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Development of a Marine Algae-Based Facial Cream Enriched with Phycoerythrin: Physicochemical Properties and Biological Activities

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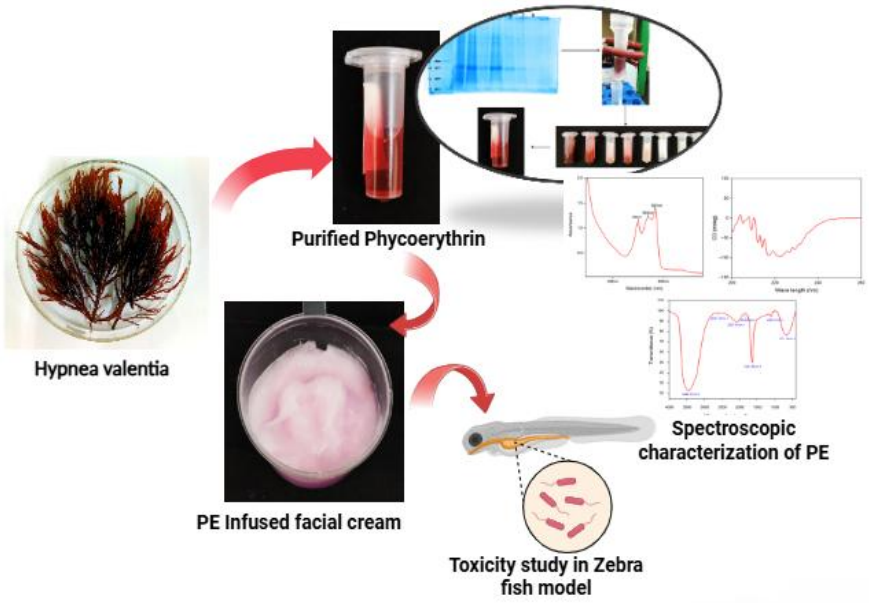
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Abstract

Phycoerythrin, a red pigment from marine algae, which has gained attention due to its unique optical properties and added biological functionality. In the present work, phycoerythrin of *Hypnea valentiae* was obtained successfully by an optimized aqueous procedure with a yield of 4.82% (w/w) of the dry biomass. The characteristic absorption peaks were verified by spectroscopic analysis, and it was confirmed that the pigment structure was not disturbed during extraction. The pigment showed strong, concentration-dependent antioxidant activity (20–100 µg/mL) in DPPH and ABTS assays, suggesting effective radical scavenging ability. It also demonstrated notable anti-inflammatory activity, with IC_{50} values comparable to diclofenac sodium, along with broad-spectrum antimicrobial effects. The pigment was added to a topical cream to investigate its potential application. The formulation was bioactive, which means it was stable in the matrix. The dermal application was suitable with respect to physicochemical properties like pH, homogeneity, and spreadability. The acute toxicity was not seen within 96 h in a zebrafish model of biosafety evaluation. In general, the research indicates the relationship between the structural integrity of the pigment and the functional performance, and contributes to the idea of the pigment as a natural colorant with the added bioactive properties in cosmetic and other applications.

Keywords: Phycoerythrin, Antioxidant activity, Anti-inflammatory activity, Sun protection factor (SPF), Cosmeceutical formulation, Zebrafish toxicity model.

Graphical abstract



Introduction

The recent growth of safe, natural, and multifunctional biomolecules has further fuelled interest in identifying bioactive compounds purified in oceans as a source of pharmaceutical and cosmeceutical molecules. Marine macroalgae, especially red seaweeds, are considered a highly effective source of structurally diverse secondary metabolites with important antioxidant, anti-inflammatory, and antimicrobial properties [1]. Pycobiliproteins are bioactive constituents that have received considerable interest owing to their potent free radical scavenging activity, biocompatibility, and therapeutic potential. Phycoerythrin (PE) is a water-soluble red pigment that belongs to the phycobiliprotein family [2]. It is abundantly found in red algae and serves as a light-harvesting protein during photosynthesis. In addition to its photobiological functionality, phycoerythrin exhibits potential pharmacological properties, including antioxidant, anti-inflammatory, hepatoprotective, and antimicrobial properties [3].

Oxidative stress is an important factor in the pathogenesis of skin aging, inflammation, microbial infections, and other degenerative states. Environmental stress factors, such as ultraviolet radiation and pollution, produce reactive oxygen species (ROS) that disrupt cellular homeostasis through lipid peroxidation, protein denaturation, and DNA damage [4]. Likewise, inflammation, which is frequently induced or exacerbated by an oxidative imbalance, is a contributor to erythema, tissue destruction, and barrier dysfunction. Thus, natural antioxidants and anti-inflammatory agents with the potential to reduce oxidative damage and stabilize protein structures are of great therapeutic importance [5]. Another in vitro system that has been significantly applied to assess the anti-inflammatory potential of any substance is protein denaturation, a well-known mechanism underlying inflammatory disorders.

Phycoerythrin has a number of benefits in comparison with other synthetic antioxidants and anti-inflammatory compounds, including low toxicity, biodegradability, and versatile bioactivity. Previous studies have reported that phycoerythrin exhibits strong radical-scavenging forces in DPPH and ABTS assays, in addition to

its ability to suppress heat-induced denaturation of proteins [6]. Moreover, there is also emerging evidence that algal pigments can have some antimicrobial effect against common skin pathogens, and this also indicates that algal pigments can be successfully used to prepare topical formulations. Nevertheless, despite increased interest, there is a shortage of research on the extraction yield, functional efficacy following formulation, and safety profile of phycoerythrin obtained using *Hypnea valentiae,* a red macroalga commonly found along tropical shorelines [7].

Creams and other topical delivery systems are efficient methods for administering bioactive compounds in a localized manner to increase retention in the skin and reduce systemic absorption [8]. However, the integration of protein-based bioactives into semi-solid matrices is associated with difficulties in terms of stability, preservation of activity, and formulation excipient compatibility. Thus, a rigorous assessment of the antioxidant effect, anti-inflammatory response, antimicrobial activity, physicochemical stability, and in vivo safety is imperative to confirm the therapeutic importance of such formulations [9].

In this present study aimed to extract and characterize phycoerythrin from *Hypnea valentiae* and determine its extraction productivity and antioxidant, anti-inflammatory, and antimicrobial properties. Moreover, a topical cream supplemented with HV-phycoerythrin was developed and evaluated based on its biological activity, physicochemical characteristics, and acute toxicity using a zebrafish model. This integrated methodology offers a methodical assessment of HV-phycoerythrin as a multifunctional bioactive compound derived from the sea, applicable to cosmetics and therapeutic products.

Materials and Methods

Phycoerythrin was extracted from the red macroalga *Hypnea valentiae,* collected from the Ramanathapuram district of Tamil Nadu, India. Ammonium sulfate, potassium dihydrogen phosphate, disodium hydrogen phosphate, DPPH, ABTS, α-amylase, β-glucosidase, bovine serum albumin, egg albumin, and MTT reagents were purchased from Sigma-Aldrich (USA). Ascorbic acid and acarbose were purchased from HiMedia Laboratory (India). The reagents used in cell culture, such as Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and trypsin EDTA, were also acquired from HiMedia Laboratories (India). Stearic acid, cetyl alcohol, and glycerol of cosmetic grade and triethanolamine were purchased from SRL. Pvt.Ltd (India).

Extraction of Phycoerythrin from *Hypnea valentiae*

H. valentia fresh wet algae were collected at Rameswaram (Mandapam; Lat: 9º16'32.56" N, long: 79º07'5.3" E) in Tamil Nadu, India. The collected algal biomass was washed with tap water followed by distilled water to eliminate attached debris and contaminants, and then stored at 4°C for future use [10]. Phycoerythrin was extracted using distilled water, 0.1 M phosphate-buffer (PBS), and 0.9% NaCl through freeze-thaw, water bath sonication, and ultrasonic sonication techniques.

In the freeze-thaw method, wet algal biomass was combined with an extraction solvent at a 1:10(w/v) ratio, subjected to three freeze-thaw cycles, and then incubated overnight at 4°C [11]. The mixture was centrifuged at 6000 rpm for 10 min, after which the supernatant was collected and purified. In the water bath sonication method, the algal biomass and solvent (1:10, w/v) were sonicated for 1440 pulses and centrifuged at 6000 rpm for 10 min [12]. Ultrasonic sonication was performed using the same biomass-to-solvent ratio for 5 min in pulse mode

(5 s on/off) at 80% amplitude, followed by centrifugation at 6000 rpm for 10 min [13]. The resulting supernatants from all extraction methods were collected and used for the subsequent purification of phycoerythrin.

Purification and Quantification of Phycoerythrin

After the extraction of H. valentia crude phycoerythrin, the purification process began with ammonium sulfate precipitation. Ammonium sulfate was gradually added to the crude extract to reach 60% saturation, and the mixture was incubated at 4°C for 12 h to precipitate the proteins [14]. The precipitated proteins were centrifuged at 8000 rpm at 4°C, and the resultant pellet was resuspended in 0.01 M phosphate-buffered saline (PBS). To eliminate traces of ammonium sulfate, dialysis was performed against the same buffer for desalination. Ion-exchange chromatography was further used to purify the extract employing a Q-Sepharose column that was run at a flow rate of 0.5 mL min -1 and phycoerythrin was eluted in a linear NaCl gradient (20 -500 mM) [15].

Characterization of Purified Phycoerythrin

Purified phycoerythrin was characterized using UV–visible, Fourier-transform infrared (FTIR), and circular dichroism (CD) spectroscopy. The spectra at 250-700 nm were measured using a UV-Vis spectrophotometer (Shimadzu UV-1800, Japan) and phosphate-buffered saline (PBS, 0.01 M, pH 7.0) as the reference blank [16]. For FTIR analysis, purified phycoerythrin was lyophilized, mixed with potassium bromide (KBr), and pressed into pellets. FTIR spectra were obtained in the 4000-400 cm -1 and room temperature using an FTIR spectrometer (Bruker Alpha II, Germany) [17]. Circular dichroism measurements were performed to evaluate the secondary structural features of the phycoerythrin. CD spectra were recorded in the far-UV region (190–260 nm) using a CD spectropolarimeter (JASCO J-815, Japan) with a quartz cuvette of 1 mm path length at room temperature [18].

