

EXTRACTION AND ANTIOXIDANT ACTIVITY OF CAROTENOID FROM ALGAE *Chaetomorpha linum*

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ABSTRACT

Carotenoids are natural pigments that are found in plants, algae and microorganisms and have antioxidant, anti-inflammatory and disease prevention properties. The effect of various factors on carotenoid extraction from *Chaetomorpha linum* by static maceration and ultrasound-assisted extraction (UAE) was investigated. The effect of five solvents (ethanol, methanol, n-hexane, petroleum ether and acetone), the static extraction time (60, 90, 120, 150 and 180 minutes), the static extraction temperature (30, 40, 50, 60 and 70 °C) and the solid to solvent ratio (1:20, 1:25, 1:30, 1:35 and 1:40 w/v) were systematically investigated. Ultrasound-assisted extraction was examined at solid-to-solvent ratios (1:30, 1:35, 1:40, 1:45 and 1:50 w/v), amplitudes (20, 25, 30, 35 and 40%) and sonication times (5, 10, 15, 20 and 25 minutes). Use of response surface methodology (RSM) followed for the optimisation of the ultrasound-assisted extraction conditions. The antioxidant activity of the extracts obtained was assessed by two different assays: FRAP (ferric reducing antioxidant power) and RP (reducing power). The ultrasound assisted extraction process produced a higher amount of carotenoids (3.016 mg/g DM) than static maceration (2.819 mg/g DM). The RSM optimum was a solid-to-solvent ratio of 1:46 w/w, 36.3% amplitude and 16.39 minutes of sonication. Antioxidant activity was confirmed by both FRAP and RP. The findings indicated that *C. linum* could be a valuable source of bioactive carotenoids and ultrasound-assisted extraction could enhance the yield and antioxidant capability for food and biomedical uses.

Keywords: Antioxidant, carotenoid, *Chaetomorpha linum*, extraction, ultrasound.

1. INTRODUCTON

Chaetomorpha linum has an unbranched, stiff appearance and can be light or dark green in colour [1]. The species is frequently seen in shrimp ponds in Mekong Delta, where it can overgrow and interfere with the process of photosynthesis, absorb dissolved oxygen and lower the water quality leading to low aquaculture productivity [2]. *C. linum* is known as a natural source of pigments which can be used as food colourants and have potential health benefits [3]. Most of the previous studies have been performed on the extraction of chlorophyll from *C. linum* [4] while carotenoid-rich microalgae such as *Dunaliella salina*, *Chlorella*, *Haematococcus* and *Spirulina* have been studied [5]. Substantial pigment accumulation has been reported, as the total chlorophyll content of *C. linum* is 37.30 ± 4.72 mg/gDM under optimal conditions [2]. But, there is still limited research about its carotenoids as compared to chlorophyll, which means that there is potential for using its natural pigments.

Carotenoids are lipophilic isoprenoid pigments found in a variety of organisms, including primarily photosynthetic organisms like plants and algae, some fungi and bacteria [6, 7]. They have antioxidant and anti-inflammatory properties and may contribute to eye protection, cardiovascular health and cancer prevention [8]. Since animals cannot synthesize carotenoids endogenously, dietary intake from natural sources is essential [9]. In this context, algae have emerged as promising alternatives to terrestrial sources due to their high carotenoid content, rapid growth, and environmental sustainability. For example, *Dunaliella salina* is widely studied for its β -carotene production and related health benefits [10, 11]. Some algal species, such as *Haematococcus pluvialis*, have been reported to

contain around 3.53 g/100 gDM of carotenoids, serving as a reference for evaluating the pigment potential of *C. linum* in this study [12].

Currently, various extraction techniques have been developed to recover bioactive compounds from plants, including supercritical CO₂ extraction, microwave-assisted extraction, enzyme-assisted extraction, and conventional solvent-based methods [13]. Static maceration is a widely used traditional method, based on the diffusion of solutes along concentration gradients. It is simple, easy to implement, and suitable for heat-sensitive compounds [14]. In contrast, ultrasound-assisted extraction (UAE) relies on cavitation, which disrupts cell walls and enhances mass transfer between the material and the solvent, thereby improving the extraction efficiency of target compounds [15]. Prokopov et al. (2017) extracted carotenoids from tomato peels using UAE and demonstrated that the application of ultrasound significantly improved the carotenoid recovery yield, shortened extraction time, and reduced solvent consumption compared to conventional maceration [16].

Oxidative stress refers to an imbalance between free radicals and the antioxidant defense system, leading to cellular damage and various diseases [17]. Electron - donating substances (reducing agents) help maintain redox balance and mitigate the harmful effects of free radicals [18]. Two common methods used to evaluate the reducing capacity of bioactive compounds are the ferric reducing antioxidant power (FRAP) assay and the reducing power (RP) assay. The FRAP assay operates on the principle of reducing Fe³⁺ to Fe²⁺ under acidic conditions, forming a colored complex with 2,4,6-tripyridyl-s-triazine (TPTZ), which reflects the antioxidant potential of the sample [17]. RP, on the other hand, measures the overall electron-donating ability by reduction of ferricyanide to ferrocyanide, which is indicative of reducing activity [19]. Thus, using both FRAP and RP results in a more complete and accurate determination of the antioxidant activity of plant extracts. Carotenoids are known to have potent antioxidant properties, and have been linked to various possible health benefits such as cardiovascular disease prevention, UV damage prevention and reducing inflammation [20, 21]. Algal carotenoids also exhibit promising antioxidant bioactivity.

While carotenoid extraction from several natural sources was investigated in previous studies, not much information is available on optimising carotenoid extraction from *C. linum* and on comparing the conventional and modern extraction methods. In this study, factors affecting extraction efficiency using conventional maceration and ultrasound-assisted extraction were investigated, and their yields were compared. RSM optimised material-to-solvent ratio, ultrasound amplitude and extraction time. The antioxidant activity of carotenoids extracted was determined by FRAP and RP assays. This study could offer the conditions for carotenoid extraction from *C. linum* algae, which is a platform for further studies related to bioactive properties of the obtained extract. Therefore, this study was conducted with the following objectives: (1) To compare the carotenoid extraction efficiency from *C. linum* using conventional static maceration and ultrasound-assisted extraction (UAE); (2) To optimize the ultrasound-assisted extraction conditions using response surface methodology (RSM), focusing on the material-to-solvent ratio, ultrasonic amplitude, and extraction time; and (3) To preliminarily evaluate the antioxidant activity of the carotenoid-rich extract obtained under optimized conditions using FRAP and RP assays.

