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Sequential extraction of *Caulerpa lentillifera* and *C. racemosa* var. *corynephora*: FTIR-based phytochemical characterization, antioxidant properties, and bioaccessibility

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ABSTRACT

Conventional single-solvent extraction yields heterogeneous bioactive profiles with limited digestive stability. This study investigated sequential extraction effects on phytochemical composition, antioxidant activity, and bioaccessibility of *Caulerpa lentillifera and C. racemosa* var. *corynephora*, edible green seaweeds consumed in Southeast Asia. Dried seaweeds were sequentially extracted using hexane, ethyl acetate, and 70% ethanol, followed by *in vitro* gastrointestinal digestion. Total phenolic content (TPC) increased substantially post-digestion, with *C. racemosa* var. *corynephora* hexane extract achieving 413.00 mg GAE/100 g DW and demonstrating the highest bioaccessible fraction. Conversely, total flavonoid content (TFC) decreased in polar extracts (68–93% loss) but remained stable in *C. lentillifera* hexane and ethyl acetate extracts (>90% retention), consistent with non-glycosylated profiles. Antioxidant responses varied by mechanism: DPPH activity was best preserved in water extracts through matrix synergy, while ABTS showed dramatic increases (3–16-fold) inversely related to baseline values, and FRAP demonstrated balanced enhancement in sequential extracts. FTIR-PCA (61–72% variance explained) confirmed chemical differentiation driven primarily by glycosylation status rather than lipophilicity, with hexane and ethyl acetate fractions co-clustering as non-glycosylated compounds. Species-specific differences included higher ash content in *C. racemosa* var. *corynephora* (49.36% *vs.* 43.58%) and elevated protein in *C. lentillifera* (18.55% *vs.* 15.01%). Sequential extraction enables targeted bioactive isolation with predictable digestive stability based on glycosylation status, supporting development of seaweed-based functional foods with enhanced nutritional value.

1. Introduction

Seaweed consumption has increased globally due to growing awareness of its nutritional and health benefits (Cofrades et al., 2013; Wu et al., 2023). Among edible seaweeds, green algae (Chlorophyta) represent an underexploited resource despite their broad commercial potential (Kopp & Lauritano, 2025). *Caulerpa* species are widely consumed in Southeast Asia (Estrada et al., 2021) and exhibit distinct chemical compositions, making them ideal for comparative studies. *C. lentillifera* contains phenolic compounds (caffeic acid, protocatechuic acid), flavonoid aglycones (quercetin, apigenin), carotenoids, and polyunsaturated fatty acids (Syakilla et al., 2022). In contrast, *C. racemosa* var. *corynephora* is characterized by glycosylated flavonoids, sulfated polysaccharides, and a higher mineral content (Intawongse et al., 2018). These compositional differences reflect fundamentally distinct phytochemical profiles: *C. lentillifera* is aglycone-rich, whereas

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C. racemosa var. *corynephora* is glycoside-rich. However, this difference has not yet been used as a guideline in the design of extraction methods to systematically classify and evaluate the properties of aglycone and glycoside compounds in the two types of algae.

The structure of bioactive compounds significantly determines their bioavailability. Aglycone flavonoids are directly absorbed via passive diffusion, whereas glycosylated flavonoids must undergo enzymatic degradation before absorption, resulting in delayed uptake and reduced immediate bioactivity (Sak, 2023; Zhang et al., 2020). Similarly, the structure of sulfated polysaccharides determines their immunomodulatory and antioxidant properties (Nagahawatta et al., 2023). Extraction methods should consequently be designed to preserve the structural integrity of bioactive compounds, rather than solely maximizing extract yield. The degree of glycosylation is therefore a critical parameter that should guide the selection of the extraction strategy, as it directly influences both compound recovery and digestive stability. Unlike red and brown algal products such as Ascophyllum nodosum, commercially available *Caulerpa* products largely lack well-established compositional data (El-Beltagi et al., 2022; Ismail et al., 2020). Establishing reliable compositional data for *Caulerpa* products is therefore increasingly urgent, given the anticipated 6.9% annual growth of the global seaweed market through 2035 (Global Market Insights, 2024). Sequential extraction has been applied to red algae, such as Chondrus crispus (Gonçalves et al., 2025), and to brown algae, including Fucus vesiculosus and kelp species (Birgersson et al., 2023; Ummat et al., 2024). However, investigations of *Caulerpa* remain scarce, as previous studies have primarily employed single-solvent extractions with ethanol or methanol, yielding limited compositional resolution (Syakilla et al., 2022).