Biological Activity Evaluation of Phycoerythrin

Antioxidant Activity

DPPH Radical Scavenging Assay

2,2-diphenyl-1-picrylhydrazyl (DPPH) was used to determine the DPPH radical-scavenging activity of the purified phycoerythrin. Phycoerythrin samples of various concentrations (20-100µg/mL)) were mixed with a newly prepared DPPH solution (0.1 mM in methanol) and kept in the dark at ambient temperature for 30 min (Afreen & Fatma 2018). A UV -1800 (Shimadzu, Japan) was used to measure the decrease in absorbance at 517 nm. Radical-scavenging efficiency was computed as a percentage of the control [19].

ABTS Radical Cation Decolourisation Assay

The antioxidant potential of phycoerythrin was assessed using the ABTS radical cation decolorization assay. ABTS radical cations ($ABTS^{\bullet+}$) were generated by reacting ABTS (7 mM) with potassium persulfate (2.45 mM) and incubating the mixture in the dark for 12–16 h at room temperature [19]. The resulting solution was diluted with phosphate-buffered saline to obtain an initial absorbance of 0.70 ± 0.02 at 734 nm wavelength. Following the addition of phycoerythrin, absorbance changes were recorded at 734 nm after 6 min, and the antioxidant activity was expressed as percentage inhibition [20].

Phosphomolybdenum Reduction Assay

A phosphomolybdenum reduction assay was used to determine the total antioxidant capacity of phycoerythrin. The phycoerythrin samples were added to the phosphomolybdenum reagent containing sulfuric acid, sodium phosphate, and ammonium molybdate and incubated at 95°C for 90 min [21]. The absorbance at room temperature was recorded at 695 nm using a UV-visible spectrophotometer after the solution cooled [20]. The antioxidant capacity was determined using ascorbic acid.

Anti-Inflammatory Activity

Protein Denaturation Assays

The anti-inflammatory activity of purified phycoerythrin was determined based on its ability to prevent the heat denaturation of proteins. Phycoerythrin solutions were adjusted to between 20 and 100 µg/mL after the addition of 100 µg/mL bovine serum albumin (BSA, 1% w/v) and 800 µg/mL phosphate-buffered saline (PBS, pH 6.4) [22]. The mixtures were incubated at 37°C for 20 min to allow interaction between phycoerythrin and BSA. The mixtures were then denatured by heating them to 70°C (5 min) and then allowing them to cool to room temperature. A UV- visible spectrophotometer (Shimadzu UV-1800, Japan) was used to measure the extent of turbidity at a wavelength of 660 nm. Diclofenac sodium was considered the reference anti-inflammatory drug, and dimethyl sulfoxide (DMSO) was used as the positive control [23]. The percentage of protein denaturation inhibition was determined relative to the control.

Egg Albumin Denaturation Assays

A heat-induced denaturation model was used to establish the inhibitory effect of phycoerythrin on the denaturation of egg albumin. Phycoerythrin and standard solutions were prepared at a concentration of 20-100 µg/mL. The reaction mixture was prepared by combining 0.2 mL of fresh egg albumin, 2.8mL of PBS (pH 6.4), and 2.0mL of fresh phycoerythrin at the respective concentrations [23]. The mixtures were incubated at 37°C for 15 min, followed by thermal denaturation at 70°C for 5 min. The absorbance values at 660 nm were measured after the mixture had cooled to ambient temperature using a UV-visible spectrophotometer [22]. Diclofenac sodium was used as the standard, and the inhibition of albumin denaturation was expressed as a percentage compared to the untreated control.

Antimicrobial Activity of Phycoerythrin

The antibacterial activity of phycoerythrin isolated from *Hypnea valentiae* against Escherichia coli (MTCC 443), Bacillus subtilis (MTCC 441), and Staphylococcus aureus (MTCC 96) was determined using the agar well diffusion method. Fresh cultures were obtained by subculturing and incubating at 37 ± 2 °C for 24 h [24]. Sterile nutrient agar plates were prepared, and the bacterial inocula were uniformly spread over the agar surface using a sterile swab technique. Aseptically, wells of the same diameter were punched into the agar using a sterile gel puncher. Phycoerythrin was added to each well at various concentrations (200, 400, 600, 800, and 1000 µg/mL). Ampicillin (1 mg/mL) was used as the positive control, and the plates were incubated at 37 ± 2 °C (24 hrs) [25]. The diameter of the inhibition zones (cm) of each well was used to determine the antibacterial activity.

Anticancer Activity of phycoerythrin on Breast Cancer Cell Line

Cell culturing

The MDA-MB-231 human breast cancer cell line and L929 mouse fibroblast cell line were procured from the National Center of Cell Science (NCCS), Pune, India, and were used to assess the anticancer effect of phycoerythrin isolated from *Hypnea valentiae* (HV-PE). The cells in the MDA-MB-231 were grown in Leibovitz L-15 medium with 10-15 % fetal bovine serum (FBS) and 2 mM L-glutamine [25]. The L929 fibroblast cell line, used as a normal control cell line, was cultured in DMEM/F12 supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin, and 10% heat-inactivated FBS. The cells were incubated at 37 °C with 5 percent CO_2.

Cells were permitted to attain a confluence of approximately 70-80% and then treated with serum-free medium for 24 h before treatment to enable synchronization [24]. Cell detachment was performed using a dissociation solution comprising 0.2 percent trypsin, 0.02 percent EDTA, and 0.05 percent glucose in phosphate-buffered saline (PBS). Viable cells were centrifuged, resuspended, seeded into 96-well plates at a density of 5×10^4 cells per well, and incubated for 24 h before treatment.

Cell Viability Assay (MTT Assay)

The cytotoxicity of phycoerythrin on cells of MDA-MB-231 and L929 was measured by MTT assay. Cells were placed in 96-well plates with a density of 5×10^4 cells per well and allowed to adhere to the plate after 24h [26]. Phycoerythrin was then added in varying concentrations and incubation of the cells was done again after 24 h. Untreated cells were used as the control, doxorubicin as the reference anticancer drug. 20 µL of MTT solution (5 mg/mL in phosphate-buffered saline, PBS) were put into each well and the plates were incubated at 37°C over a period of 4 h. The culture medium was cautiously removed and the obtained formazan crystals were dissolved in dimethyl sulfoxide (DMSO) [24]. Absorbance was measured at 570 nm using a microplate reader (Bio-Rad iMark™, USA). Cell viability was expressed as a percentage relative to the untreated control, and IC_{50} values were calculated from the dose–response curves.

Morphological Assessment

Phase-contrast microscopy was used to analyze morphological changes that were caused by the treatment with phycoerythrin. In the given case, MDA-MB-231 cells and L929 cells were incubated in 6-well plates and reacted with phycoerythrin at the concentrations of the IC_{50} value during 24 h [25]. Cellular morphology detected under an inverted phase-contrast microscope (Olympus CKX53, Japan) was observed after the treatment. Cell shrinkage, membrane blebbing, rounding and detachment were observed and noted as cytotoxic and apoptotic responses.

Formulation of the Base Facial Cream

A modified oil-in-water emulsion technique was used to prepare the base cream, which consisted of an oil phase and an aqueous gel phase. The oil phase was comprised of cetyl alcohol (3%), olive oil (10 %), glycerol monostearate (2%) and stearic acid used as emollients and stabilizing agents [26]. Aqueous gel phase was obtained by adding Carbopol-980 (5%), and agar in distilled water in a gentle heating until a homogenous gel was formed as is mentioned above. The oil and gel phases were made separately and heated to around 70°C to make sure that the components are fully melted and mixed together. Gradually aqueous gel phase was added to the oil phase, which was stirred continuously within a mechanical homogenizer with a speed of 2000 rpm leading to the formation of a smooth and stable emulsion [27]. The emulsion was then left to cool at room temperature where the formulation became thicker over time.

The dropwise addition of triethanolamine to the cooled emulsion was used to adjust the pH of the formulation to 7.0-72, which is within the range of the human skin [28]. The resulting formulation was then employed as the base cream into which phycoerythrin was further added.

Incorporation of Phycoerythrin into the Facial Cream

Phycoerythrin was then dissolved in distilled water to achieve a concentration of 1 mg/mL and gently added to the base cream in 1:1 (v/v) ratio and stirred in slow mode to attain homogenous distribution without creating protein denaturation. The phycoerythrin-infused facial cream (HV-PE cream) was preserved under airtight conditions to undergo additional physicochemical, biological, and safety tests.

Physicochemical Characterization of PE-Infused Facial Cream

pH Measurement

The pH of the prepared phycoerythrin (PE)-impregnate facial cream was determined with a digital pH meter calibrated. Before the analysis, the instrument was equilibrated using buffer solutions of pH 4.0 and 7.0. To determine pH, 1 g of the cream sample was dissolved in 10 mL of distilled water, stirred with it, and left to stabilize after 30 min at room temperature (25 ± 2 ° C) [29]. Electrode was then put into the cream dispersion and pH values were recorded when the readings became stable. All the measurements were in triplicates and the results were in mean pH values.

Viscosity Analysis

A Brookfield digital viscometer (Model DV-II+, Brookfield Engineering, USA) using a spindle number 64 was used to determine the viscosity of the phycoerythrin (PE)-infused facial cream. The cream sample (approximately 50 g) was scooped into a clean beaker and the viscosity was measured at 25 ± 1°C of rotational speeds of 10 to 100 rpm [30]. The values of the viscosity were noted when a constant torque was obtained. The values were reported in centipoise (cP). Every measurement was done in three times and the average viscosity values were provided to determine the uniformity, flow characteristics, and stability of the formulation in storage and use [31].