2. MATERIALS AND METHODS

2.1. Materials

Chaetomorpha linum algae were collected once from shrimp ponds located in Can Duoc District, Long An Province, in April 2024, the algae were transported to the laboratory in insulated foam boxes. The morphological identification of *Chaetomorpha linum* was based on previous descriptions reporting its unbranched filamentous thalli composed of cylindrical cells, sometimes forming ball-like aggregates in calm waters [22]. Molecular identification and voucher specimen deposition will be conducted in future studies to confirm species authenticity. Basic environmental parameters such as salinity, pH, and water temperature were observed during sampling, but exact values were not recorded. Upon arrival, the samples were thoroughly washed several times with tap water to remove impurities such as snail shells, sand, and stones. This washing process was followed by a final rinse with deionized to remove residual salt. Cleaned algae were then dried at 50 °C with an air velocity of 1 m/s for 10 h until constant weight was achieved to ensure the moisture content dropped below 10% [23]. To prevent photodegradation, the drying and storage processes were conducted under light-restricted conditions.

The dried material was ground using a mechanical blender (DE-500, Philips), and sieved through an 80-mesh screen (equivalent to 177 μm particle size) to ensure sample homogeneity. The resulting powder was stored in light-resistant zip-lock bags and kept in a freezer at 5 °C until further analysis.

The chemicals used in this study included ethanol (99.5%, Merk, Vietnam), n-hexane (99.8%, China), methanol (99.5%, Sigma-Aldrich, China), petroleum ether (99.5%, Sigma-Aldrich, China), acetone (99.5%, China), butylated hydroxytoluene (BHT, 99.5%, Sigma-Aldrich, China), Trolox (97%, USA), and 2,4,6-tripyridyl-s-triazine (TPTZ, Sigma-Aldrich, Germany).

The equipment used in this study included a UV-Vis spectrophotometer (V-730, Kern, Germany), a thermostatic water bath (Mettler WNB10, Germany), an ultrasonic processor (HS-S20-500, Hiesco, Korea).

2.2. Method

2.2.1. Study on the effects of solvent on the carotenoid extraction

1 gram of dried algal powder was accurately weighed and placed in a 100 mL glass beaker. Extraction solvents were used at 60% concentration: methanol, ethanol, n-hexane, acetone and petroleum ether and butylated hydroxytoluene (BHT) was added at 0.1% (w/w). Solid-to-solvent ratios of 1:20, 1:25, 1:30, 1:35 and 1:40 (w/v), temperatures of 30, 40, 50, 60 and 70 °C and extraction times of 60, 90, 120, 150 and 180 minutes were examined. A thermostatic water bath (Mettler WNB10, Germany) was used to keep the samples at the temperature of extraction. The mixtures were then filtered through filter paper to obtain clear extracts and carotenoid content in the filtrates was determined by using UV-Vis spectrophotometry at wavelengths of 470, 645 and 662 nm. Each experiment was repeated three times.

2.2.2. Study on the effects of ultrasound-assisted extraction on the carotenoid recovery process

One gram of algal powder (dried) was placed in a 100 mL glass beaker and 60% ethanol was added to it containing 0.1% BHT (w/w). The extraction was conducted at solid-to-solvent ratios of 1:30, 1:35, 1:40, 1:45 and 1:50 (w/v), ultrasound amplitudes of 20%, 25%, 30%, 35% and 40%, and extraction times of 5, 10, 15, 20 and 25 minutes. After ultrasonic treatment the samples were kept in a thermostatic water bath (Mettler WNB10, Germany) at 50 °C for 1 h. The mixtures were filtered through filter paper to get clear extracts. Carotenoids were determined spectrophotometrically at 470, 645 and 662 nm. Each experiment was repeated three times.

2.2.3. Optimization of extraction process

The extraction of carotenoid from *C. linum* was optimized by Response Surface Methodology (RSM) including a three-factor Box-Behnken Design (BBD). Based on preliminary single factor experiments, the most efficient extraction method and main influencing factors were identified and then chosen for the further optimization of the extraction process. The carotenoid content (mg/gDM) was the response variable and the relationship between the independent variables and the response variable was represented by the following second-order polynomial regression equation:

$$Y = \beta_0 + \sum_{i=1}^3 \beta_i X_i + \sum_{i=1}^3 \beta_{ii} X_i^2 + \sum_{i=1}^3 \sum_{j=i+1}^3 \beta_{ij} X_i X_j$$

In this equation, Y represents the response variable, while X_i and X_j denote the coded levels of the independent variables, reflecting their effects on the response. β_0 is the intercept, and β_i , β_{ii} và β_{ij} are the coefficients of the linear, quadratic, and interaction terms, respectively. The optimal conditions were predicted by the model and experimentally validated. Experimental design and statistical analysis were performed using JMP software version.

2.2.4. Evaluation of antioxidant activity by FRAP and RP assays

The antioxidant capacity of the carotenoid extract was evaluated using the FRAP assay. This method is based on the reduction of the ferric-tripyridyltriazine (Fe^{3+} -TPTZ) complex to its ferrous (Fe^{2+}) form. The FRAP reagent was prepared by mixing 25 mL of acetate buffer (300 mM, pH 3.6), 2.5 mL of TPTZ solution (10 mM), and 2.5 mL of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (20 mM). A 0.1 mL aliquot of carotenoid extract at different concentrations (25, 50, 75, 100, 125 $\mu\text{g/mL}$) was added to 2.9 mL of FRAP reagent

and incubated in the dark for 30 minutes. The reducing capacity was determined by measuring the absorbance at 593 nm. The same procedure was performed for the Trolox standard control at concentrations of 2, 4, 6, 8 and 10 µg/mL, respectively. For each concentration, 0.1 mL of Trolox solution was mixed with 2.9 mL of FRAP reagent under the same conditions as the samples, and the absorbance was measured at 593 nm. The calibration curve (absorbance vs. Trolox concentration) was used to calculate the antioxidant capacity of the samples and expressed as µmol Trolox equivalents (TE)/g extract.