Sequential extraction employs solvents selected for their progressively higher polarity, such as hexane (polarity index ~0.1), ethyl acetate (4.4), and 70% ethanol (6.34), consistent with biorefinery principles (Matos et al., 2021). Such an approach allows selective separation of compounds based on their solubility characteristics. Nonpolar solvents such as hexane extract lipids and carotenoids, moderately polar solvents such as ethyl acetate recover phenolic compounds, and highly polar solvents such as 70% ethanol solubilize glycosides and sulfated polysaccharides (Ferreira & Sarraguça, 2024; Nawaz et al., 2020). In the present study, tray-drying at 50 °C was selected as the starting material preparation method to maximize the retention of heat-sensitive phenolic compounds and aglycone profiles (Cascais et al., 2021; Rodríguez-Bernaldo de Quirós & López-Hernández, 2021; Tahar et al., 2025). Although freeze-drying better preserves thermolabile compounds, its high cost and limited scalability restrict industrial application (Ashworth et al., 2024). Tray-drying at 50 °C therefore represents a practical compromise between compound preservation and commercial feasibility. The selected temperature is also suitable for industrial-scale production, offering cost-effectiveness and commercial applicability (López-Hortas et al., 2021; Rodríguez-Bernaldo de Quirós & López-Hernández, 2021; Tahar et al., 2025).

The present study provides the first comparative investigation of how sequential solvent extraction differentially preserves structure-dependent bioactivities in two compositionally distinct *Caulerpa* species, namely the aglycone-rich *C. lentillifera* and the glycoside-rich *C. racemosa* var. *corynephora*, with explicit evaluation of bioaccessibility through simulated gastrointestinal digestion. No prior study has systematically compared how sequential solvent fractionation differentially affects the digestive stability of bioactive compounds in compositionally distinct *Caulerpa* species, nor has the bioaccessibility of sequentially extracted green algal fractions been evaluated. The present study aimed to quantify phenolic compounds, flavonoids, and antioxidant activities using sequential extraction and to evaluate their bioaccessibility through *in vitro* digestion. The findings provide mechanistic insights to inform the development of functional foods and nutraceutical products.

2. Material and methods

2.1. Experimental design

This study employed a factorial design to evaluate the effects of seaweed species, extraction solvent, and digestion condition on phytochemical composition, antioxidant activity, and bioaccessibility. Independent variables included seaweed species (2 levels: *C. lentillifera* and *C. racemosa* var. *corynephora*), extraction solvent (4 levels: water as control, hexane as 1st sequential extraction, ethyl acetate as 2nd sequential extraction, and 70% ethanol as 3rd sequential extraction), and digestion condition (2 levels: pre-digestion and post-digestion) as a within-treatment condition to assess bioaccessibility. Dependent variables measured after each treatment included total phenolic content (TPC, mg GAE/100 g DW), total flavonoid content (TFC, mg QE/100 g DW), DPPH radical scavenging activity (EC_{50}, mg/mL; with kinetic profiles at 0–40 min), ABTS radical scavenging capacity (mg TE/100 g DW), ferric reducing antioxidant power (FRAP, mg TE/100 g DW), and FTIR spectral profiles subjected to principal component analysis (PCA). All experimental treatments were conducted in three independent replicates (n = 3).

2.2. Sample preparation

Seaweed samples of *C. lentillifera* and *C. racemosa* var. *corynephora* were collected from Sikao District, Trang Province, Thailand. Species identity was verified using diagnostic morphological characteristics (Silva, 2003). *C. racemosa* var. *corynephora* was identified by distinctive cylindrical branchlets with a characteristic branching pattern and basal rhizoid attachment. *C. lentillifera* was confirmed by its articulated fronds and the arrangement of its bead-like branchlets. Both species were further authenticated by a taxonomic expert based on comparison with published taxonomic records from Thai *Caulerpa* populations (Charoendat et al., 2019). The samples were cleaned with distilled water, drained, and dried in a tray dryer (HS-169, O.V.D. Series 10 Trays, AT Packing, Thailand) at 50 °C until constant weight (approximately 5–6 h). The dried seaweed was ground using a blender (Waring 7009S, Waring, Japan) and passed through a 60-mesh sieve (250 μm) to obtain a uniform particle size. The powdered samples were packed in aluminum foil bags and stored in a desiccator at 25±2 °C before analysis.

2.3. Proximate analysis

The proximate composition of seaweed samples was analyzed according to AOAC (2000) standard methods: moisture content (Method 925.10), ash content (Method 900.02A), fat content (Soxhlet extraction), and protein content (Kjeldahl method).

