suggested aromatic chromophores and protein folding. These findings clarified PE's structural composition which were important for understanding its stability and potential pharmacological applications.

Fourier Transform Infrared (FTIR) spectroscopic analysis

The FTIR spectral analysis of purified PE from *H. refugium* revealed crucial details about its molecular structure thus establishing it as a glycoprotein compound (Fig. 3C). The strong and broad absorption band at 3377 cm^{-1} confirmed the presence of hydrogen-bonded hydroxyl and amine groups which supported both protein-related functional groups and water molecules bound to the structure[34]. Two weak absorbance peaks at 2602 cm^{-1} and 2097 cm^{-1} indicated potential chemical interactions between aliphatic or lipid components and chromophore sides inside the protein structure. The 1639 cm^{-1} peak functioning as the amide I band served as an essential marker of peptide bonds found within protein backbones those appeared in well-ordered protein complexes. A similar FTIR band pattern was previously reported for PE found in *Porphyra* and *Gracilaria* species[10]. The bands at 1077 cm^{-1} indicated C–O stretching vibrations that were typical for phycobiliproteins because they contained glycoprotein components and carbohydrates which might aid the glycosylation processes. The functional groups in PE may played fundamental roles in its biochemical properties because they enable binding interactions with biological targets including enzymes, membranes and nucleic acids[16].

Free radical scavenging activity

The human body generates unstable molecules referred to as free radicals as a reaction to environmental hazards and oxidative stress events[21,23]. Cellular damage due to free radical encounter can be delayed by antioxidants. The antioxidant efficacy of PE extracted from *H. refugium* was evaluated using three well-established *In vitro* assays namely DPPH, ABTS, and phosphomolybdate, in order to capture different mechanisms of antioxidation (Fig. 4)[19]. PE showed an exceptional inhibition rate of 91.2% as given in the (Suppl. Table S1 and Fig. 4A). Although the DPPH radical scavenging assay with IC_{50} values of 45.01 µg/mL which was slightly elevated from the performance of ascorbic acid's IC_{50} measurement of 41.94 µg/mL. The extracted pigment demonstrated robust electron donating capacity through its flavonoid and phenolic components that readily donated hydrogens to stabilize free radicals[38]. The results correlated with the previous investigation with Microchaete PE that showed an equivalent antioxidant performance (IC_{50} = 43 µg/mL)[38].

The ABTS assay was conducted to measure electron-based antioxidant capacity. The results indicated thatPE demonstrated 81.5% inhibitory capacity with an IC_{50} value of 63.07 µg/mL, shown in the (Suppl. Table S1 and Fig. 4B), which was significantly higher than that of ascorbic acid (IC_{50} = 44.85 µg/mL). The extracted pigment demonstrated superior antioxidant potential because it efficiently

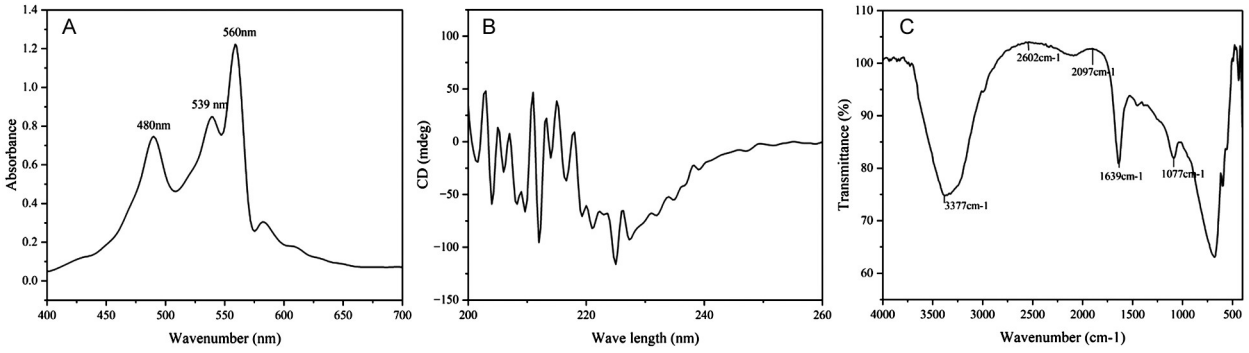

Fig. 3 — Characterization of PE. (A) UV- Visible spectrum depicting the presence of PE; (B) CD spectrum to demonstrate the protein structure in PE; and (C) FTIR spectral graph of PE illustrating the functional moieties in PE