The general ferric reducing power was assessed using the RP assay. A volume of 1 mL of carotenoid extract at various concentrations (25, 50, 75, 100, 125 µg/mL) was mixed with 2.5 mL of phosphate buffer (0.2 M) and 2.5 mL of potassium ferricyanide solution (1%). The mixture was incubated at 50 °C for 20 minutes. After incubation, 2.5 mL of trichloroacetic acid (10%) was added, and the mixture was centrifuged at 3,000 rpm for 10 minutes. Then, 2.5 mL of the supernatant was collected and mixed with 2.5 mL of distilled water and 0.5 mL of FeCl₃ solution (0.1%), followed by thorough shaking. The absorbance was measured at 700 nm. Trolox was used as a positive control. The same procedure was performed for the Trolox standard control at concentrations of 2, 4, 6, 8 and 10 µg/mL, respectively. Similarly, a Trolox standard curve was prepared at 2–10 µg/mL, and absorbance was measured at 700 nm. The results were expressed as µmol Trolox equivalents (TE)/g extract based on the calibration curve.

2.2.5. Analysis methods

Determination of carotenoid content: Carotenoid content was determined by UV-Vis spectrophotometry. The absorbance was recorded at 470, 645 and 662 nm which are the characteristic absorption peaks of the carotenoid pigments in the extract. The absorbance values are a measure of intensity of pigment or concentration of carotenoid in the solution. The carotenoid content was calculated from these measurements, according to the following formula [24]:

$$\text{Chlorophyll a} = (11.75 \times A_{662}) - (2.53 \times A_{645})$$

$$\text{Chlorophyll b} = (18.61 \times A_{645}) - (3.96 \times A_{662})$$

$$\text{Total Carotenoid } (\mu\text{g/gDM}) = ((1000 \times A_{470}) - (2.27 \times \text{Chl.a}) - (81.4 \times \text{Chl.b}))/227$$

2.2.6. Data analysis

All the experiments were repeated three times and results are reported as mean ± standard deviation. Data analysis was carried out using the software Excel 2016 (Microsoft Corporation, USA) and Minitab 20 (Minitab, LLC, USA). Data were subjected to one-way ANOVA at 95% confidence level, the differences of treatments were determined by LSD test. Graphs were created using Microsoft Office Excel 2016.

3. RESULTS AND DISCUSSION

3.1. Effects of solvent on the carotenoid extraction

The extraction was carried out using static maceration with the addition of 0.1% BHT as an antioxidant. The effects of solvent type, solid-to-solvent ratio (S/S ratio), extraction temperature, and extraction time were investigated to determine the optimal extraction conditions. The results are presented in Figure 1.

The carotenoid content depends on the type of extraction solvent and the prevention of oxidation when combined with BHT (Figure 1A). The performance of the solvent is influenced by the solid-to-solvent ratio (S/S), temperature, and time. The carotenoid content increased gradually when using solvents in the following order: n-hexane, petroleum ether, acetone, methanol, ethanol (from 0.311 ± 0.016 mg/gDM to 2.014 ± 0.011 mg/gDM), representing an approximately 6.47-fold [25]. Static maceration from algae is usually carried out from dried biomass through maceration and thermal extraction using organic solvents, depending on the polarity of the target compounds to be extracted. Therefore, it is necessary to select an appropriate solvent system based on the target carotenoids. These results are consistent with the study of Parveen et al. on *C. reinhardtii*, in which ethanol extraction yielded the highest carotenoid content [26]. Ethanol is more effective than methanol possibly due to its polarity being more compatible with

carotenoids and its lower tendency to cause oxidation. In addition, ethanol is safer and approved for use in food products [26]. In addition to its high extraction efficiency, ethanol is safer than toxic solvents such as acetone and hexane. As a food-grade solvent, ethanol is more suitable for applications related to food and pharmaceuticals [27]. Thus, ethanol solvent will be used in the following experiments.

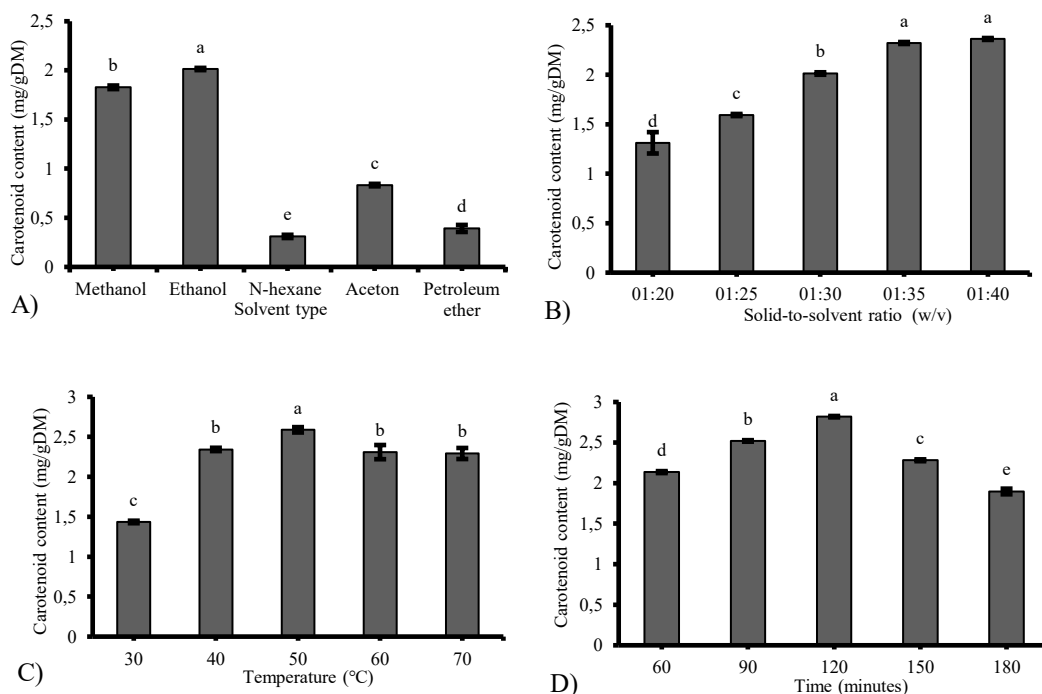


Figure 1. Effects of solvent type (A), solid-to-solvent ratio (B), temperature (C), and extraction time (D) on carotenoid yield by maceration.

For each investigated factor, different letters (a-e) above the bars indicate statistically significant differences at $p < 0.05$, as determined by ANOVA analysis.