2.4. Sequential extraction

Sequential extraction was performed using three solvents with increasing polarity (hexane, ethyl acetate, and 70% ethanol), adapted from Yusri et al. (2012). The first extraction step involved extracting the algae sample with hexane at a ratio of 1:20 (w/v) by shaking for 24 h at room temperature (25±2 °C). The extract was then filtered through filter paper (Whatman No 1, Whatman International Ltd., Maidstone, UK). Afterwards, the filtrate was evaporated by rotary evaporator (Büchi Rotavapor R-300, BÜCHI Labortechnik AG, Flawil, Switzerland) at 40 °C until completely dry, and the dried extract was dissolved in 50% ethanol (1:20 w/v) for analysis of phenolic compounds, flavonoids, and antioxidant activity.

The residue was subjected to a second extraction with ethyl acetate (1:20 w/v) following the same procedure as the first extraction. For the final extraction, the residue from the second step was extracted with 70% ethanol (1:20 w/v) using identical conditions. All dried extracts were dissolved in 50% ethanol (1:20 w/v) prior to chemical analyses.

2.5. *Determination of total phenolic content*

The quantification of total phenolic compounds was conducted following a modified Folin-Ciocalteu procedure (Sigma-Aldrich, USA) based on the method of Singleton et al. (1999). The 2.5 mL of the seaweed extract was mixed with 200 µL of Folin-Ciocalteu's phenolic reagent, then maintained under dark conditions at room temperature (25±2 °C) for 5 min. Afterwards, 2 mL of a sodium carbonate solution (Na_2CO_3, 7% w/v) (Loba Chemie, India) was added to the mixture, and incubated in the dark at room temperature for 90 min. Spectrophotometric analysis was performed at 760 nm using a double-beam UV–Vis spectrophotometer (UH5300, Hitachi, Japan). The analytical standard employed was gallic acid, prepared at different concentrations between 0 and 100 µg/mL to establish a standard calibration curve. Results for total phenolic content were expressed as milligrams of gallic acid equivalents (GAE) per 100 *g* of dry weight sample (mg GAE/100 g DW).

2.6. *Determination of total flavonoid content*

The assessment of total flavonoid content was performed using a protocol adapted from Zhishen et al. (1999). In the procedure, 1.5 mL of the seaweed extract was combined with 2.8 mL of deionized water, followed by the addition of 100 µL of an aluminum chloride solution (10% w/v in methanol) (Loba Chemie, India) and 100 µL of a potassium acetate buffer (1 M). The mixture was incubated in the dark at room temperature (25±2 °C) for 10 min. Subsequently, the mixture was measured for the absorbance at 415 nm using the spectrophotometry method (UH5300, Hitachi, Japan). Quercetin (Sigma-Aldrich, MO, USA) was used as the reference standard, and the flavonoid concentration was reported as milligrams of quercetin equivalents per 100 *g* of sample dry weight (mg QE/100 g DW).

2.7. *Antioxidant activity*

2.7.1. *DPPH radical scavenging assay*

The DPPH radical scavenging assay was performed using a protocol modified from Brand-Williams et al. (1995) and included kinetic analysis. Seaweed extract samples (300 µL at various concentrations: 0.5–3.0 mg/mL) were mixed with DPPH solution (1.5 mL, 0.2 mM in 95% ethanol) and incubated in darkness at room temperature (25±2 °C). Absorbance at 517 nm was measured at multiple time intervals: 0 (immediately after mixing), 5, 10, 15, 20, 30, and 40 min using a spectrophotometer (UH5300, Hitachi, Japan). The kinetic analysis enabled differentiation between fast-reacting antioxidants (plateau by 5 min, *e.g.*, Trolox) and slow-reacting samples (sustained increase 5–40 min, *e.g.*, seaweed extracts), characterizing complex antioxidant mechanisms. Kinetic curves were generated by plotting inhibition percentage *versus* time to determine the rate of free radical scavenging. The DPPH inhibition percentage was calculated at each time point using equation [1]. EC_{50} values (effective concentration required to achieve 50% of the maximum DPPH inhibition, Emax) were determined from the inhibition percentage at 40 min (when the reaction approached equilibrium) using linear regression. EC_{50} was expressed in mmol Trolox equivalent (TE)/g dry weight (dw). EC_{50} was used instead of IC_{50} for seaweed extracts due to their weak antioxidant activity (Emax < 50%). Trolox IC_{50} values were obtained from the literature (Brand-Williams method). Trolox (0.5–3.0 mg/mL) served as a positive control. Antioxidant activity was expressed as mg Trolox equivalent (TE)/100 g dry weight (dw).

2.7.2. *Ferric reducing antioxidant power (FRAP) assay*

The FRAP assay was conducted using a modified procedure derived from the method established by Benzie and Strain (1996). The FRAP working solution was prepared immediately before use by combining sodium acetate buffer (300 mM, pH 3.6), ferric chloride solution (20 mM $FeCl_3$), and 2,4,6-tripyridyl-s-triazine (TPTZ) (10 mM in 40 mM HCl) in volumetric proportions of 10:1:1, respectively. For the analysis, 150 µL of seaweed extract and 1.5 mL of FRAP working solution were mixed and incubated in the dark at room temperature (25±2 °C) for 20 min. The absorbance of the reaction mixture was measured at 593 nm using a spectrophotometer (UH5300, Hitachi, Japan). Trolox (Sigma-Aldrich, USA) was employed as the reference standard, with the antioxidant capacity results expressed as milligrams of Trolox equivalents per 100 g of dry weight sample (mg TE/100 g dw).