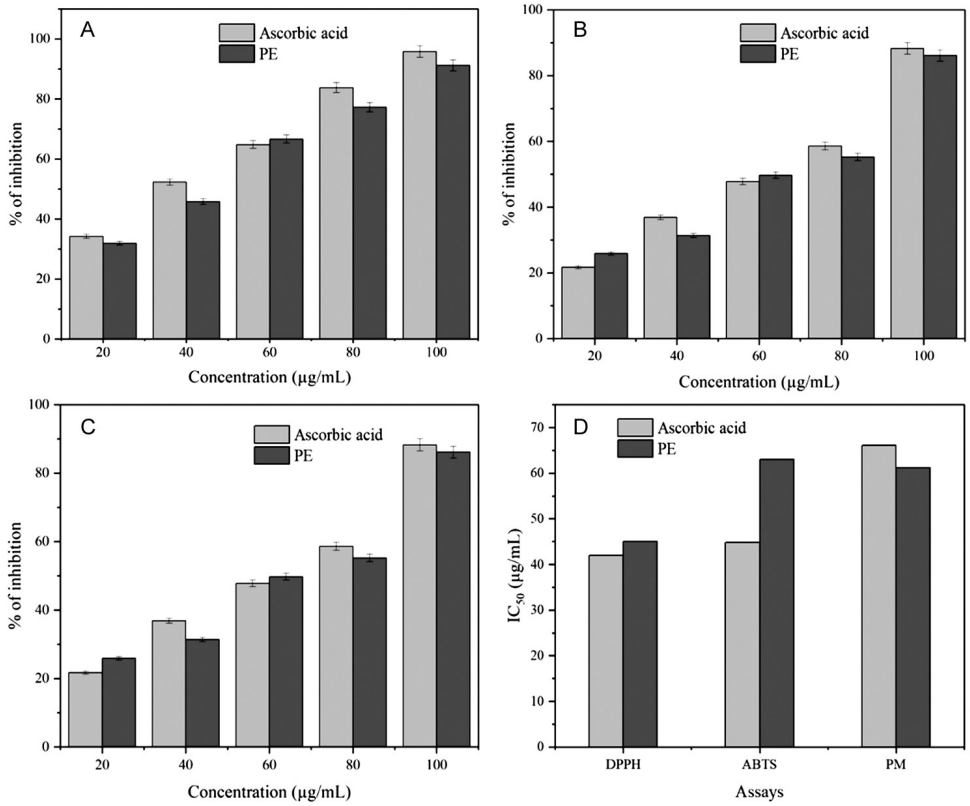

Fig. 4 — Antioxidant activity of PE. The graph illustrates the scavenging activity of (A) DPPH; (B) ABTS; (C) phosphomolybate (PM) at varying tested concentrations; and (D) at their respectively IC_{50} values

reduced the radical cation ABTS. An earlier study reported a higher activity value (IC_{50} = 23 µg/mL) for PE from Microchaete yet the current findings contrast with previous reports due to differences in extraction procedures, pigment purity conditions and the influence of phycobilin on biological response[38].

H. refugium PE showed antioxidant capabilities which were validated through the phosphomolybdate assay that reduced Mo (VI) into Mo (IV) species. Tests revealed 86.15% activity of the sample at an IC_{50} value of 61.19 µg/mL in (Suppl. Table S1 and Fig. 4C) which showed very similar results when compared to the reference ascorbic acid standard with an IC_{50} value of 49.63 µg/mL[37]. The resulting data showed that the functional components such as the phenolics and proteins in the pigment aided metal ion reduction and maximum scavenging effect was determined through phosphomolybdate assay as illustrated in (Fig. 4D).