Spreadability Test

The spreadability of the phycoerythrin (PE)-infused facial cream was evaluated to determine its ease of application and ability to form a uniform film on the skin surface using the slip–drag method. Accurately weighed cream samples (1 g) were placed between two clean, dry glass slides. The upper slide was then weighed with a standard weight of 500 g and allowed to stay 5 min as indicated to spread the formulation into a uniform and thin film [32]. After the formation of the film, the top glass slide was drawn through the horizontal direction using a thread connected to a predetermined weight of 50g. A digital stopwatch was used to measure the time taken by the upper slide to travel through a given distance of 10 cm in seconds [31]. Each measurement was conducted thrice to achieve reproducibility and mean values were analysed.

Spreadability (S) was calculated using the following equation:

$$S = \frac{M \times L}{T}$$

S =spreadability (g cm/s), M=weight applied to the upper slide(g), L= distance moved by slide(cm), T=time taken to move through the given distance(s).

Smaller values of spreading time and increased values of spreading were regarded as the evidence of better spreading properties, ease of application, and a smoother texture of the prepared PE-infused facial cream [33].

Colorimetric Analysis

The color attributes of the phycoerythrin (PE)–infused facial cream were quantitatively analyzed using a portable colorimeter (Konica Minolta CR-400, Japan) operating under the CIE Lab* color space system. The instrument was then calibrated using the instructions provided by the manufacturer on a standard white calibration plate before analysis. The measurements were conducted at a room temperature (25 ± 2 ^{o}C) and the cream sample was smeared to provide a smooth, opaque surface without any air bubbles [34].

The readings on the CIE Lab were noted, with L* being the lightness on a scale of 0 (black) to 100 (white), a being the chromatic axis of green (−a*) to red (+a*) and b* being the chromatic axis of blue (-b*) to yellow (+b*). Chroma (C*) and hue angle (h)were also determined using the following equations to describe the color saturation and color hue further:

$$C^* = \sqrt{(a^2 + b^2)}$$

$$h^* = \tan^{-1}(b^*/a^*)$$

The overall color difference (ΔE*) between the PE-infused cream and the control formulation was determined using the equation:

$$\Delta E^* = \sqrt{[(L_2 - L_1)^2 + (a_2 - a_1)^2 + (b_2 - b_1)^2]}$$

where the subscripts 1 and 2 correspond to the control and PE-infused cream samples, respectively [35]. All measurements were conducted in triplicate, and the mean values were reported to ensure reproducibility and analytical accuracy.

Sun Protection Factor (SPF) Analysis

The in vitro sun protection factor (SPF) of the phycoerythrin (PE)-infused facial cream was obtained by UV spectrophotometric method, using a commercial sunscreen cream as the reference [36]. In short, 1 g of cream formulation was weighed properly and placed into a 100 ml volumetric flask. An ethanol-distilled water mixture (40: 60, v/v) was added to the sample to dilute it to its volume and ultrasound was applied to the sample (5 minutes) to extract all the UV absorbing components.

The resulting dispersion was filtered through Whatman No. 1 filter paper, and the first 10 mL of filtrate was discarded to eliminate particulate interference. From the clear filtrate, 5.0 mL was transferred into a 50 mL volumetric flask and diluted to volume with the same ethanol–water solvent system (40:60, v/v). Subsequently, 5.0 mL of this solution was further diluted to 25 mL in a volumetric flask using the identical solvent mixture [37].

The absorbance of the final diluted solution was measured using a UV–Visible spectrophotometer in the wavelength range of 290–320 nm at 5 nm intervals, employing the ethanol–water mixture (40:60, v/v) as the blank. Each measurement was performed in triplicate to ensure reproducibility.

$$SPF = CF \times 290 \sum 320 [EE(\lambda) \times Abs(\lambda)]$$

where EE(λ) represents the erythemal effect at a given wavelength and Abs(λ) is the measured absorbance of the sample. The calculated SPF values reflect the UVB protective potential of the PE-infused facial cream [38].

Drug release study of the PE-infused facial cream

A dialysis diffusion method was used to study the in vitro release profile of phycoerythrin (PE) in the PE-infused facial cream. The accurate amount of the formulation of cream with a fixed content of PE was precisely dispersed in the phosphate-buffered saline (PBS, pH 7.4) and loaded into a previously wetted dialysis membrane (molecular weight cut-off 12-14 kDa) [39]. A dialysis bag was put in a beaker that contained PBS and was stirred with constant gentle stirring at 37°C to replicate physiological skin conditions.

Aliquots of the release medium were taken out at timed intervals (0, 1, 2, 4, 8, 12, and 24 h), then substituted by an equal amount of fresh PBS in order to maintain sink conditions. A UV-visible spectrophotometer was used to measure the quantity of PE released into the receptor medium at the required wavelength (characterized by phycoerythrin) (Schlupp et al., 2011). Equivalent concentrations of free PE solution were exposed to the same release condition to compare them. The release percentage of PE was plotted as a function of time and was computed cumulatively. They were repeated three times, and the results were presented in mean and standard deviation.

In Vivo Evaluation Using Zebrafish (Danio rerio)

The acute toxicity of the PE-infused facial cream was assessed using adult zebrafish (Danio rerio) according to OECD Guideline 203. The study was carried out at Mass Biotech Pvt. Ltd., Chennai, with approval from the Institutional Animal Ethics Committee (IAEC Approval No. MB. /IAEC/25/02/06) and in accordance with CPCSEA guidelines, Government of India. Prior to the experiment initiation, the zebrafish were acclimated over seven days in controlled laboratory environments, such as a 12 h light/12 h dark cycle, continuous stirring, and a regular feeding schedule. Only the healthy zebrafish were chosen and randomly selected into control and treatment groups [40].

The single concentration (100 mg/L) of the PE-infused facial cream was added to the treatment group, whereas the control group was kept in dilution water only. The exposure was conducted under static conditions for 96 h without renewal of the test solution.

Observation of Clinical Signs and Mortality

During the exposure period, zebrafish in both control and treatment groups were observed every 6 h for behavioral changes and mortality. Observable physical deformities, loss of balance, abnormal swimming behavior, difficulties in breathing, change in body color, and other clinical signs were noted [41]. No opercular movement and unresponsiveness to soft touch of the skin were taken to be mortality.

Monitoring of Water Quality Parameters

Water quality parameters were monitored throughout the experiment to maintain stable exposure conditions. The pH of both control and treatment media was measured at 0, 6, and 24 h during the initial phase and at defined intervals over the 96-h exposure period. Temperature was recorded every 6 h on the first day and periodically thereafter to ensure suitable conditions for zebrafish survival and normal behavior [42]. All pH and temperature measurements were carried out using a calibrated ACETEQ pH/temperature meter.

Results and Discussion

Extraction Yield and Purity of Phycoerythrin from *Hypnea valentiae*

The extraction and purification of phycoerythrin (PE) of *Hypnea valentiae* were optimized by evaluating the various methods of extraction and solvents used to get the maximum yield of the pigment without causing destruction of the pigment. The freeze-thaw procedure with phosphate-buffered saline (PBS) yielded the most PE (6.35mg/g of wet biomass) [Fig-1]. This improved recovery is mainly due to repeated ice crystal formation during freeze–thaw cycles, which breaks the algal cell walls and releases intracellular PE, as reported for other red macroalgae [30].

The extraction solvent had a strong influence on PE recovery. PBS performed better than distilled water and saline, likely because its buffering capacity maintains a stable, neutral pH and protects the protein from denaturation during extraction. The same findings were obtained with buffered extraction systems to extract red algae, in which the stability of the pigments and their own solubility improved relative to unbuffered solvents [43]. Other physical techniques have also been described to be used, including liquid nitrogen grinding and mechanical homogenization, but both are difficult to use on a basic scale of laboratory extraction because of the extra steps involved in their preparations (Kumar et al 2025).

Recent studies have highlighted green extraction technologies, such as high-pressure homogenization (HPH) and ultrasound-assisted extraction (UAE), as efficient alternatives for PE recovery. Although HPH has been reported to give greater PE yields in certain red algal species, the freeze-thaw system employed in this experiment gave a greater yield with H. valentiae under mild conditions and undergoing minimal processing, making it useful in the laboratory [9]. Purification of PE was carried out using ammonium sulfate precipitation (60% saturation), followed by dialysis and ion-exchange chromatography. Ammonium sulfate precipitation effectively concentrated the pigment and removed a large proportion of unwanted proteins and polysaccharides. Dialysis was also effective in increasing purity and eliminating residual salts and low-molecular-weight impurities, which contributed to a better stability of the pigment [25]. A further enrichment of PE was achieved in ion-exchange chromatography with a column of Q-Sepharose, which is indicated by the presence of clear elution peaks and the typical reddish-pink color of the purified fractions.