When increasing the S/S ratio from 1:20 to 1:40 (w/v), the carotenoid content continuously increased (from 1.312 ± 0.108 mg/gDM to 2.362 ± 0.010 mg/gDM), equivalent to a 79.9% (Figure 1B). Although the carotenoid content peaked at the S/S ratio of 1:40 (w/v) at 2.362 ± 0.010 mg/gDM, at the ratio of 1:35, the carotenoid content reached 2.321 ± 0.010 mg/gDM, which was not significantly different ($p < 0.05$). Choosing the ratio of 1:35 helps reduce the amount of solvent used while achieving the desired carotenoid content. When a certain amount of solvent is reached and the compounds have fully diffused from the cells into the medium, the carotenoid content will no longer increase significantly, even if the solvent volume continues to increase [28]. Figure 1B also clearly shows that if the amount of solvent continues to increase, the carotenoid content tends to remain unchanged. Therefore, the S/S ratio of 1:35 (w/v) will be used for the following experiments.

In theory, the higher the temperature, the more efficient the extraction, as the release of compounds increases, and the solvent has a greater ability to dissolve substances at higher temperatures. At the same time, the surface tension and viscosity of the solvent decrease, improving sample wetting and penetration into the corresponding compounds. Moreover, temperature is one of the important parameters that can cause carotenoid degradation by enhancing molecular vibrations, responsible for breaking intramolecular bonds [29]. The efficiency of carotenoid extraction at different temperatures under the same S/S ratio showed that increasing the temperature from 30 to 50 °C led to a gradual increase in carotenoid content, reaching a maximum of 2.588 ± 0.033 mg/gDM (30.4% increase) at 50 °C. When the temperature was further increased to 60 °C, the carotenoid content decreased due to its sensitivity to heat and light (Figure 1C). These results are consistent with the study of Tran Xuan Hien et al. on the extraction of carotenoids from *L. cerasus*, where 50 °C yielded the highest carotenoid content in the range of 40–90 °C [30]. Therefore, the temperature of 50 °C will be used for the following experiments.

Depending on the extraction time, the carotenoid content varied to different extents (Figure 1D). The highest carotenoid content was achieved at 120 minutes, reaching 2.819 ± 0.008 mg/gDM. At times shorter than 120 minutes, the carotenoid content was not very high because the extraction time was insufficient for the carotenoids to fully dissolve into the solvent. At 150 and 180 minutes, carotenoids were still well dissolved into the solvent; however, other co-extracted compounds may also be present, which can contribute to the occurrence of isomerization during the extraction process, which reduced the carotenoid content. This result is similar to the study by Al-idee et al. on carotenoid extraction from orange peel, where 120 minutes yielded the highest carotenoid content in the tested time range [31]. Thus, the optimal extraction time is 120 minutes.

3.2. Effects of ultrasound-assisted extraction on the carotenoid recovery process

The effect of ultrasound on carotenoid content was investigated based on key factors including solid-to-solvent ratio (S/S), amplitude, and sonication time. The experimental parameters were as follows: S/S ratios (1:30, 1:35, 1:40, 1:45, 1:50 w/v), amplitudes (20, 25, 30, 35, 40%), and sonication times (5, 10, 15, 20, 25 minutes), to determine the optimal extraction conditions. During all ultrasound-assisted extraction experiments, the samples were subsequently incubated at 50 °C for 1 h to allow further diffusion of carotenoids into the solvent, thereby enhancing the extraction efficiency. The results are presented in Figure 2.

The interaction between the substrate and the surrounding solvent environment enhances extraction efficiency. As shown in Figure 2A, the S/S ratio significantly affected the carotenoid yield. Higher S/S ratios led to greater solvent contact and solubility of the compounds, resulting in more efficient extraction. When the ratio increased from 1:30 to 1:45 (w/v), the carotenoid content rose from 0.926 ± 0.005 mg/gDM to 1.939 ± 0.004 mg/gDM. However, further increasing the S/S ratio caused excessive solvent volume, leading to inefficient dissipation of ultrasonic-induced heat. The resulting temperature rise in the solvent may denature or degrade carotenoids, reducing the final carotenoid content [16]. Therefore, ultrasound-assisted extraction with an S/S ratio of 1:45 (w/v) was selected for further investigations.

As illustrated in Figure 2B, ultrasound amplitude can be used to boost the extraction efficiency by producing strong mechanical oscillations by ultrasonic waves. Unfortunately, too high an amplitude can lead to a significant amount of cell disruption, releasing too many compounds, and hence lower carotenoid yield and activity. However, at low amplitude, ultrasound energy may not be enough to destroy algal cell structure and thus extraction efficiency is low. Beyond a certain amplitude, carotenoid content decreased slightly due to denaturation [32]. The highest carotenoid content was found at the amplitude of 35% (1.965 ± 0.002 mg/gDM). Lara-Abia et al. also reported that 35% provided the highest carotenoid content in single-factor extraction studies [33]. Therefore 35% was chosen for further experiments.

As shown in Fig. 2C, the carotenoid content was the highest in the sonicated extract at 15 min (3.016 ± 0.006 mg/gDM) and this was statistically different from the other extracts at the 95% level of significance. This was greater than that reported for other strains of *Scenedesmus* sp. (0.61 ± 0.05 mg/gDM) and *D. flabellata* (1.763 mg/gDM) [34, 35]. The carotenoid contents decreased with increasing sonication time. This can be explained by the fact that longer sonication enhances the compression and expansion effects on the material's cells, facilitating compound release and increasing extraction efficiency. However, extended sonication time also raises the system temperature. Additionally, prolonged sonication may cause more non-target compounds to dissolve into the extract, negatively affecting carotenoid content. These results are in line with previous studies on carotenoid extraction from carrots, where the optimal time was around 17 minutes [36]. Therefore, a sonication time of 15 minutes was selected.