2.7.3. *ABTS radical scavenging capacity assay*

The ABTS assay was carried out according to Re et al. (1999) with slight modifications. ABTS solution was prepared by mixing 7 mM ABTS solution (Sigma-Aldrich, USA) with 2.45 mM potassium persulfate ($K_2S_2O_8$) (Q RëC, New Zealand) at equal volumes (1:1, v/v). The mixture was incubated in the dark at room temperature (25±2 °C) for 12 h to allow for complete radical formation. The resulting ABTS solution was subsequently diluted with 95% methanol to achieve an optical density of 0.700±0.05 at 734 nm. For the analysis, 20 µL of the extract was added to 1.98 mL of the prepared ABTS+ solution and maintained in the dark at room temperature (25±2 °C) for 5 min. Absorbance measurements were then recorded at 734 nm using spectrophotometric analysis. Quantification employed Trolox (Sigma-Aldrich, USA) as the reference compound, with results expressed as milligrams of Trolox equivalents per 100 *g* of dry weight (mg TE/100 g dw).

2.8. *Bioaccessibility analysis*

The assessment of extract bioaccessibility was conducted using a static *in vitro* gastrointestinal digestion model adapted from the INFOGEST standardized protocol (Minekus et al., 2014). This approach simulated the physiological conditions and sequential digestive processes occurring in the oral, gastric, and intestinal compartments.

For the oral digestion phase, the procedure involved combining the extract sample (5 mL) with simulated salivary fluid (5 mL, pH 7.0) containing α-amylase at a final concentration of 75 U/mL. The mixture was gently shaken at 37 °C for 2 min. For the gastric phase, the oral bolus (10 mL) was mixed with simulated gastric fluid (10 mL, pH 3.0) containing porcine pepsin (2000 U/mL) and calcium chloride (0.075 mM). The pH was adjusted to 3.0 with 1 M HCl, and the mixture was incubated at 37 °C with gentle shaking for 2 h. The intestinal digestion phase was initiated by combining the gastric chyme (20 mL) with simulated intestinal fluid (20 mL, pH 7.0) containing pancreatin (calibrated to provide a final trypsin activity of 100 U/mL), fresh porcine bile (providing a final concentration of 10 mM bile salts), and calcium chloride (final concentration 0.3 mM). The pH was adjusted to 7.0 using 1 M NaOH, followed by incubation at 37 °C for 2 h with continuous gentle agitation. After intestinal digestion, the mixture was centrifuged at 5000×g for 10 min at 4 °C, and the supernatant was filtered through a 0.45 µm membrane filter to obtain the bioaccessible fraction. Total phenolic content, flavonoid content, and antioxidant capacity were determined using the methods described in Sections 2.5, 2.6, and 2.7. Bioaccessibility was calculated using equation [2]:

$$\text{Inhibition}(\%) = [(\text{Controlabsorbance} - \text{Sampleabsorbance})/\text{Controlabsorbance}] \times 100 \quad (1)$$

$$\text{Bioaccessibility}(\%) = (\text{C}_{\text{bioaccessible}} / \text{C}_{\text{initial}}) \times 100 \tag{2}$$

where Cbioaccessible is the concentration in the bioaccessible fraction and Cinitial is the concentration in the extract before digestion.

2.9. Fourier-transform infrared (FTIR) spectroscopy analysis

FTIR analysis was performed using the drop-coating deposition method. Extract solutions (5–10 μL) were deposited on barium chloride ($BaCl_2$) windows and vacuum-dried to form thin films. Spectra were acquired in transmission mode using an FT-IR microscope (Hyperion 2000, Bruker Optics, Germany) equipped with an MCT detector. Data were collected over a wavelength range of 4000–600 cm^{-1} with a resolution of 4 cm^{-1}, using 64 scans, a 15× objective, and an aperture of 50 × 50 $μm^2$. A minimum of 50 spectra per sample with absorbance <1.0 were collected using OPUS 7.8 software and analyzed using The Unscrambler X 10.5 (CAMO Analytics, Norway).