Anti-diabetic activity of PE

Biological compounds obtained from macroalgae block the activity of α-amylase enzyme preventing starch hydrolysis in food substances through α-1,4-glycosidic bond cleavage. α-amylase enzyme inhibition together with restricted glucose absorption through this process assists the maintenance of blood sugar stability after food intake[22]. Results demonstrated that *H. Refugium* PE inhibited α-amylase by 88.30% at 100 µg/mL following an initial value of 33.96% at 20 µg/mLas represented in (Fig. 5A and Suppl. Table S2). Standard acarbose demonstrated the inhibition patterns against α-amylase with maximum efficiency ranging between 32.08% and 95.85% when the concentrations ranged between 20 and 100 µg/mL, respectively. When comparing IC_{50} values of PE to the standard, the assay demonstrated a higher inhibitory effect of PE than acarbose with values of 35.14 µg/mL and 30.81 µg/mL respectively (Suppl. Table S2).The hydroxyl, carboxyl and chromophoric moieties in PE might have assisted the bonding with enzyme molecules. The results demonstrated that PE possessed promising potential to act as an α-amylase blocking agent and may as well be tested for its efficacy in for controlling type 2 diabetes mellitus[22].

The study evaluated the β-glucosidase inhibitory properties of *H. refugium.* PE against acarbose.

β-glucosidase breaks down glycosidic linkages in glucose leading to its release in the small intestine. This is crucial for postprandial hyperglycaemia control. Beta-glucosidase inhibition stands as a fundamental method to manage postprandial hyperglycaemic conditions[22]. The inhibition effect of PE on β-glucosidase was compared to acarbose at a rate of 100 µg/mL, causing 86.81% inhibition by acarbose and 91.06% inhibition by PE, yet PE demonstrated a lower IC_{50} value of 36.34 µg/mL than acarbose (IC_{50} = 42.13 µg/mL)as represented in (Fig. 5B and Suppl. Table S2). PE demonstrated that the optimal enzyme-binding performance to β - glucosidase through chromophore and functional group characteristics enabled hydrogen bonding and hydrophobic interactions [22]. The overall study results suggested that the higher inhibition was shown upon β-glucosidase than α-amylase with respect to their IC_{50} values (Fig. 5C). Hence it was suggested from this experiment that PE operated as an inhibitor through non-competitive or mixed inhibitory mechanisms demonstrating essential potential in developingit for antidiabetic purposes.

Anti-inflammatory activity of PE

The anti-inflammatory potential of PE extracted from *H. refugium* was analyzed using the protein denaturation assay, a standard *In vitro* method for assessing anti-inflammatory activity. PE exhibited a concentration-dependent inhibition of protein denaturation, achieving 25.05% inhibition at 20 µg/mL and 94.09% at 100 µg/mL, with an IC_{50} value of 44.62 µg/mL. In comparison to the standard, diclofenac sodium, a conventional non-steroidal anti-inflammatory drug (NSAID), showed inhibition ranging between 35.23% and 80.92% over the same concentration range in (Suppl. Table S3), with an IC_{50} of 46.22 µg/mL (Fig. 6A). The inhibition effect of PE at higher concentrations suggested a robust interaction with protein structures, potentially stabilizing them against denaturation [23,24].

The anti-inflammatory efficacy of PE was quantitatively evaluated via the egg albumin denaturation assay and compared with the standard NSAID, diclofenac sodium[23]. PE exhibited a concentration-dependent inhibition of thermally induced protein denaturation, with percent inhibition