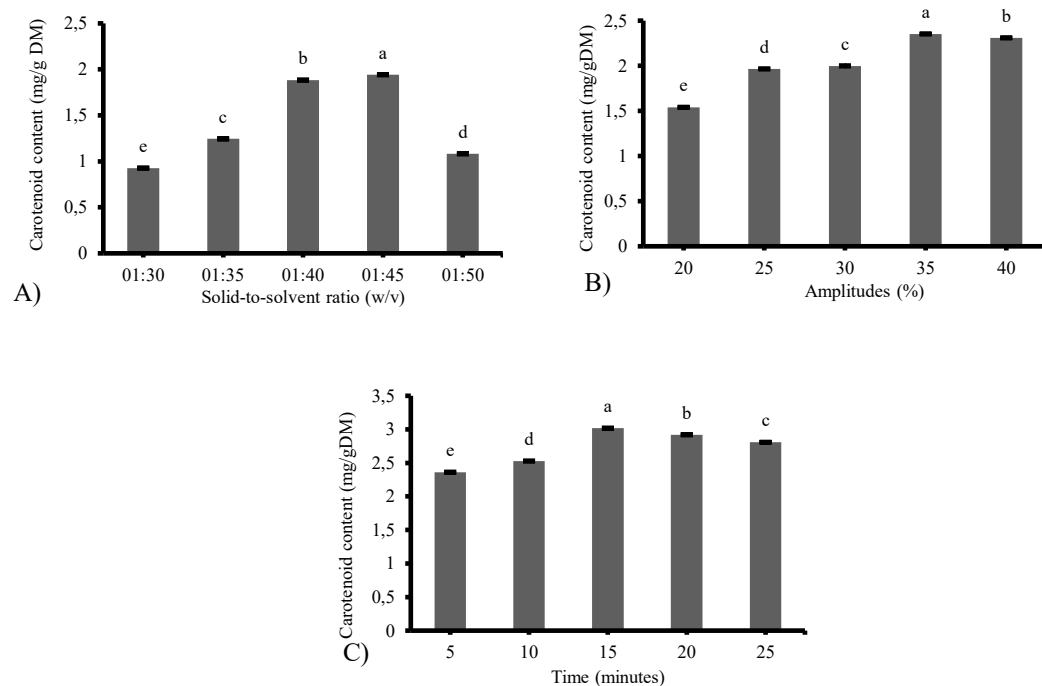


Figure 2. The effects of solid-to-solvent ratio (A), ultrasonic amplitude (B), and extraction time (C) on the ultrasound-assisted extraction of carotenoids were investigated. For each investigated factor, different letters (a-e) above the bars indicate statistically significant differences at $p < 0.05$, as determined by ANOVA analysis

These factors may interact with each other. Increasing the ultrasound amplitude can shorten the extraction time while simultaneously affecting the extraction efficiency and the carotenoid content obtained. Therefore, considering the interactions among these factors is essential for optimizing the extraction efficiency. Compared to static maceration, the recovery efficiency of carotenoids was 1.07 times higher, offering substantial practical advantages by reducing the extraction time from several hours to 15 minutes, decreasing solvent consumption, and minimizing thermal degradation of carotenoid.

3.3. Optimization of carotenoid extraction

RSM was used to optimize ultrasound-assisted carotenoid extraction from *C. linum*. Three key factors involved in the extraction of the material were material-to-solvent ratio (X_1 , mL/gDM), ultrasonic amplitude (X_2 , %) and extraction time (X_3 , minutes). The Box-Behnken Design (BBD) was used to design experiments and the experimental matrix was generated by JMP software version 10.0 (SAS Institute Inc, USA). Preliminary screening experiments identified suitable boundary values for each independent variable, as shown in Table 1.

Table 1. Coded levels of the independent variables used for the optimization experiment.

Variable	Symbol	Unit	Coded levels		
			-1	0	1
Material-to-solvent ratio (mL/gDM)	X_1	mL/gDM	40	45	50
Ultrasonic amplitude (%)	X_2	%	30	35	40
Extraction time (minutes)	X_3	minutes	10	15	20

The effects of extraction parameters, including material-to-solvent ratio (X_1), ultrasonic amplitude (X_2), and extraction time (X_3), on the total carotenoid content are presented in Table 2.

Table 2. Experimental design matrix and results for carotenoid extraction using Box-Behnken Design

Run	Coded variable			Actual variable			Total carotenoid content (mg/gDM)
	X ₁	X ₂	X ₃	Material-to-solvent ratio (mL/gDM)	Ultrasonic amplitude (%)	Extraction time (minutes)	
1	-1	-1	0	40	30	15	2.745
2	-1	1	0	40	40	15	3.482
3	1	-1	0	50	30	15	2.776
4	1	1	0	50	40	15	3.675
5	0	-1	-1	45	30	10	2.953
6	0	-1	1	45	30	20	3.124
7	0	1	-1	45	40	10	3.155
8	0	1	1	45	40	20	3.726
9	-1	0	-1	40	35	10	3.013
10	1	0	-1	50	35	10	3.143
11	-1	0	1	40	35	20	3.334
12	1	0	1	50	35	20	3.875
13	0	0	0	45	35	15	4.385
14	0	0	0	45	35	15	4.25
15	0	0	0	45	35	15	4.46

3.3.1. Total carotenoid content

After data processing using JMP software version 10.0, the results of the analysis of variance (ANOVA) and the estimated regression coefficients of the model are presented in Table 3 and Table 4, respectively.

Table 3. Analysis of Variance (ANOVA) results

Source of variation	Degrees of freedom	Sum of squares	Mean square	F Ratio
Model	9	4.3788347	0.486537	16.6344
Error	5	0.1462443	0.029249	Prob > F
Total	14	4.5250789		0.0032*

*Indicates statistically significant coefficients at $p < 0.05$

Table 4. Estimation of regression coefficients to evaluate the effects of variables on the response function

Coefficient	Estimate	Std Error	t Ratio	Prob> t
Intercept	4.365	0.09874	44.21	<.0001*
X ₁	0.111875	0.060466	1.85	0.1235
X ₂	0.305	0.060466	5.04	0.0040*
X ₃	0.224375	0.060466	3.71	0.0138*
X ₁ ×X ₂	0.0405	0.085511	0.47	0.6557
X ₁ ×X ₃	0.10275	0.085511	1.20	0.2833
X ₂ ×X ₃	0.1	0.085511	1.17	0.2949
X ₁ ×X ₁	-0.546875	0.089003	-6.14	0.0017*
X ₂ ×X ₂	-0.648625	0.089003	-7.29	0.0008*
X ₃ ×X ₃	-0.476875	0.089003	-5.36	0.0030*

*Indicates statistically significant coefficients at $p < 0.05$

According to Joglekar and May, the coefficient of determination (R^2) indicates the goodness of fit of the model and should be at least 0.80 [37]. Additionally, the adequacy of the model can be assessed by the F-value of the lack-of-fit test. The p-value is used to evaluate the significance of each regression coefficient; specifically, factors with $p < 0.05$ are considered to have a significant effect on the response.