2.10. Statistical analysis

All analyses were conducted in triplicate and reported as mean ± standard deviation. One-way and two-way ANOVA were used to evaluate the effects of seaweed species (C. lentillifera and C. racemosa var. corynephora), extraction sequence (1st, 2nd, and 3rd), and *in vitro* digestion condition (pre- and post-digestion) on phytochemical composition and antioxidant activities. Group mean comparisons were evaluated using Duncan's multiple range test at $p < 0.05$ in SPSS Statistics version 27 (IBM Corp., Armonk, NY, USA). Principal component analysis (PCA) of FTIR spectroscopic data was carried out using The Unscrambler X 10.5 (CAMO Analytics, Oslo, Norway) to assess phytochemical differentiation among extraction fractions.

3. Results and discussion

3.1. Proximate analysis

The proximate composition of dried *C. lentillifera* and *C. racemosa* var. *corynephora* is presented in Table 1.

Proximate composition analysis revealed significant species-specific differences between *C. lentillifera* and *C. racemosa* var. *corynephora* (Table 1). *C. lentillifera* exhibited significantly higher fat (0.26±0.03% *vs.* 0.16±0.02%, $p < 0.001$) and protein content (18.55±2.08% *vs.* 15.01±0.49%, $p < 0.05$), while *C. racemosa* var. *corynephora* showed significantly higher ash content (49.36±0.50% *vs.* 43.58±1.26%, $p < 0.001$). No significant differences were observed between species for moisture (10.25±0.30% *vs.* 10.10±0.06%) and fiber content (38.20±1.66% *vs.* 37.75±0.98%). Compared to previous studies, ash content of *C. lentillifera* (43.58±1.26%) fell within the upper range for *Caulerpa* species (22.2–48.9% DW; De Gaillande et al., 2017), while both species showed values 2–9 times higher than those reported by Premarathna et al. (2022) for *C. racemosa* from different locations (5.41–20.72% DW), reflecting geographical variations in mineral availability. Protein levels (15.01–18.55% DW) aligned with reported ranges (11.8–26.3% DW; De Gaillande et al., 2017), with the observed difference between species indicating nitrogen metabolism variations. The moderate protein levels likely reflected environmental conditions at the sampling site, including nitrogen availability, water salinity, and seasonal factors, as well as methodological considerations. The drying process (50 °C) may have induced some protein denaturation compared to freeze-drying, which better preserves protein structure in seaweeds (Obluchinskaya & Daurtseva, 2020).

Table 1
Proximate composition of dried *C. lentillifera* and *C. racemosa* var. *corynephora.*

Proximate composition	*C. lentillifera*	*C. racemosa* var. *corynephora*	t-value	Sig.
Moisture (%)	10.25±0.30	10.10±0.06	t = 0.839	ns
Ash (%)	43.58±1.26	49.36±0.50	t = −7.385	***
Fat (%)	0.26±0.03	0.16±0.02	t = 4.804	***
Protein (%)	18.55 ±2.08%	15.01±0.49	t = 2.982	*
Fiber (%)	38.20±1.66	37.75±0.98	t = 0.462	ns

Remark: Values represent mean ± standard deviation (n = 3). Different significance levels (*** $p < 0.001$, * $p < 0.05$, ns = not significant, $p \geq 0.05$) were determined by independent *t*-test comparing proximate composition between the two seaweed species.

The moisture content of both species remained below 12% (10.25 ±0.30% and 10.10±0.06%, respectively), ensuring suitable storage stability by maintaining water activity levels that inhibit microbial proliferation (Tolstorebrov et al., 2024). Fiber contents (37.75–38.20% DW) aligned with typical green seaweed levels (30–60% DW; De Gaillande et al., 2017; Syakilla et al., 2022). The comparable fiber contents between species suggest similar cell wall structures primarily composed of ulvan, a sulfated polysaccharide characteristic of Ulvophyceae (Ciancia et al., 2020). Fat contents (0.16–0.26% DW) were characteristically low for green seaweeds. However, both species contain valuable PUFAs, including omega-3 and omega-6 fatty acids (Syakilla et al., 2022).

The complementary nutritional profiles of both species, characterized by high mineral content, moderate protein, substantial fiber, and bioactive PUFAs, support their potential as functional food ingredients. *C. racemosa* var. *corynephora* may be particularly suited for mineral supplementation, while *C. lentillifera* offers advantages for protein enrichment.

3.2. Total phenolic content and total flavonoid content

Sequential extraction using solvents of varying polarity was employed to fractionate bioactive compounds from both *Caulerpa* species. The total phenolic content (TPC) and total flavonoid content (TFC) of extracts obtained from each extraction step were analyzed and compared before and after simulated *in vitro* gastrointestinal digestion (Table 2).

All samples exhibited significant TPC increases after *in vitro* digestion ($p < 0.05$). Water extracts of *C. lentillifera* and *C. racemosa* var.

Table 2
Effect of sequential extraction and *in vitro* gastrointestinal digestion on TPC and TFC of *Caulerpa* species.