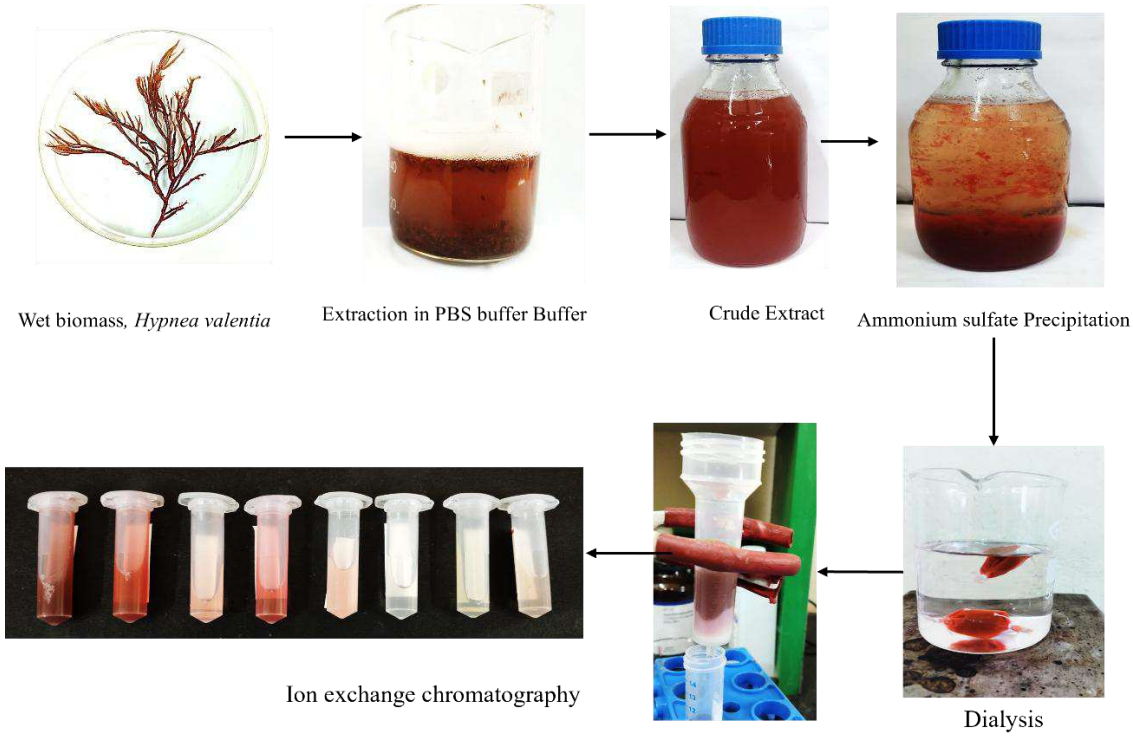


Fig 1. Extraction and purification of the phycoerythrin from *H.valentia*

Fig 1 shows the stepwise extraction and purification of phycoerythrin from *Hypnea valentiae*. Wet algal biomass was extracted using phosphate-buffered saline (PBS) to obtain a crude pigment extract. Proteins were precipitated using ammonium sulfate, followed by dialysis to remove salts and impurities. The extract was then further purified using ion-exchange chromatography, yielding distinct red fractions containing phycoerythrin.

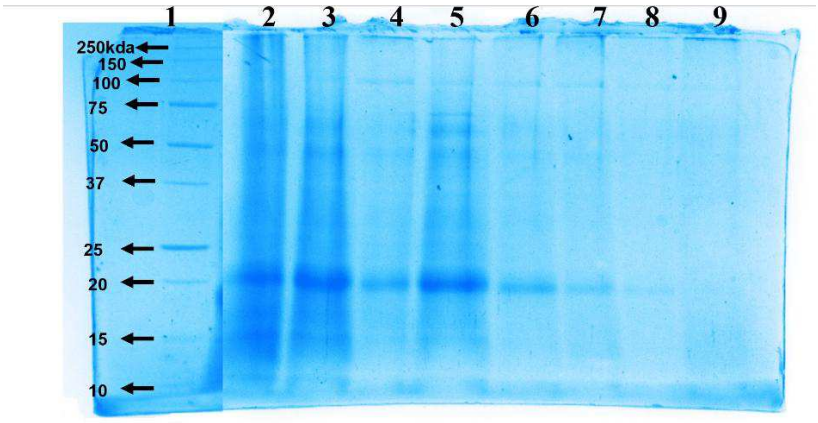


Fig2. SDS PAGE Analysis of purified phycoerythrin

SDS–PAGE revealed a prominent protein band corresponding to approximately 20 kDa, confirming the presence of phycoerythrin. 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 8: eluent 4; lane 9: NaOH wash. The distinct band observed in the elution fractions indicates successful purification of PE.

The analysis of a protein by the SDS-PAGE method revealed a specific protein band at about 20-22 kDa corresponding to the known molecular weight of PE subunits in red algae shown in the [Figure – 2][44]. The fact that there was a gradual fading of this band in subsequent fractions was a confirmation of the successful separation and purification of PE. Purity of PE is usually measured as Purity Index (PI), which is determined as a ratio of absorbance at the PE-specific wavelength and absorbance at 280 nm. According to the literature standards, the purification strategy employed in the current study resulted in PE of a high enough purity to be used in cosmetic and biomedical purposes [18]. In general, *Hypnea valentiae* was found to be a good source of phycoerythrin, offering a compromise between extraction efficiency and purification efficiency, and having a potential for scale-up and subsequent application development.

Purity of PE is usually measured as Purity Index (PI), which is determined as a ratio of absorbance at the PE-specific wavelength and absorbance at 280 nm. According to the literature standards, the purification strategy employed in the current study resulted in PE of a high enough purity to be used in cosmetic and biomedical purposes [45]. In general, *Hypnea valentiae* was found to be a good source of phycoerythrin, offering a compromise between extraction efficiency and purification efficiency, and having a potential for scale-up and subsequent application development.

Spectroscopic and Structural Characterization of PE (UV–Vis, FTIR, CD)

The identity and purity of phycoerythrin extracted from *Hypnea valentiae* (HV-PE) were confirmed by UV- Vis spectroscopy. The purified pigment showed distinct absorption peaks at approximately 498, 539, 560, and a dominant peak at 587 nm [Fig 3]. These absorption bands are typical of R-phycoerythrin, and these are due to the availability of phycoerythrobilin chromophores and phycourobilin chromophores attached to the protein [21]. The high absorption of 540-590 nm indicates that the chromophore is absorbing light well in the green-yellow region, and is a sign that the structure is intact. The sharp and clear peaks also indicate that not much protein denaturation occurred during extraction and purification [46]. The absorption profile that has been obtained is closely related to that of the reported algal phycoerythrin, which means that the pigment is purified and stable.

The secondary structure of HV-PE was analyzed by circular dichroism (CD). A negative band was observed with the spectrum of CD at the wavelength of 210- 220nm, and the negative band is characteristic of proteins containing a high concentration of alpha-helical structure [47]. Analysis revealed that the predominant structure was that of alpha-helix with a lesser proportion of beta-strand, beta-sheet, and random coil structure [Fig -3]. The fact that the protein existed in α-helical content is a positive sign that the protein was in its natural folded form following the purification process. Protein folding is directly related to optical properties and biological activity, so preservation of secondary structure is significant to phycoerythrin [48]. The fact that it had a clear signal at the close of 230 nm also indicates that the aromatic amino acids and chromophores were left in the right position in the folded protein.

Structural integrity of HV-PE was also supported using FTIR spectroscopy. The extensive absorption band at 3448 cm-1 is related to O -H and N -H vibrations, which shows that it has hydrogen bonds and that the

protein backbone is stable [17]. The sharp peak at around 1621 cm -1 indicates the existence of the amide I band, which proves the existence of ordered secondary structures, i.e., alpha-helix and beta-sheet sown in the [Fig-3]. Other bands at 1082 cm -1 were also linked to C -O and C -N vibrations, and this indicated the presence of polysaccharide-protein interactions and functional side chains. The maximum of 671 cm -1 is related to aromatic C-H bending, and it is characteristic of phycobiliprotein chromophores [24]. These FTIR characteristics are all in agreement with reported spectra of native phycoerythrin, and it is clear that the purification procedure did not alter the pigment functionality or the structure of the protein. In general, the overall UV-Vis, CD, and FTIR findings indicate that phycoerythrin extracted from *Hypnea valentiae* retains its native chromophoric and structural values [26]. The retained optical characteristics and maintained protein structure reveal that HV-PE is of decent quality and can be used in further cosmetic and medical applications

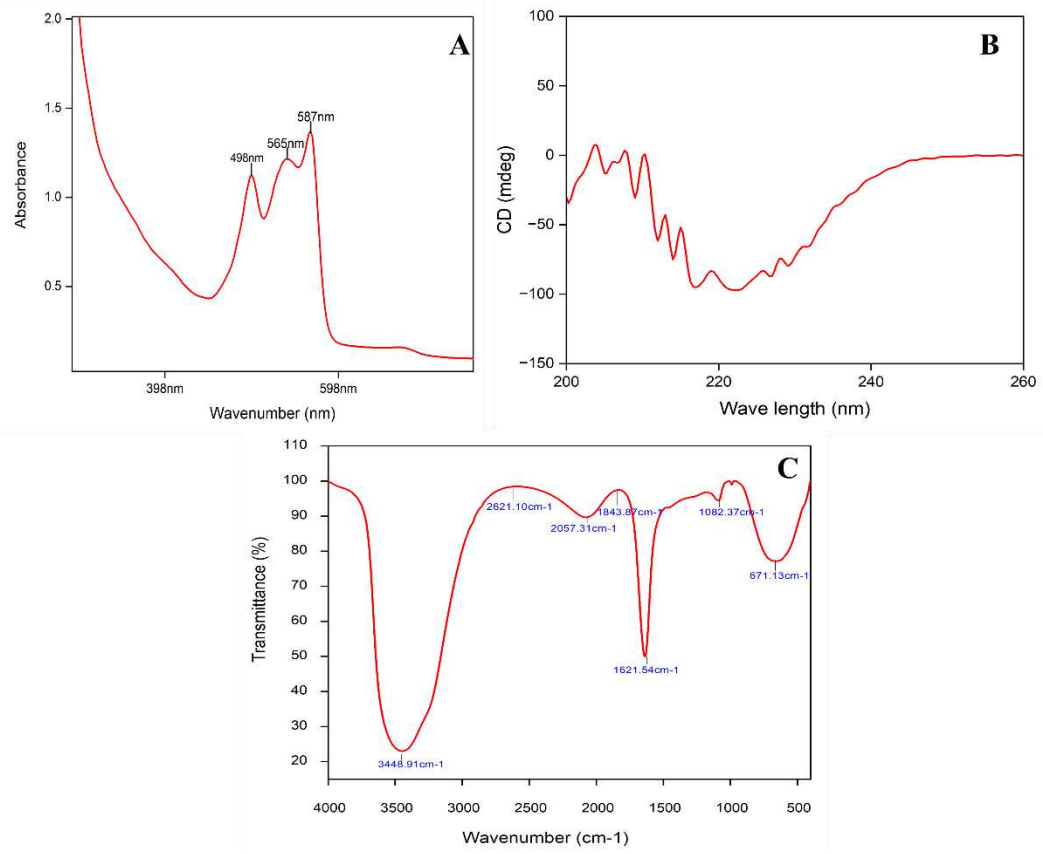


Figure 3. Spectroscopic characterization of purified HV-PE (A) UV–Visible spectrum depicting the characteristic absorption peaks of PE; (B) CD spectrum demonstrating the protein secondary structure of PE; and (C) FTIR spectrum illustrating the functional groups present in PE.