Based on the experimental results, $R^2 = 0.968 > 0.80$ indicates that the model is well fitted. From the results in Table 4, the following regression equation describing the effect of the variables on the total carotenoid content was obtained:

$$Y_1 = 4.365 + 0.111875X_1 + 0.305X_2 + 0.224375X_3 + 0.0405X_1X_2 + 0.10275X_1X_3 + 0.1X_2X_3 - 0.546875X_1^2 - 0.648625X_2^2 - 0.476875X_3^2$$

Based on the regression model and the analysis of variance results presented in Table 4, the solvent-to-solid ratio, amplitude, and extraction time were all found to have a significant effect on the total carotenoid content ($p < 0.05$).

However, different processing factors exhibit varying directions of influence. If the regression coefficient is positive, it means the corresponding variable is increasing the response and the opposite is true if the regression coefficient is negative. The regression model showed that the individual effect of amplitude (X_2) and extraction time (X_3), and the quadratic term of the solvent-to-solid ratio (X_1^2), amplitude (X_2^2) and time (X_3^2) were statistically significant for the total carotenoid content. The effect of the solvent to solid ratio (X_1) alone was not significant. Amplitude (X_2) and time (X_3) showed positive influence which means that the carotenoid yield increased with increase in these variables. By contrast, the negative quadratic terms (X_1^2), (X_2^2) and (X_3^2) showed that excessive values reduced carotenoid content (Y_1). Regression coefficients for other interaction terms did not differ significantly ($p > 0.05$) from zero, indicating that the effects of the interaction terms were not important. Notably, the regression coefficients of amplitude and time were of similar magnitude, implying that both factors had comparable and statistically significant impacts on the total carotenoid yield. The results also demonstrated that the use of the antioxidant BHT contributed to an enhanced carotenoid extraction efficiency, which is consistent with findings from previous studies [38].

The response surface and contour plots illustrating the effects of the factors (solvent-to-solid ratio, mL/g DM) and (amplitude, %) on the response variable are presented in Figure 3.

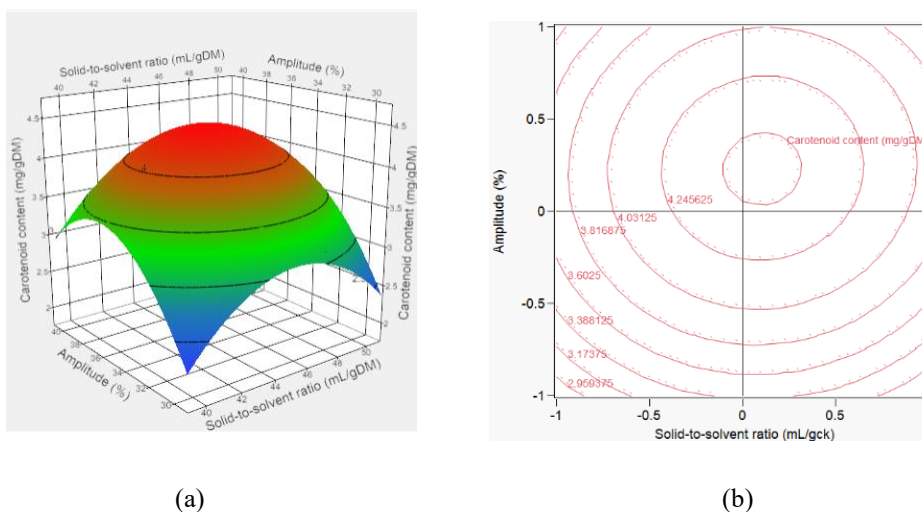


Figure 3. The response surface (a) and contour plots (b) illustrate the effects of the factors (solvent-to-solid ratio, X_1 ; amplitude, X_2) on the total carotenoid content.

The response surface and contour plots showing the effects of the factors (solvent-to-solid ratio, mL/g DM; time, minutes) on the response variable are presented in Figure 4.

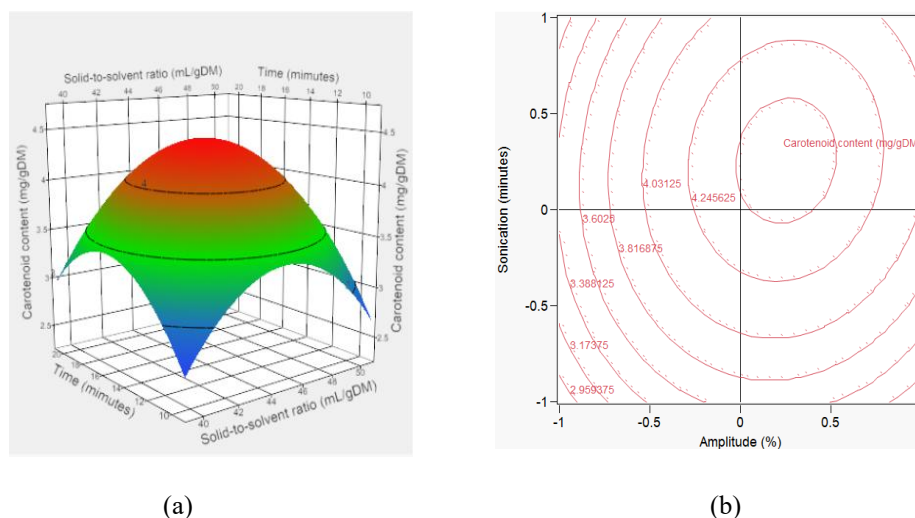


Figure 4. The response surface (a) and contour plots (b) illustrate the effects of the factors (solvent-to-solid ratio, X_1 ; time, X_3) on the total carotenoid content

The response surface and contour plots showing the effects of the factors (amplitude, %; time, min) on the response variable are presented in Figure 5.