Sample	Extraction	TPC (mg GAE/100 g DW)		TFC (mg QE/100 g DW)	
		Before *in vitro*	After *in vitro*	Before *in vitro*	After *in vitro*
CL	Control	60.25 ± 5.32^{c}	201.75 ± 10.15^{e}	1722.00 ± 24.25^{a}	348.93 ± 16.44^{b}
CR	Control	80.75 ± 3.77^{b}	201.25 ± 3.54^{e}	1872.67 ± 35.91^{b}	593.87 ± 14.48^{a}
CL	1st	36.75 ± 6.41^{d}	296.50 ± 4.52^{c}	170.67 ± 26.63^{e}	178.67 ± 9.24^{c}
CR	1st	105.75 ± 3.97^{a}	413.00 ± 11.06^{a}	83.33 ± 17.01^{f}	92.13 ± 6.70^{d}
CL	2nd	52.00 ± 7.28^{c}	355.75 ± 11.38^{b}	193.33 ± 32.02^{e}	201.27 ± 12.93^{c}
CR	2nd	59.00 ± 4.99^{c}	286.75 ± 8.30^{c}	130.67 ± 25.32^{ef}	79.13 ± 2.72^{d}
CL	3rd	54.11 ± 2.88^{c}	279.50 ± 9.99^{c}	634.00 ± 33.29^{d}	72.67 ± 8.90^{d}
CR	3rd	56.25 ± 4.56^{c}	249.08 ± 3.47^{d}	889.33 ± 29.28^{c}	67.07 ± 3.86^{d}

Note: CL = *C. lentillifera*; CR = *C. racemosa* var. *corynephora*. Control = single step water extraction; 1st = hexane extraction; 2nd = ethyl acetate extraction; 3rd = 70% ethanol extraction. Values are mean ± standard deviation (n = 3). Different superscript letters (a-f) within the same column indicate significant differences (Duncan's multiple range test, $p < 0.05$).

corynephora increased similarly (60.25→201.75 and 80.75→201.25 mg GAE/100 g DW, respectively). Among sequential extractions, *C. racemosa* var. *corynephora* hexane exhibited the highest TPC both before (105.75 mg GAE/100 g DW) and after digestion (413.00 mg GAE/100 g DW), while *C. lentillifera* ethyl acetate showed the highest post-digestion TPC (355.75 mg GAE/100 g DW). Ethanol extracts (70%) produced comparable post-digestion TPC for both species (279.50 and 249.08 mg GAE/100 g DW for *C. lentillifera* and *C. racemosa* var. *corynephora*, respectively). Statistical analysis revealed that post-digestion TPC values differed significantly among extraction methods ($p < 0.05$), with sequential extractions generally yielding higher TPC than water extraction alone, particularly for *C. racemosa* var. *corynephora* hexane (413.00 mg GAE/100 g DW, group 'a') and *C. lentillifera* ethyl acetate extracts (355.75 mg GAE/100 g DW, group 'b').

TFC exhibited contrasting behavior, with values varying widely depending on extraction solvent polarity (83.33–1872.67 mg QE/100 g DW before digestion). Water extracts contained the highest TFC (1722.00–1872.67 mg QE/100 g DW), while hexane extracts contained significantly lower levels (83.33–193.33 mg QE/100 g DW), reflecting selective extraction of glycosylated *versus* non-glycosylated flavonoids. The superior stability of non-polar extracts during digestion (>90% retention) compared to polar extracts (68–93% loss) highlights the importance of glycosylation status in determining flavonoid bioavailability. It should be noted that observed TFC reduction may reflect both actual degradation and reduced reactivity with the $AlCl_3$ reagent (*e.g.*, complexation with bile salts or loss of hydroxyl groups). Future HPLC-MS studies could distinguish between structural modifications and complete degradation.

The contrasting TPC and TFC behaviors indicate differential stability under gastrointestinal conditions. Phenolic acids and phlorotannins, predominant in seaweeds, exhibit greater stability and are progressively released from protein-bound and cell wall-bound complexes during digestion, contributing to increased TPC (Subbiah et al., 2024). Conversely, flavonoid glycosides undergo acid-catalyzed hydrolysis (gastric pH 2–3) followed by oxidation and ring-fission reactions, leading to partial degradation (Wojtunik-Kulesza et al., 2020). Flavonoid stability in hexane and ethyl acetate extracts can be attributed to three mechanisms: (1) selective extraction of stable aglycones rather than unstable glycosides (Rodríguez De Luna et al., 2020); (2) co-extraction of lipophilic protective matrices preventing oxidation (Chaves et al., 2020); and (3) removal of pro-oxidant factors including transition metals and oxidative enzymes. Conversely, 70% ethanol preferentially solubilized flavonoid glycosides, which underwent rapid hydrolysis under gastric pH (1.5–2.0), resulting in 88–93% degradation. This differential stability has implications for functional food formulation where maximizing flavonoid bioavailability is critical (Makarewicz et al., 2021).