Anticancer Activity of PE on Breast Cancer Cells

To determine cytotoxicity selectivity against MDA-MB-231 human breast cancer cells, the cytotoxic activity of *Hypnea valentiae* phycoerythrin (HV-PE) was assayed against the target cells and compared with normal L929 fibroblast cells. HV-PE stimulated a distinct dose-dependent decrease in cell viability of the cancer cells in 48 h of treatment. The morphological changes of treated MDA-MB-231 cells were characterized by severe cell shrinkage, diminished cell density, and cell adherence loss in evidence of cytotoxic stress and cell death shown in the Figure – 4 [24]. The quantitative assessment indicated that HV-PE has strong anticancer effects with an

IC50 of about 25 ug/mL in the MDA-MB-231 cells. However, HV-PE was much less toxic to L929 fibroblast cells, and the IC50 of this agent was around 79.4 µg/mL. The selective cytotoxic activity of HV-PE on the cancer cells and the almost three-fold increased IC50 in normal cells show the selective cytotoxic effect of HV-PE on cancer cells and indicate a good safety margin [48].

The selective anticancer activity of HV-PE is probably accomplished by the ROS-controlled mitochondrial death. The basal ROS levels in cancer cells remain high, and additional ROS production caused by phycoerythrin can surpass the antioxidant capacity, which results in mitochondrial membrane depolarization and the initiation of intrinsic apoptotic pathways. Earlier research findings indicate that phycoerythrin stimulates apoptosis through ROS accumulation, caspase activation, chromatin condensation, and DNA fragmentation, which justify the mechanistic nature of the current results [26]. The reduced cytotoxicity of L929 cells could be explained by the fact that the antioxidant defense mechanisms of the normal cells are intact and, thus, allow them to maintain the levels of oxidative stress and prevent apoptosis at the levels of cytotoxicity to the cancer cells. This selectivity by ROS largely increases the therapeutic applicability of HV-PE [25].

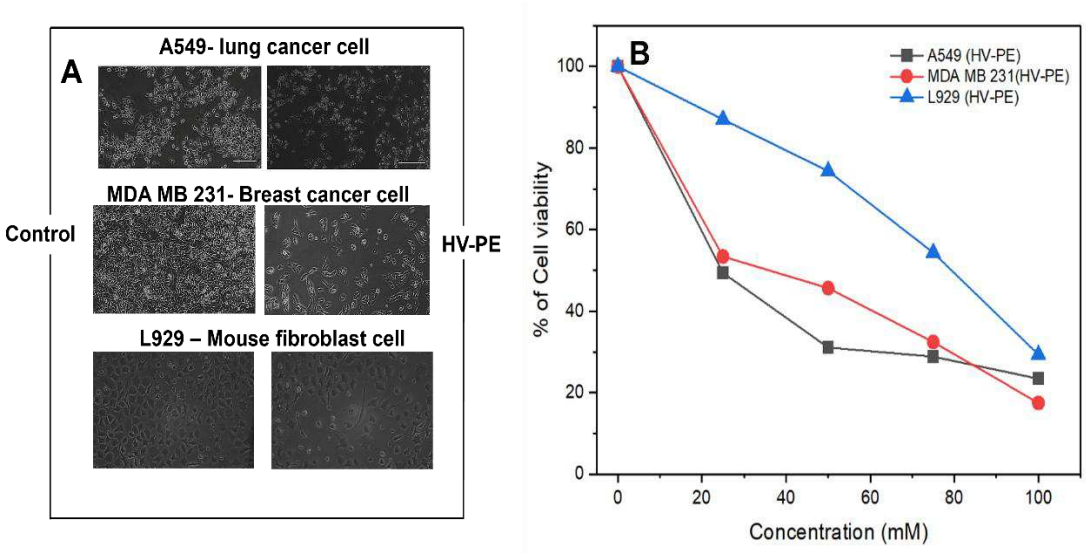
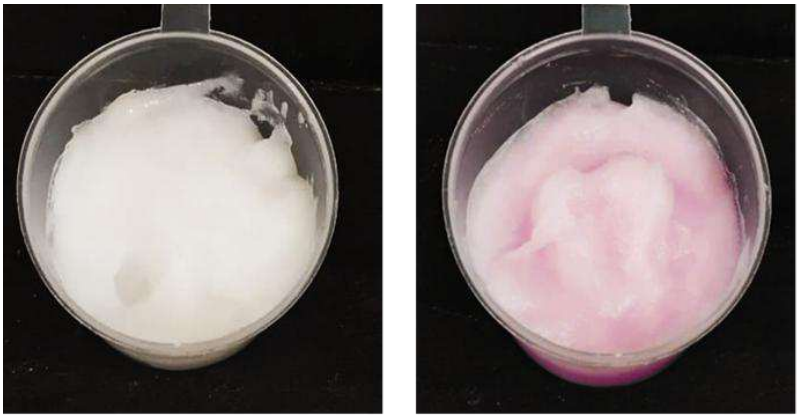


Figure 4 Cytotoxic activity of HV-phycoerythrin (HV-PE). (A) Morphological changes in A549, MDA-MB-231, and L929 cells after 48 h of HV-PE treatment compared with control. (B) Dose-dependent decrease in cell viability of cancer cells, with lower cytotoxicity observed in L929 normal fibroblast cells.

These findings are aligned with previous reports that show cytotoxicity in phycoerythrin-stimulated cancer models in lung (A549), liver (HepG2), colorectal cancer (HCT-116, SW620, CaCo-2) and leukemia (HL-60), and apoptosis, cell-cycle arrest, and activation of mitochondrial pathway were found to be the dominant mechanisms [24,49]. All these results make HV-PE a promising marine-derived bioactive pigment, which can be developed as an anticancer therapeutic or drug-lead candidate.

Characterization of PE-Infused Facial Cream



Control Cream PE infused facial cream

Figure 5 formulation of PE infused facial cream

pH

Topical facial creams are typically advised to maintain a pH between 4.6 and 6.7 in order to be similar to the natural pH of the skin surface (4.0 to 6.0) and reduce irritation, without affecting the stability of ingredients [50]. The control cream has a pH of 7.48, and the PE-infused cream had a slightly lower value of 7.12, as indicated in the present study were shown in the Figure 5. The slight reduction in the addition of phycoerythrin shows that there is good compatibility with the base formulation and the presence of no destabilizing influence on the emulsion [30]. The pH did not vary significantly, although it was a little higher than the normal acidic skin range (5.5–6.7); this indicates that the pH was constant and comparable to a range of plant-extract creams published in literature (5.1–5.4), which experienced little change over time, indicating that the formulation was stable and topically suitable.

Viscosity

The pseudoplastic (shear-thinning) effect is regularly observed with cosmetic creams, with viscosity declining with applied shear, allowing them to be very easily spread when applying [51]. The viscosities of herbal and antioxidant creams are reported to be varied (around 600-9000 cP) in accordance with the content of the oil phase and the level of emulsifier. The viscosity in this experiment varied between 3510 cP (control) and 3060 cP (PE cream) at 100 rpm (LV-04, spindle number 64), and the torque values were found to be within the acceptable measurement values [Table -1]. Reduction in such occurrences following the addition of bioactive extracts has been reported and is commonly associated with the alterations in the emulsion droplet structure [52]. The obtained values fall within the realistic range of facial creams, which implies structural stability with enhanced flow properties.

Table 1- Viscosity of the facial cream

Parameter	Control Cream	PE infused facial cream
Torque	58.5%	51.0%
Viscosity	3510 cP	3060 cP

Spindle	LV-04	LV-04
Spindle multiplier	#64	#64
Speed	100 RPM	100 RPM

Spreadability

The major quality parameter that affects consumer acceptance and the even coverage of the skin is spreadability. The values of literature on the spread diameter and spreadability indices of creams spread widely depending on the composition and temperature, and a negative correlation with the viscosity is well documented [52]. In line with the same direction, PE-infused cream exhibited higher spreadability (80.71 g cm/s) as compared to the control (74.79 g cm/s), with a slight increase in spread diameter and reduction in the time of spreading [Table -2]. The enhancement implies better application performance after PE incorporation, which was probably caused by the slight decrease in viscosity. Improved diffusion facilitates easy topical application and film development.

Table 2- Spreadability of the facial cream

	Diameter(cm)	**Time (s)**	**Length (cm)**	**Spreadability (g.cm/s)**
Control Cream	6.9	13.37	10	74.79
PE infused facial cream	7	12.39	10	80.71

Colorimetric and SPF Enhancement by PE

Color measurement is important in cosmetic products to ensure good appearance and color stability. It is usually evaluated using the CIELAB system, where L* indicates lightness, a* shows red–green tone, and b* shows yellow–blue tone. Many studies report that natural pigments from plants and marine sources remain stable during storage and can safely replace synthetic colors. Phycoerythrin and phycocyanin are marine pigments that are particularly useful due to offering color and antioxidant properties [49].

Colorimetric analysis was used in the current research, and the effect of HV-PE incorporation on the cream's appearance was clear. The PE-impregnated cream was found to be lighter (L* =38.97) compared to the control (35.57) and had a greater ΔL (5.42) than the 2.02, portraying better brightness. a* value moved to the red area and b* changed, which proved that phycoerythrin was expected to contribute to the red-yellow tonality [53]. Chroma and angle of hue variations also helped in the enrichment of the pigments, and the overall color change (ΔE) was significantly greater in the PE cream (5.53) compared to the control (2.09), proving the presence of visually discernible color additions to the formulations [Table – 3] [51]. The HV-PE extract was found to possess a high chromaticity, which further indicates that it is a good natural cosmetic colorant with functional bioactivity.