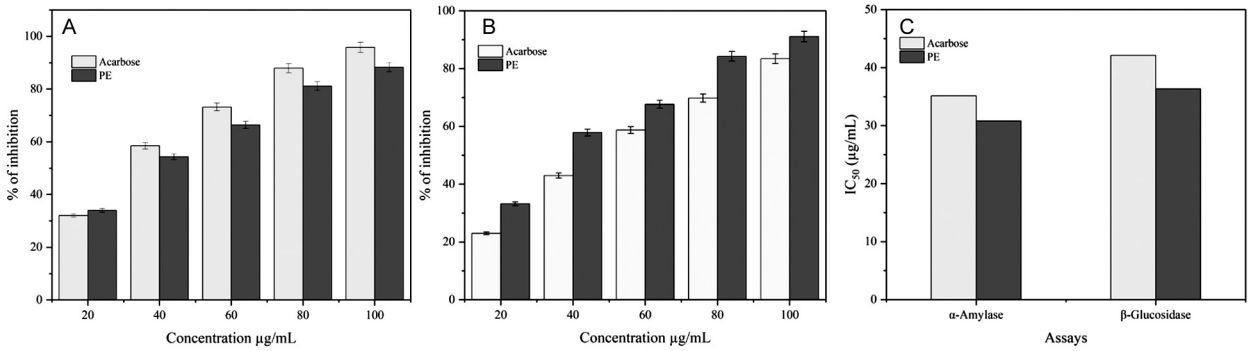

Fig. 5 — Anti diabetic efficiency of PE. Graphs represent the effect of PE on inhibiting starch hydrolysis enzymes (A) α-amylase; (B) β-glucosidase at tested concentrations; and (C) inhibition at their optimized IC_{50} values

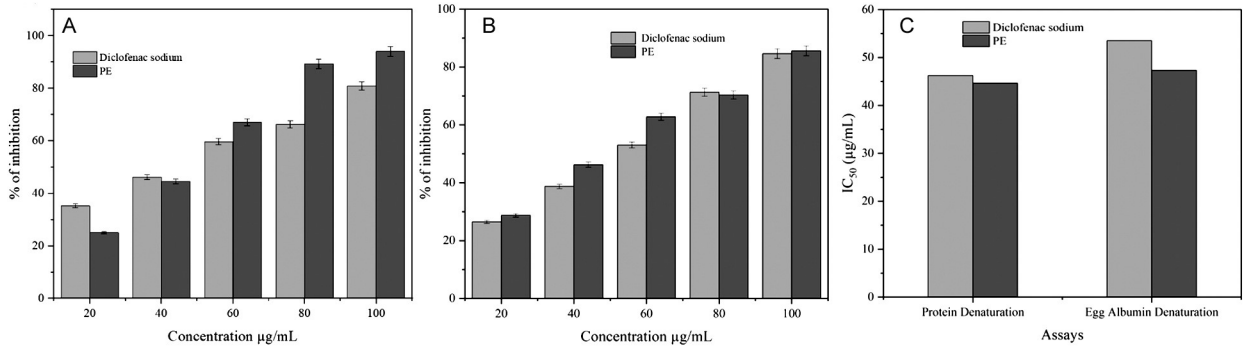

Fig. 6 — Anti inflammatory activity of the PE. Graphs illustrating the capability of PE in preventing the denaturation of proteins via (A) Protein denaturation assay; (B) Egg albumin assay at varying concentrations and; and (C) comparison of the assays based on their IC_{50} values

Table 1 — Zones of Inhibition (cm) for different concentrations of PE

Culture	Control	Concentration (µg/mL)				
		200	400	600	800	1000
S. aureus	2.17±0.1	-	-	0.2±0.12	0.51±0.31	0.76±0.21
B. subtilis	1.4±0.3	-	-	0.17±0.17	0.31±0.15	0.68±0.48
E. coli	0.95±0.2	-	0.1±0.2	0.12±0.1	0.23±0.09	0.17±0.94

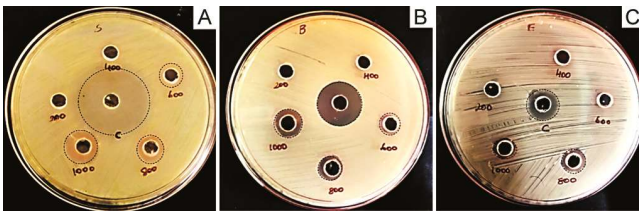

Fig. 7 —Anti-microbial activity of the PE. The plates of bacterial pathogens, (A) *S. aureus*; (B) *B. Subtilis*; and (C) *E.* coli, depicting the zones of clearance (marked as dashed black circles) when exposed to various concentrations of PE and positive control, C