Species-specific differences in TFC varied by extraction polarity, with *C. racemosa* var. *corynephora* showing higher TFC in polar extracts and *C. lentillifera* in non-polar extracts. These patterns may reflect compositional differences: *C. racemosa* var. *corynephora*'s higher ash content (49.36% *vs.* 43.58%) potentially influences mineral-phenolic interactions, while *C. lentillifera*'s higher protein content (18.55% *vs.* 15.01%) may affect phenolic binding. Sequential extraction enabled selective isolation based on digestive stability: hexane extracts preserved flavonoids during gastrointestinal transit (>90% retention in *C. lentillifera*), while polar extracts (water, ethanol) maximized TPC release. For functional food applications, extract selection should balance TPC bioaccessibility *versus* flavonoid preservation through appropriate solvent polarity or encapsulation strategies.

3.3. Antioxidant activities

The antioxidant activities of *C. lentillifera* and *C. racemosa* var. *corynephora* before and after *in vitro* gastrointestinal digestion, including DPPH and ABTS radical scavenging activities and ferric reducing antioxidant power (FRAP), are presented in Table 3.

Antioxidant activities exhibited mechanism-dependent responses across DPPH, ABTS, and FRAP assays (Table 3). DPPH radical scavenging activity, expressed as IC_{50} values, showed optimal performance in water extracts post-digestion: *C. lentillifera* achieved the lowest IC_{50} of 0.08±0.02 mg/mL (from 0.15±0.01 mg/mL) and *C. racemosa* var. *corynephora* improved from 0.25±0.01 to 0.12±0.02 mg/mL. Sequential extractions generally decreased DPPH activity after digestion, with hexane extracts exhibiting the poorest performance (IC_{50} increased: *C. racemosa* var. *corynephora* from 0.27 to 0.43 mg/mL; *C. lentillifera* from 0.19 to 0.30 mg/mL), while ethanol extracts showed species-specific improvements. ABTS activity increased significantly across all samples, with control extracts showing substantial increases (3.4–13.8-fold). The dramatic 13.8-fold increase in *C. lentillifera* control extract correlated with its higher protein content (18.55% *vs.* 15.01%, Table 1), suggesting that protein hydrolysis liberates bioactive peptides with radical scavenging capacity (Garcia-Mora et al., 2016). Non-polar fractions demonstrated maximum fold-increases (10.0–16.1-fold), while ethanol fractions showed modest increases (2.9–3.6-fold). FRAP showed contrasting patterns: control water extraction resulted in decreases (*C. lentillifera* 40% reduction; *C. racemosa* var. *corynephora* 57% reduction), while sequential extractions significantly enhanced activity, with the highest increase in *C. racemosa* var. *corynephora* hexane (87.5%, from 0.48 to 0.90 mg TE/g DW). The increased FRAP in sequential extractions indicates enzymatic release of bound phenolic compounds during digestion (Wojtunik-Kulesza et al., 2020).

The antioxidant activities observed were comparable to previous reports on Malaysian *Caulerpa* species. Ismail et al. (2020) reported DPPH EC_{50} values of 24.16–62.88 mg/mL, FRAP values of 11.18–27.09 mg TE/100 g, and H_2O_2 scavenging of approximately 80% in methanolic extracts from Port Dickson and Merambong. Aroyehun et al. (2020) found total antioxidant capacity of 0.31 mg AAE/g, ferric reducing capacity of 6.24 µg AAE/mL, and H_2O_2 scavenging of 80.55% in ethyl acetate extracts of cultured *C. racemosa* from Johor. The substantial

Table 3

DPPH and ABTS radical scavenging activities and FRAP values of *C. lentillifera* and *C. racemosa* var. *corynephora* before and after *in vitro* gastrointestinal digestion.