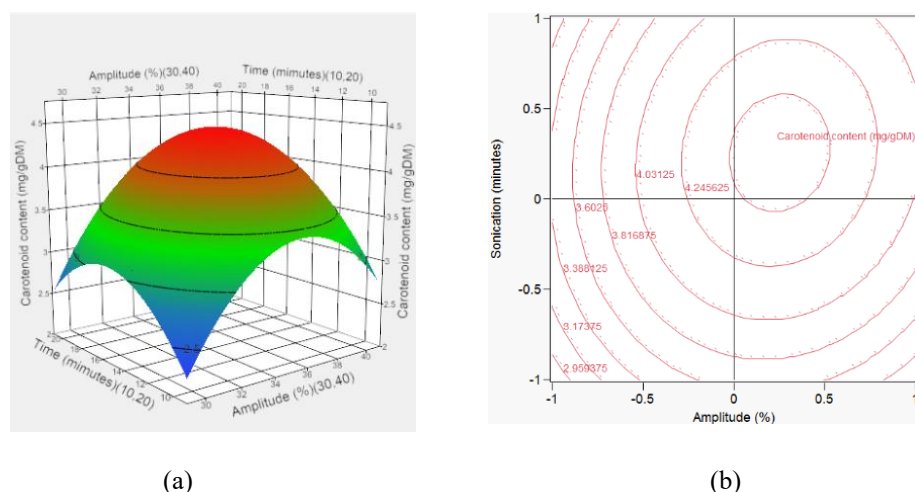


Figure 5. The response surface (a) and contour plots (b) illustrate the effects of the factors (amplitude, X_2 ; time, X_3) on the total carotenoid content.

3.3.2. Effect of process parameters on total carotenoid content

After performing ANOVA analysis using JMP, the predicted optimal extraction conditions were solvent-to-solid ratio of 1:46 mL/gDM, ultrasonic amplitude of 36.3%, and ultrasound time of 16.39 minutes, resulting in a total carotenoid content of 4.444 mg/g DM. Figure 6 shows the predicted optimum extraction model as a result of the combined effect of the experimental factors. Carotenoids are easily released from cell structures by ultrasound-induced cavitation, which creates strong mechanical impulses and localized heating in the cells. A sufficiently high solvent-to-solid ratio maintains a steep concentration gradient so as to limit local saturation and promote diffusion. Moderate amplitude can be used to disrupt cells without over-heating, avoiding degradation or isomerization of carotenoids. The recovery of the maximum carotenoid content is possible with a relatively short ultrasound duration, which also saves time and energy, without degradation mechanisms taking place. All three of these factors work together to enhance carotenoid stability and extraction efficiency. These parameters are consistent with other studies optimizing UAE for natural compounds, such as carotenoids from mango (30% amplitude, 30 min) and papaya pulp (60% amplitude, 10 minutes) [33, 39]. The similarity in optimal conditions indicates that

moderate ultrasonic amplitudes (30–60%) enhance mass transfer without causing pigment degradation, and short extraction times (approximately 15 minutes) are sufficient for efficient recovery while minimizing oxidation or isomerization. Therefore, the selected UAE parameters 36.3% amplitude, 16.39 minutes are both reasonable and in line with previous research.

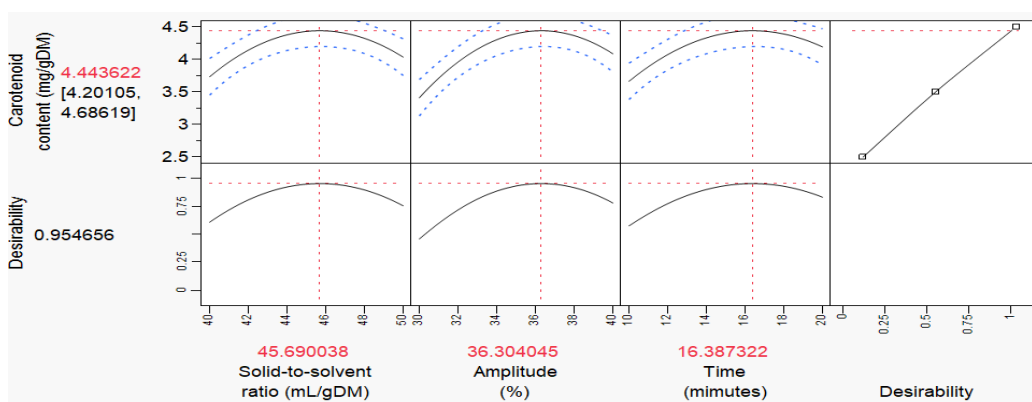


Figure 6. Response surface model showing the effects of process parameters on the carotenoid content of the extract obtained at the optimal point

A verification experiment was conducted under the optimal conditions, yielding a total carotenoid content of 4.442 mg/gDM. The difference from the predicted optimal value was less than 5%, indicating that the predicted optimum is reliable.

However, this study has some limitations. The *C. linum* samples were collected from a single source, and seasonal or geographical variations were not assessed. Only a limited set of ultrasonic extraction parameters was optimized, while factors such as temperature and pH were not considered.

3.4. Evaluation of antioxidant activity using the FRAP and RP assays

The antioxidant capacity of carotenoids was evaluated using two commonly employed assays, FRAP and RP. The combined results are presented in Figure 7.

The FRAP assay is based on the principle of reducing the Fe^{3+} -TPTZ complex to Fe^{2+} -TPTZ at low pH, resulting in the formation of a blue-colored complex. The results indicate a high antioxidant capacity of the carotenoids at various concentrations through their ability to reduce Fe^{3+} -TPTZ. At 25 $\mu\text{g/mL}$, carotenoids showed an absorbance of 0.039, corresponding to a Fe^{3+} reduction of 25.30%. When the concentration increased to 125 $\mu\text{g/mL}$, over 90% of Fe^{3+} was reduced, with an optical density of 0.412. The regression equation allowed the determination of the IC_{50} value for the extract as 57.45 $\mu\text{g/mL}$. In comparison, the standard antioxidant Trolox exhibited an IC_{50} of 4.43 $\mu\text{g/mL}$, indicating that the antioxidant activity of the extract was approximately 13 times lower than that of Trolox under the same experimental conditions. Excess free radicals circulating in the body can oxidize LDL, making it potentially lethal. Free radicals can also accelerate aging and are associated with various severe diseases, such as stroke, diabetes, rheumatoid arthritis, Parkinson's disease, Alzheimer's disease, and cancer. Physiologically, oxidative free radicals are among the most significant types. Chemical and physical phenomena may initiate oxidation, which continues in the presence of suitable substrates until a protective mechanism intervenes [40]. The study by Aluc *et al.* (2022) demonstrated that *Phormidium* sp. exhibited antioxidant activity with an IC_{50} value of 25.105 mg/mL in the FRAP assay [41].