Table -3 Colorimetric analysis of the formulated cream

COLORIMETRIC ANALYSIS									
	Control Cream			PE infused facial cream			HV-PE		
Parameter	Value	Δ (Difference)	Interpretation	Value	Δ (Difference)	Interpretation	Value	Δ (Difference)	Interpretation
L* (Lightness)	35.57	ΔL* = 2.02	Light++	38.97	ΔL* = 5.42	Light++	34.14	ΔL* = 0.59	Light++
a* (Green-Red)	-2.26	Δa* = 0.17	-	-1.91	Δa* = 0.52	Red++	1.95	Δa* = 4.38	Red++
b* (Blue-Yellow)	0.59	Δb* = 0.48	Yellow+	1.07	Δb* = 0.96	Yellow++	3.76	Δb* = 3.65	Yellow++
C* (Chroma)	2.34	ΔC* = -0.10	-	2.19	ΔC* = -0.24	-	4.24	ΔC* = 1.80	-
h* (Hue angle)	165.28	Δh* = -0.50	-	150.72	Δh* = -1.07	-	62.61	Δh* = -5.41	-
ΔE* (Color difference)	-	2.09	-	-	5.53	-	-	5.73	-

The Factor of Sun Protection (SPF) is a UVB protection factor that is typically measured in vitro by UV spectrophotometry by using the Mansur formula between the wavelengths of 290 and 320 nm were shown in the Table -4. Reported literature on SPF indicates that it can be enhanced by the use of physical filters (TiO_2, ZnO), marine bioactives like mycosporine-like amino acids, and plant extracts containing antioxidants, and that antioxidant combinations are synergistic in enhancing the photoprotection. Yet, there is variability between labeled and measured SPF in commercial products, which demonstrates the importance of formulation-level validation [53]. As per these principles, PE-enriched cream in this experiment showed a better UV absorbance throughout the UVB spectrum and a calculated SPF of approximately 24, as opposed to approximately 12 in the control base. This is due to the chromophoric protein structure of phycoerythrin that produces greater UV absorption and antioxidant defense against photo-induced oxidative stress. In this way, HV-PE is a dual-action cosmetic ingredient, providing both natural color enhancement and a quantifiable increment of the SPF, which justifies its usefulness in the multifunctional bioactive skincare preparations.

Table 4. Calculation of sun protection factor (SPF) using the Mansur equation based on UV absorbance values of control and HV-PE test formulation across wavelengths 290–320 nm.

Wavelength (nm)	**EE × I**	**Absorbance (Control)**	**EE × I × Abs (Control)**	**Absorbance (Test)**	**EE × I × Abs (Test)**
290	0.0150	0.90	0.0135	1.95	0.0293

295	0.0817	1.05	0.0858	2.10	0.1716
300	0.2874	1.25	0.3593	2.45	0.7041
305	0.3278	1.30	0.4261	2.60	0.8523
310	0.1864	1.20	0.2237	2.38	0.4436
315	0.0839	1.00	0.0839	2.05	0.1720
320	0.0180	0.85	0.0153	1.72	0.0310
Σ (EE × I × Abs)			**1.2076**		**2.404**

Correction factor (CF) = 10

- **SPF (Control)** = 10 × 1.2076 = **12.08**
- **SPF (PE-Test Cream)** = 10 × 2.404 = **24.04**

In Vitro Bioactivity of the PE-Infused Cream

Antioxidant Efficiency of the Formulated Cream

The antioxidant activity of phycoerythrin (PE) extract and the PE-enriched facial cream was evaluated using DPPH and ABTS radical scavenging assays, which together provide a reliable estimate of free-radical neutralization capacity under different reaction environments were shown in the figure- 6 [54]. Both assays demonstrated a clear concentration-dependent antioxidant response.

In the DPPH assay, PE extract produced progressive radical scavenging from 32.4% at 20 µg/mL to 78.6% at 100 µg/mL, with an estimated IC_{50} of ~46.8 µg/mL, indicating strong hydrogen-donating ability. PE-impregnated cream also maintained significant activity with an inhibition range of 28.1 -69.3 percent in the same concentration range, implying that addition into the emulsion matrix did not significantly affect antioxidant activity. The same trend was noticed in the ABTS assay [55]. PE extract inhibited 35.7, 49.8, 63.5, 74.2, and 82.1 at 20-100 ug/mL, respectively, and the IC 50 was approximately 41.3 ug/mL. The PE cream exhibited an inhibition percentage of 30.4-71.6, which validates that the active antioxidant ingredients were bioavailable post-formulation. The reaction of DPPH and ABTS leads to the conclusion that PE antioxidants can be used in both hydrophilic and mixed radical systems [19].

These findings are similar with a report of earlier findings on red algal phycoerythrins, which often exhibit DPPH and ABTS scavenging activities of 55-80% at high concentrations. The differences in activity reported in different studies can be explained by the purity of pigments, mode of extraction, and thermal stability. Overall, the available results can confirm the usefulness of PE as a strong natural antioxidant that could be used topically and in cosmetics [20].

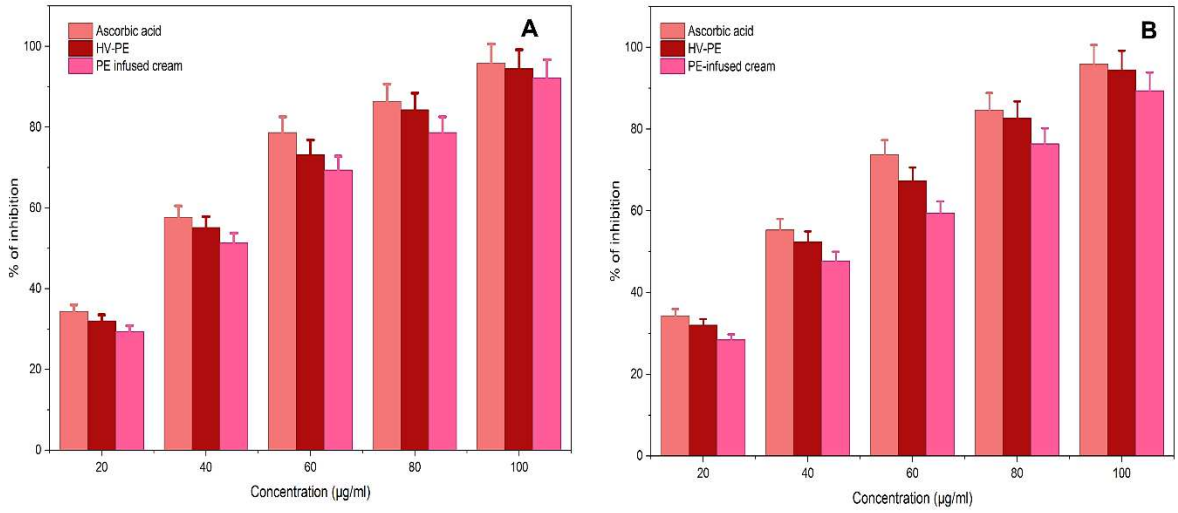


Figure 6 Antioxidant activity of HV-phycoerythrin (HV-PE) and PE-infused cream. (A) DPPH radical scavenging activity and (B) ABTS radical scavenging activity

Anti-Inflammatory Potential of the Formulated Cream

The anti-inflammatory activity of HV-phycoerythrin (HV-PE) extract and its cream formulation was tested using both the egg albumin denaturation and the protein denaturation assays at a concentration of 20-100 µg/mL. Both experimental models had a distinct concentration-dependent inhibition of heat-induced protein denaturation in all the tested samples were shown in the Figure -7 [20]. Diclofenac sodium presented a percent inhibition of 14.06% in 20 µg/mL of the egg albumin, and a percent inhibition of 88.32% in 100 µg/mL of the egg albumin. The HV-PE extract was found to have a similar level of inhibition, with the value increasing up to 19.19% and then 87.13% at the different concentrations of the extract, and the PE-infused cream had an inhibition of between 15.30 and 81.64%, respectively, with the latter demonstrating a high degree of retention of bioactivity after formulation [22]. The same trends of inhibition were observed during the protein denaturation assay, which also supported the reproducibility and reliability of the observed anti-inflammatory response.

The obtained IC50 values were 46.6 ug/ml of diclofenac sodium, 52.1 ug/ml of HV-PE extract, and PE-infused cream: 60.5 ug/ml. The IC50 of HV-PE extract was very similar to the reference drug, showing a strong anti-inflammatory activity [23]. The cream formulation had a comparatively higher IC 50 value, but the activity was nevertheless high, indicating that the functional bioactivity is well preserved in the topical matrix, and a small loss can be only explained by diffusion limitations associated with the formulation.

The current results are supported by the earlier studies that reported phycoerythrin to be a powerful natural anti-inflammatory agent. B-phycoerythrin and R-phycoerythrin have both been shown to have a strong in vitro anti-inflammatory effect, and R-phycoerythrin has been reported to also have antioxidant, antibacterial, and hepatoprotective properties [56]. Anti-inflammatory solutions. Red macro-algae have been identified as useful sources of bioactive phycobiliproteins, including Palmaria palmata, Kappaphycus alvarezii, and Solieria filiformis, which support the biological importance of algal-derived pigments. These observations are also supported by clinical evidence of natural topical preparations. Fermented pomegranate peel extracts in creams have demonstrated a considerable decrease in redness of the skin under prolonged use, whereby the increased

bioactivity of aglycone flavonoids produced in the fermentation process is attributed to the effects [23]. Similarly, recipes that include pomelo peel extract and polyherbal preparations of aloe, neem, turmeric, and mint have been reported to be very well tolerated dermally in humans, and no irritation was observed, which serves to indicate the safety and effectiveness of plant-derived anti-inflammatory agents.