Table 2 — Minimum inhibitory / Bactericidal Concentration (MIC/MBC) of PE

Culture	MIC (µg/mL)	MBC (µg/mL)
S. aureus	62.25	62.25
B. subtilis	500	250
E. coli	250	62.25

values increasing from 28.72% at 20 µg/mL to 85.57% at 100 µg/mL (Fig. 6B). Diclofenac sodium displayed inhibition values ranging between 26.46% and 84.62% across the same concentration gradient. The calculated IC_{50} for PE was 47.31 µg/mL, lower than that of diclofenac sodium (53.51 µg/mL) in (Suppl. Table S3), indicating accelerated inhibitory potency[24].

The mechanism of action was likely mediated through the interaction of PE's hydrophilic polypeptide backbone and covalently bound chromophores with denatured protein residues. These interactions, involving hydrogen bonding and electrostatic stabilization, contribute to the maintenance of protein tertiary structure under denaturing conditions[24]. The data suggested that PE effectively suppressed protein aggregation associated with inflammatory responses, positioning it as a promising marine-derived anti-inflammatory agent with potential application in pharmaceutical formulations targeting inflammation-mediated disorders.

Antimicrobial analysis of PE

Agar well diffusion method

The bactericidal activity of PE was analysed against *S.aureus*, *B.cereus* and *E. coli* and the ampicillin was served as a control. The zones of inhibition were given in the (Table 1 and illustrated in Fig. 7). The antibacterial activity was demonstrated to be higher in Gram positive bacteria (*S. aureus, B. subtilis*) than that of Gram-negative bacteria (*E. coli*), suggesting that PE was more effective against peptidoglycan rich cell walls and also the dose dependant activity was observed with higher concentration showing stronger inhibition, indicating that PE exhibited concentration dependent bactericidal or bacteriostatic properties[39].

Minimum inhibitory / Bactericidal Concentration (MIC/MBC)

The bactericidal properties of PE extracted from *H. refugium* were evaluated quantitatively using MIC and MBC tests. PE exhibited strong activity against *Staphylococcus aureus*, with both MIC and MBC values determined at 62.25 µg/mL, indicating effective bactericidal action at minimal concentrations (Table 2). PE demonstrated superior antimicrobial properties against Gram-positive bacteria structures because the basic cell architecture of bacteria was able to interact immediately with amphipathic compounds[22]. *Bacillus subtilis* demonstrated higher resistance to PE as it can form spores while secreting proteolytic enzymes that break down protein antimicrobials[39]. While PE retains the ability to kill bacteria, the presence of microbial resistance mechanisms—such as extracellular enzyme production or cell wall modifications—can reduce its overall effectiveness. The Gram-negative bacteria *Escherichia coli* demonstrated promising results with PE showing moderate susceptibility to the compound (MIC: 250 µg/mL) but a lower MBC value of 62.25 µg/mL which suggested that intracellular activity can lead to significant damage[28]. The dual-stage antimicrobial mechanism of PE demonstrates its suitability as a membrane-active compound that performs first by blocking entry into cells and subsequently killing intracellular bacteria. Previous studies on phycobiliproteins had shown that PE destroyed bacterial cells by destabilizing their membranes and inducing oxidative damage to cellular