In the RP assay, the reducing power was assessed based on the antioxidant capacity of the purified extract to reduce Fe^{3+} to Fe^{2+} , which results in a color change from yellow to blue [42]. Fe^{3+} reduction indicates electron-donating ability, a fundamental mechanism of antioxidants. This assay is based on the principle that the higher the absorbance of the reaction mixture the higher the reducing power of the tested sample [43]. The RP assay is used to determine the H-donating capacity of compounds to stop the free-radical chain reaction. The carotenoid extract contains antioxidants which reduce ferricyanide (Fe^{3+}) to ferrocyanide (Fe^{2+}) [44].

It was found that the absorbance of carotenoid extract was proportional to its concentration in the

RP assay [45]. The absorbance values of the test samples showed that absorbance increased from 0.013 to 0.201 as the concentration rose from 0 to 125 $\mu\text{g/mL}$ (approximately 15.46-fold) showing concentration dependent antioxidant activity. The regression equation derived showed that the extract had an IC_{50} of 60.48 $\mu\text{g/mL}$ while the standard anti-oxidant Trolox had an IC_{50} of 4.67 $\mu\text{g/mL}$ which showed that the extract had approximately 13-fold less anti-oxidative activity under the same experimental conditions. However, the sample exhibited higher OD than Trolox (10 $\mu\text{g/mL}$) suggesting that the sample possessed higher antioxidant activity than the control. The RP assay is based on the principle that the more reducing power, the more antioxidant capacity, which is indicated by higher absorbance at 700 nm. The increase in absorbance is due to the formation of the ferrocyanide complex as antioxidants donate hydrogen to reduce Fe^{3+} in potassium ferricyanide ($\text{K}_3[\text{Fe}(\text{CN})_6]$) to Fe^{2+} in potassium ferrocyanide ($\text{K}_4[\text{Fe}(\text{CN})_6]$) resulting in the change of colour from yellow to blue which is directly proportional to the concentration [46]. Figure 7 shows that in general the samples had an increased reduction potential with increasing concentration of carotenoid extract. Likewise, Similarly, Rostami et al. (2016) reported that carotenoid-rich pigment extracts from *Rhodotorula glutinis* exhibited antioxidant activity, with higher antioxidant potential than pigment extracts from *Micrococcus roseus* across the assays evaluated [47].

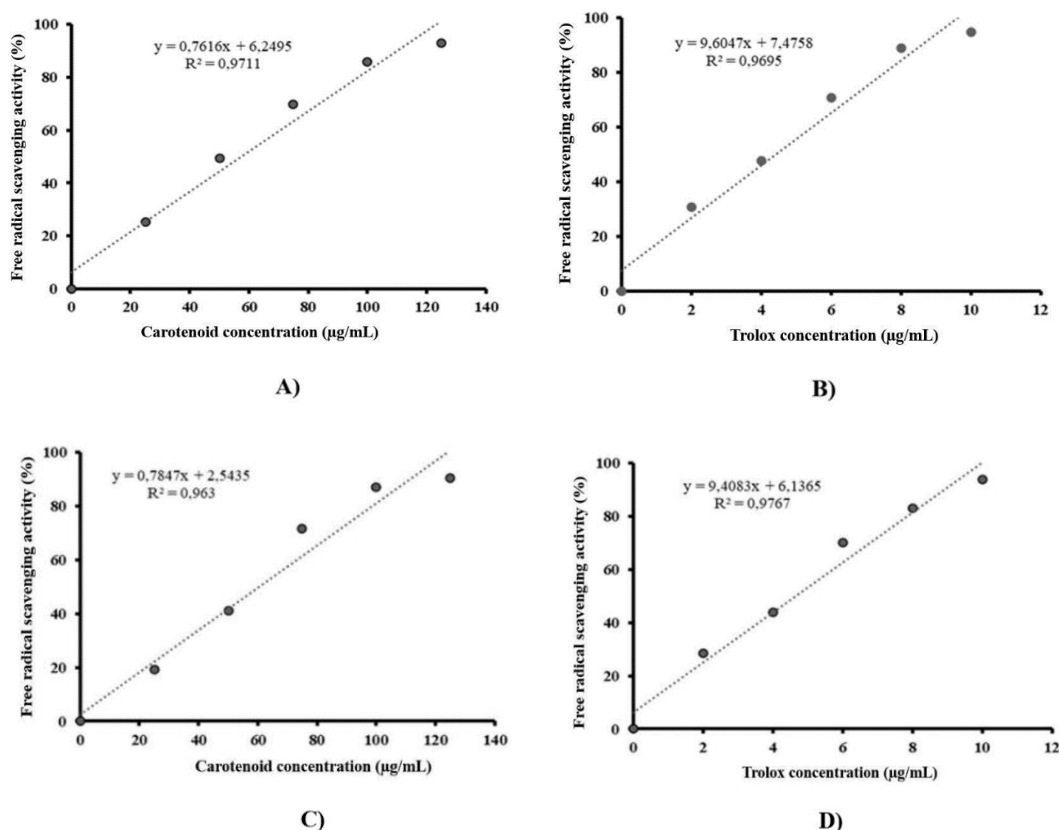


Figure 7. FRAP antioxidant activity of carotenoids (A) and Trolox (B), RP antioxidant activity of carotenoids (C) and Trolox (D)

4. CONCLUSION

The study showed that ultrasound-assisted extraction was more efficient in the extraction of carotenoids than the conventional maceration extraction method from *Chaetomorpha linum*. Response surface methodology was used to determine the optimal extraction conditions: a material-to-solvent ratio of 1:46 (w/v), an ultrasonic amplitude of 36.3% and a sonication time of 16.39 minutes, producing the highest extraction yield. The antioxidant activity of extracts was found to be significant and thus *C. linum* can be considered a potential source of antioxidants in pharmaceutical and functional food industries. It is suggested that future research efforts should be directed towards the isolation and

structural elucidation of the individual carotenoids, and the assessment of the bioactivity of the same using *in vivo* models.

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Additional information

Author contributions

Ngo Phuong Thao: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. Truong Nguyen Viet Ngan: Conceptualization, Data curation. Nguyen Thi Thao Minh: Validation, Visualization. Hoang Thi Ngoc Nhon: Supervision, Validation, Visualization, Writing – review & editing. All authors have read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest and no affiliations with or involvement in any organization or entity with any financial interest in the subject matter or materials discussed in this manuscript.

Artificial Intelligence (AI) use

ChatGPT (OpenAI) and Grammarly were used solely for spelling and grammar correction. The authors reviewed all edits and take full responsibility for the manuscript's content.

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