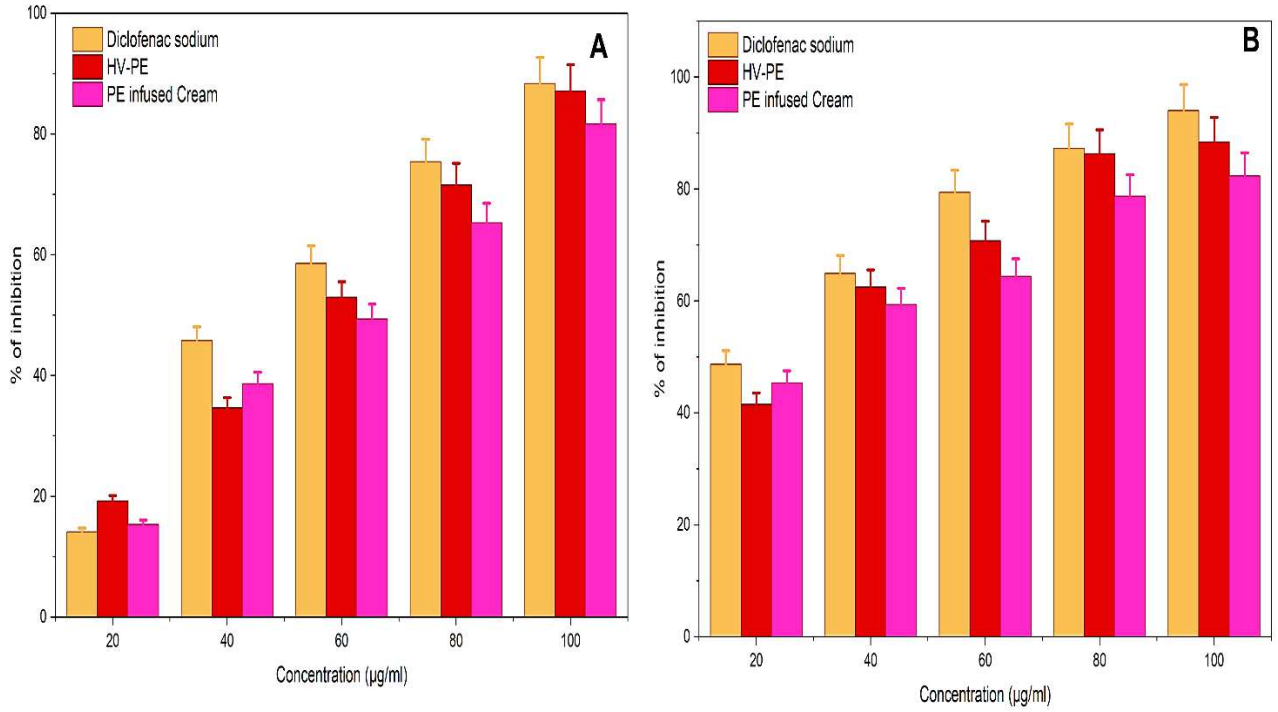


Figure 7 Anti-inflammatory activity of HV-phycoerythrin (HV-PE) and PE-infused cream. (A) Inhibition of egg albumin denaturation and (B) inhibition of protein denaturation

The marine-derived bioactives, including fucoidan, ectoine, fucoxanthin, and phlorotannins, have been reported to regulate the inflammatory mechanisms through reinforcing the barrier integrity and oxidative stress, as well as inhibiting the activities of matrix-degrading enzymes, collagenase, and elastase. Similar protective effects are displayed by phycoerythrin, especially when it comes to maintenance of membrane integrity and cell metabolic activity during oxidative stress conditions [57]. Moreover, phytochemicals, including the α-mangostin and bioactive compounds of the Aloe vera and turmeric, can have synergetic effects when they are included in topical delivery systems. Taken together, these results make HV-PE a promising marine-based bioactive with a high translational potential to be used as a pharmaceutical and cosmeceutical anti-inflammatory agent.

Antimicrobial Activity of the PE-Loaded Cream

The agar well diffusion method was used to determine the antimicrobial activity of HV-phycoerythrin (HV-PE) extract and PE-impregnated facial cream against the microbes Staphylococcus aureus, Bacillus subtilis, Escherichia coli, and Staphylococcus epidermidis, and to measure the zones of inhibition in centimeters (mean ± SD) in Figure 8 and Table 5 &6. The HV-PE extract was found to have concentration-dependent antibacterial activity within the range of 200-1000 µg/mL [24]. Inhibition was not observed against S. aureus, with a higher concentration of 0.10 + 0.25 cm at 600 µg/mL, 0.16 + 0.23 cm at 800 µg/mL, and 0.37 + 0.73 cm at 1000 µg/mL,

which implies moderate susceptibility. B. subtilis, in its turn, was highly sensitive to the extract, as the size of inhibition zones grew to 1.62 ± 0.25 cm (600 µg/mL), 1.86 ± 0.17 cm (800 µg/mL), and 1.98 ± 0.21 cm (1000 µg/mL). In the case of E. coli, the inhibition was observed in concentrations of 400 µg/mL and above, and zones of 0.10 cm, 0.36 cm, 1.53 cm, and 1.23 cm were found to be 0.40, 0.60, 0.80, and 1.0, respectively, showing moderate antibacterial activity. All in all, the extract showed greater activity against Gram-positive strains, especially B. subtilis, which could be explained by the fact that the outer lipopolysaccharide membrane, which usually prevents the penetration of bioactive compounds into Gram-negative bacteria, was absent

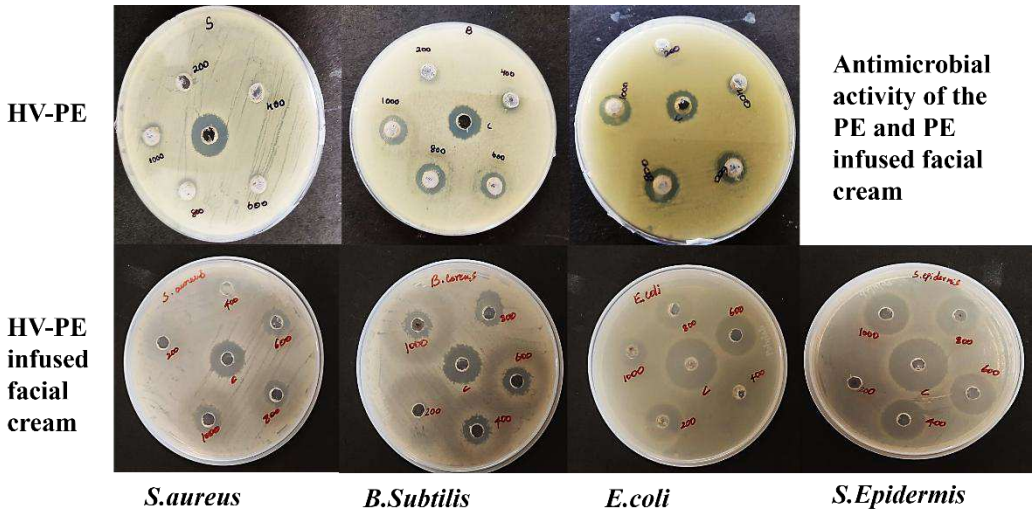


Figure 8 Antimicrobial activity of HV-phycoerythrin (HV-PE) and HV-PE–infused facial cream.

The antimicrobial activity of the PE-impregnated facial cream was better and more consistent than that of the crude extract [58]. Inhibition zone against S. aureus rose gradually with an increase in concentration in each case from 1.21 cm to 0.1 cm (400 µg/mL) to 1.77 cm to 0.73 cm (1000 µg/mL). In case of B. subtilis, the inhibition level was 1.17 + -0.6 cm at 2000 µg/ml and 1.81 + -0.21 cm at 1000 µg/ml, which confirms the long-term antibacterial efficacy.

Table 5 Antimicrobial activity of the HV-PE

It is important to note that the cream was quite active towards E. coli, as the inhibition zones expanded to 1.28± 0.5 cm (200 µg/mL) and then to 1.96 ± 0.92 cm (1000 µg/mL), indicating the enhanced diffusion and solubility of the active compound in the formulation matrix. The maximum activity was seen in the case of S. epidermidis, with the inhibition zone of 1.31 +/- 0.4 cm (200 ug/mL) to 2.11 +/- 0.4 cm (1000 ug/mL), which indicates high activity in connection with microorganisms of the skin.

The increased antimicrobial efficacy of the PE-impregnated cream could possibly be explained by the improved stabilization, dispersion, and sustained release of phycoerythrin in the topical base that might result in better contact with the microbial cell membranes [59]. Phycoerythrin has been reported to have antimicrobial action by disrupting the membrane, interfering with cellular metabolism, and causing oxidative stress in bacterial cells. The high activity against the Gram-positive bacteria is consonant with the structural differences in bacterial cell walls that are easily penetrated by proteinaceous bioactives [60]. All these data indicate that HV-PE has great antibacterial properties, and its integration into a topical cream-form preparation makes it more applicable in practice as a multifunctional cosmeceutical agent that can both provide antimicrobial effects and possess anti-inflammatory effects that have been shown before.

Table 6 Antimicrobial activity of PE infused facial cream

In Vivo Performance in Zebrafish

Observation of Clinical Signs and Mortality

Antimicrobial activity of HV-PE [Zone of inhibition (cm)]						
	Antimicrobial activity of PE infused facial cream [Zone of inhibition (cm)]					
	Control	**200 µg/ml**	**400 µg/ml**	**600 µg/ml**	**800 µg/ml**	**1000 µg/ml**
	Control	**200 µg/ml**	**400 µg/ml**	**600 µg/ml**	**800 µg/ml**	**1000 µg/ml**
S.aureus	2.12±0.1	-	-	0.1±0.25	0.16±0.23	0.37±0.73
S.aureus	1.7±0.2	-	1.21±0.1	1.32±0.25	1.56±0.23	1.77±0.73
B.Subtilis	1.8±0.3	0.12±0.6	0.19±0.5	1.62±0.25	1.86±0.17	1.98±0.21
B.Subtilis	2.1±0.3	1.17±0.6	1.21±0.5	1.34±0.25	1.76±0.17	1.81±0.21
E.coli	1.72±0.2	-	0.1±0.3	0.36±0.54	1.53±0.07	1.23±0.92
E.coli	2.4±0.2	1.28±0.5	1.42±0.3	1.68±0.54	1.81±0.07	1.96±0.92
S.Epidermis	2.6±0.3	1.31±0.4	1.54±0.4	1.77±0.7	1.98±0.1	2.11±0.4

Zebrafish in control and treatment groups were closely observed during the time (96 h) of exposure during the first phase and periodically until the end. There were no behavioural abnormalities, including erratic swimming, hyperactivity, lethargy, loss of equilibrium, surface gasping, or abnormal opercular movement. The fish retained normal shoaling behaviour and vigorous swimming occurrences, which demonstrated that the fish had no neurotoxic or respiratory distress [40].