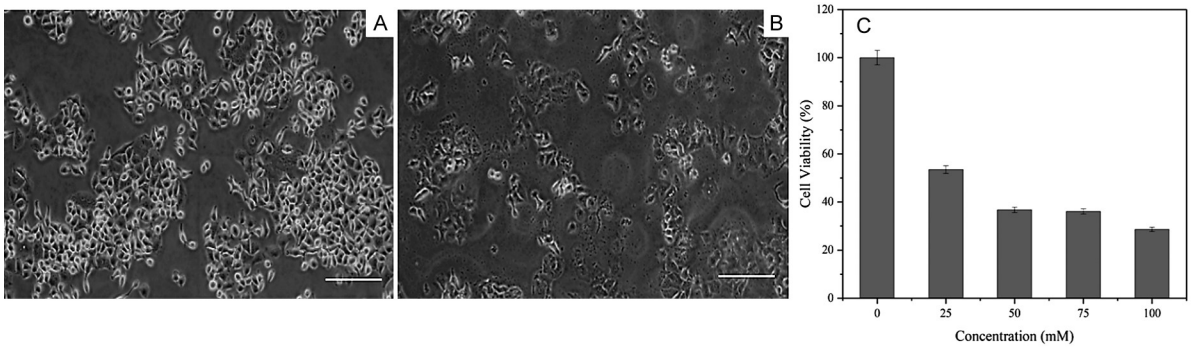

Fig. 8 — Cytotoxic effect of PE. Microscopic images of the (A) untreated; (B) treated A549 cells after 48 h (20 X magnification; scale bar – 2 mm); and (C) the quantitative MTT cytotoxic assessment of PE on A549 cell line at varying concentrations

components[22,28]. The data supported its broad-spectrum bactericidal potential, particularly against clinically relevant Gram-positive pathogens.

Cytotoxicity analysis

The MTT assay revealed that PE decreased the viability of A549 cells, (as illustrated in Fig. 8A and 8B), in a concentration-dependent manner over a 48 h period, demonstrating efficacy at concentrations between 25 mM to 75 mM (Fig. 8C). Further elevation of PE concentration led to cellular saturation along with early apoptotic signal activation which resulted in a decline of cytotoxicity at high concentrations. Endogenous oxidative stress created by PE compounds together with apoptotic pathways appeared to be the reason for this toxic mechanism. While PE exhibits the antioxidant activity in healthy cells, experimental evidence suggested which may allow it to function as a pro-oxidant in the cancer cells by generating excessive reactive oxygen species (ROS), which subsequently triggered mitochondrial-mediated apoptosis[21,23]. The ROS accumulation in tumour cells could then lead to mitochondrial membrane depolarization, caspase activation, and DNA fragmentation. According to Safaei M *et al.* 2019 the apoptosis process mediated by PE involved both pro-apoptotic and anti-apoptotic gene expression regulation through control of genetic factors BAX, BCL-2 and Caspase-3[31]. PE possesses activity that suppresses the PI3K/Akt and MAPK pathways which control lung cancer cell survival and proliferation [22]. Cellular repair processes exhibit reduced effectiveness when these paths are inhibited leading to higher susceptibility against ROS damage which results in cell growth arrest and programs cell death. The research findings supported the present results by demonstrating PE's dual mechanism against cancer through ROS production alongside destruction of mitochondria and molecular signalling blockage. Further validation of this mechanism is warranted using annexin V staining, ROS quantification assays, mitochondrial membrane potential analyses, and Western blotting of pathway-associated proteins.

Conclusion

This research illustrated PE from H. refugium as a therapeutic bioactive constituent suitable to be applied in diverse biomedical applications. By maximizing protein recovery and purification through freeze-thaw treatment accompanied by phosphate buffer, the research not only improved protein yield but also maintained its structural integrity and bioactivity. The proposed purification procedure made targeted recovery of PE in a purer form appropriate for biomedical applications feasible. The compound demonstrated robust antioxidant behaviour due to its high flavonoid and phenolic content enabling it to serve as a promising natural substitute for synthetic antioxidants. Experimental studies had shown that PE successfully inactivated *Staphylococcus aureus* and *Bacillus subtilis* indicating its potential use in microbial infection therapy because of increasing antibiotic resistance. PE also demonstrated dose-dependent cell death activity against A549 human lung cancer cells. The findings from this investigation create opportunities to investigate PE's mechanisms of action which could enable the advancement of specific treatments for cancer management alongside the control of infections and inflammations. This investigation established itself as a significant study of marine-derived PE's pharmaceutical potentials while demonstrating its clinical promise for future medical applications.

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Conflicts of interest

All authors declare no conflict of interest.

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