Besides, there were no external morphological defects, pigmentary changes, or observable lesions. There was no alteration in the reactions to light touch, and the opercular movements did not differ during the period of observation. Notably, neither of the groups exhibited any mortality in the 96-h exposure. These results indicate that acute toxic effects were not caused by the tested formulation in the experimental conditions used.

Monitoring of Water Quality Parameters

Water quality parameters were systematically monitored to ensure stable environmental conditions and to eliminate confounding stress factors. The pH of both control and treatment media was measured at 0, 6, and 24 h during the initial phase and at defined intervals throughout the 96-h exposure. Temperature was recorded every 6 h on the first day and periodically thereafter. The pH and temperature values remained within the acceptable physiological range for zebrafish survival and normal metabolic function. No abrupt fluctuations were detected between control and treatment groups. Measurements were performed using a calibrated ACETEQ pH/temperature meter, ensuring precision and reliability. Maintaining stable physicochemical conditions is critical in acute toxicity testing, as deviations in pH or temperature can alter metabolic rate, oxygen consumption, and toxicant bioavailability [41].

Table.7 Summary of Acute Toxicity Observations in Zebrafish (96-h Exposure)

Parameter	Control Group	Treatment Group	Observation
Mortality	0%	0%	No deaths recorded during 96 h
Swimming behaviour	Normal	Normal	No erratic or lethargic movement
Equilibrium	Maintained	Maintained	No loss of balance observed
Opercular movement	Normal	Normal	No respiratory distress
Response to touch	Responsive	Responsive	No signs of neurological impairment
External deformities	Absent	Absent	No visible abnormalities
Pigmentation changes	None	None	Normal body coloration
pH	Within optimal range	Within optimal range	No significant fluctuation
Temperature	Stable	Stable	Maintained within recommended range

Histopathological Evaluation

The histopathological examination was conducted to determine any possible tissue-level changes that may have occurred after acute exposure. Zebrafish of both control and treatment groups were sacrificed at the end of the experimental period of 96 hours and processed for microscopic analysis of key organs (gills, liver, kidney, and muscle tissue). Normal histoarchitecture was observed in all organs of the control group in Fig-9[61]. The gills showed intact primary and secondary lamellae lacking any signs of epithelial lifting, fusion, hyperplasia, and necrosis [40]. The hepatocyte architecture in the liver was normal with intact cytoplasm and centrally placed nuclei, but no vacuolation or inflammatory infiltration. Renal tissue was normal; glomeruli and renal tubules were not degenerated or congested. Muscle fibers were not disoriented and structurally intact with no evidence of myofibrillar damage or inflammatory alteration [42].

In the same way, no significant histopathological changes were observed in the treatment group receiving the test formulation (100mg/L) in relation to the control group. There was no edema, epithelial damage, or excess secretion of mucus in the gill lamellae. The hepatic tissue had normal cellular morphology, hepatocyte integrity, and no fatty necrosis or degeneration. The kidney had normal glomerular structure and tubular arrangement, and muscle tissue did not have degenerative alteration [40]. The lack of tissue-level abnormality supports the clinical observations and mortality data and proves that acute exposure to the test substance did not cause structural or pathological loss in the organs of zebrafish. Respondent histopathological results thus support the positive safety profile of behavioural and survival tests, which confirms the non-toxicity of the formulation when exposed to acute conditions [41].

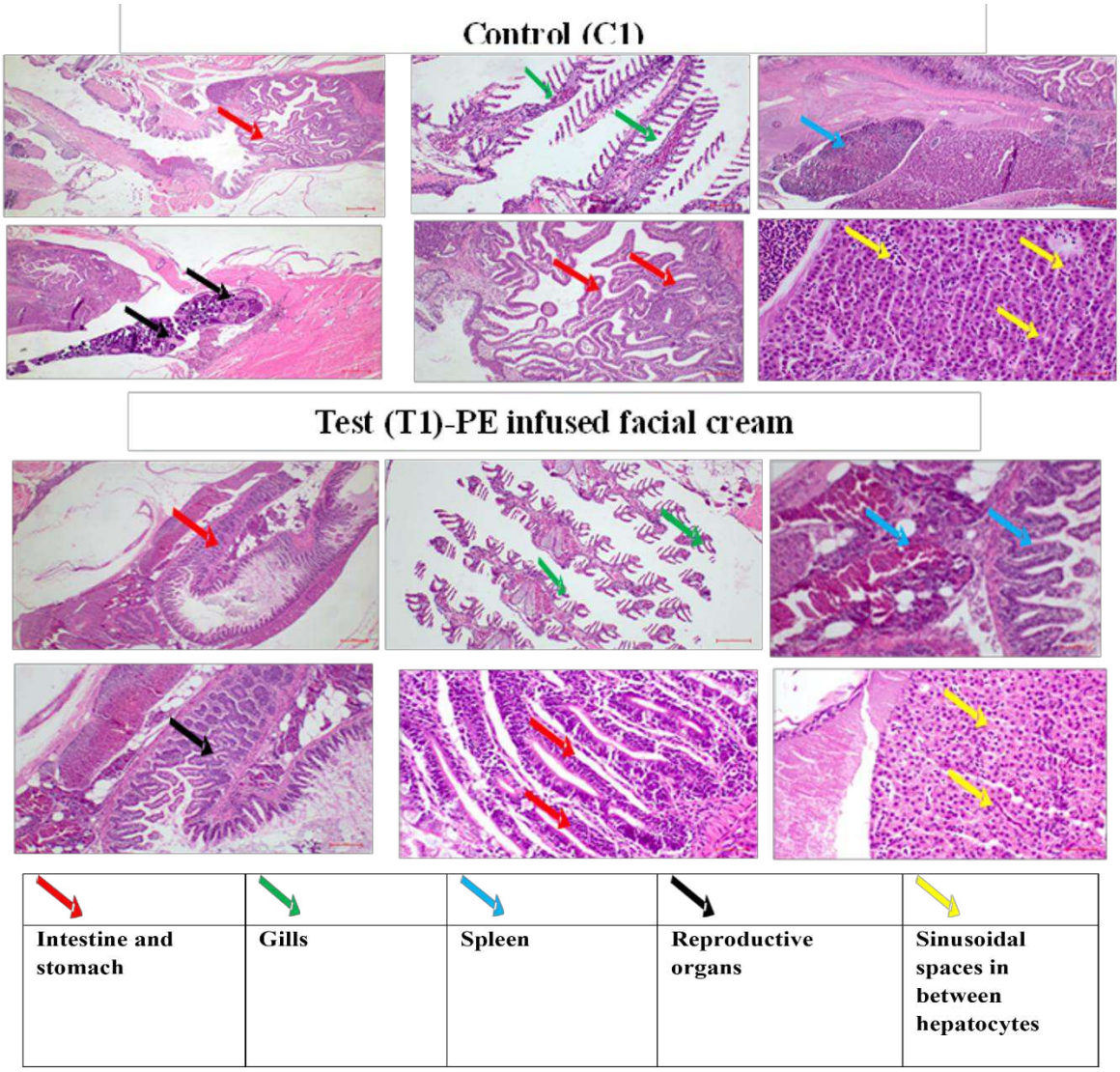


Figure 9 Histopathological evaluation of zebrafish tissues

Conclusion

Hypnea valentiae (HV-PE) phycoerythrin was extracted and attempted to be incorporated into a topical cream formulation, with its characterization and extensive functional and biological assessment carried out. The developed cream had reasonable physicochemical properties, such as homogeneous consistency, topical pH, sufficient spreadability, and formulation stability, which would allow its use in topical application without interfering with the integrity of the bioactive protein.

The HV-PE antioxidant activity was validated using the DPPH and ABTS radical scavenging assays, and it was shown that HV-PE was active at high concentration in both cases. The IC 50 values of the extract were found to be high in free radical neutralizing capacity, and more importantly, the antioxidant activity remained high after being incorporated into the cream matrix. The agreement between the results of DPPH-ABTS concordance indicates that HV-PE is a versatile antioxidant in terms of its ability to scavenge hydrophilic and mixed-system radicals. These results support the present antioxidant capacity of phycobiliproteins as powerful natural macromolecules.

Further analyses of the biological effectiveness of HV-PE were made by anti-inflammatory testing using egg albumin and protein denaturation assays. The extract had strong in vitro anti-inflammatory activity as it showed considerable inhibition of heat-induced protein denaturation with IC 50 values equal to diclofenac sodium. Despite the marginal increase of the cream formulation in terms of IC50 as compared to the pure extract, a significant activity could be maintained, and it was confirmed that the HV-PE remained stable and could be used with the topical vehicle without losing its functionality. The outcome shows the therapeutic importance of phycobiliproteins that are marine-derived as the inherent inflammatory response modulators. The use of SPF assessment showed an improvement in UV-protective properties of HV-PE-enriched formulation as compared to those of the base cream, indicating that phycoerythrin is involved in photoprotective activity by the chromophoric protein scaffold. The overall antioxidant, anti-inflammatory, and UV-absorbing effects show the existence of a multifunctional protective effect that is useful in dermal practice.

The analysis of acute toxicity through the model of zebrafish did not demonstrate any severe mortality, behavioral changes, or histopathological changes in groups with treatment, which proved the biocompatibility and safety of HV-PE at assessed concentrations. The consistency of the toxicity evaluation was also confirmed by consistent environmental conditions during the exposure.

On the summary of the results indicate that phycoerythrin of the *Hypnea valentiae* is a biologically active marine macromolecule with high antioxidant, anti-inflammatory, and photoprotective capacities, and has a positive safety profile. The ability of HV-PE to be incorporated in a stable cream formulation is another indication of its promise as a multifunctional, natural ingredient that can be used in cosmeceutical and biomedical research. The translational potential of its use in advanced dermal formulations will be further proven by future studies on the mechanistic pathways and clinical validation.

Credit statement

Sowmiya Kadalmani: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft. **Sirajunnisa Abdul Razack:** Methodology, Validation, Writing – review & editing. **Ravishankar Krishnan:** Resources, Visualization, Data interpretation, Writing – review & editing. **Renganathan Sahadevan:** Supervision, Project administration, Validation, Writing – review & editing.

Conflict of 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.

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