Sample	Extraction	IC_{50} (mg/mL sample)		ABTS (mg TE/g DW)		FRAP (mg TE/g DW)	
		Before *in vitro*	After *in vitro*	Before *in vitro*	After *in vitro*	Before *in vitro*	After *in vitro*
CL	Control	0.15 ± 0.01^{c}	0.08 ± 0.02^{d}	2.34 ± 0.04^{c}	32.21 ± 0.82^{a}	0.60 ± 0.02^{b}	0.36 ± 0.08^{d}
CR	Control	0.25 ± 0.01^{b}	0.12 ± 0.02^{d}	5.46 ± 0.46^{a}	18.63 ± 0.94^{b}	1.18 ± 0.01^{a}	0.51 ± 0.14^{c}
CL	1st	0.19 ± 0.03^{c}	0.30 ± 0.03^{b}	1.20 ± 0.02^{d}	11.99 ± 0.45^{d}	0.47 ± 0.10^{c}	0.59 ± 0.03^{c}
CR	1st	0.27 ± 0.03^{b}	0.43 ± 0.04^{a}	1.05 ± 0.08^{d}	16.86 ± 0.29^{bc}	0.48 ± 0.06^{c}	0.90 ± 0.06^{a}
CL	2nd	0.31 ± 0.01^{a}	0.32±0.03b	1.54 ± 0.12^{d}	16.04 ± 0.68^{bc}	0.51 ± 0.03^{c}	0.75 ± 0.14^{b}
CR	2nd	0.18 ± 0.02^{c}	0.27 ± 0.02^{b}	1.17 ± 0.09^{d}	13.69 ± 0.85^{cd}	0.41 ± 0.03^{d}	0.50 ± 0.05^{c}
CL	3rd	0.22 ± 0.01^{c}	0.20 ± 0.05^{c}	3.54 ± 0.20^{b}	10.16 ± 0.71^{e}	0.30 ± 0.02^{e}	0.37 ± 0.03^{d}
CR	3rd	0.33 ± 0.02^{a}	0.18 ± 0.03^{c}	3.77 ± 0.23^{b}	13.56 ± 0.34^{cd}	0.53 ± 0.03^{b}	0.58 ± 0.02^{c}

Note: CL = *C. lentillifera*; CR = *C. racemosa* var. *corynephora*. Control = single step water extraction; 1st = hexane extraction; 2nd = ethyl acetate extraction; 3rd = 70% ethanol extraction. Values are mean ± standard deviation (n = 3). Different superscript letters (a-e) within the same column indicate significant differences (Duncan's multiple range test, $p < 0.05$).

enhancement after *in vitro* digestion, particularly ABTS (3–16-fold) and FRAP (up to 87.5%), demonstrates effective release of bound antioxidants during gastrointestinal transit (Subbiah et al., 2024).

The contrasting responses across assays revealed mechanism-specific behaviors. DPPH activity was best preserved in water extracts through protective matrix synergy (Hejna et al., 2024), contrasting with TPC patterns where sequential extractions yielded higher values. The lack of correlation between TPC and DPPH demonstrates that structural characteristics and molecular interactions, rather than absolute quantity, determine radical scavenging capacity (Hatami et al., 2014). ABTS and FRAP demonstrated maximum enhancement in sequential extracts through enzymatic liberation of bound phenolics and protein-bound antioxidants, reflecting fundamentally different detection mechanisms (Cano et al., 2023). The ABTS assay detects both hydrophilic and lipophilic antioxidants, capturing contributions from diverse bioactive compounds including peptides, free amino acids, and Maillard reaction products generated during digestion (Bermúdez et al., 2024; Serena-Romero et al., 2023). These findings underscore the critical importance of multi-assay approaches for comprehensive antioxidant characterization. Analysis of DPPH radical scavenging activity across dose-response concentrations (0.5–3.0 mg/mL) at the 40 min endpoint revealed significant differences in potency between extraction methods and species (Table 4).

C. lentillifera demonstrated superior overall antioxidant potency, with average EC$_{50}$ approximately 21.5% lower than *C. racemosa* var. *corynephora* (0.5352±0.0663 vs 0.6783±0.1236 mg/mL). The most potent extract was *C. lentillifera* hexane (EC$_{50}$ = 0.4597 mg/mL), followed by *C. racemosa* var. *corynephora* hexane (EC$_{50}$ = 0.4715 mg/mL). Extraction method profoundly influenced antioxidant potency in both species. For *C. racemosa* var. *corynephora*, hexane extraction yielded the most potent extract, whereas 70% ethanol extraction was the least potent (EC$_{50}$ = 0.7591 mg/mL). Similarly, *C. lentillifera* demonstrated the extraction-dependent trends, with hexane extraction showing superior potency compared to other extraction methods. This extraction-dependent difference reflects selective recovery of bioactive compounds by different solvent polarities. Hexane and ethyl acetate preferentially recover lipophilic and semi-polar phenolic compounds with enhanced antioxidant capacity. Conversely, polar solvents (70% ethanol) recover more hydrophilic glycosidic compounds with lower potency (Ferreira & Sarraguça, 2024; Nawaz et al., 2020).

Table 4
DPPH Radical scavenging activity (Dose-response at 40-minute incubation).

Sample	Extraction	DPPH Inhibition (%)				EC$_{50}$
		0.5 mg/ mL	1.0 mg/ mL	2.0 mg/ mL	3.0 mg/ mL (E$_{max}$)	
CL	Control	3.59 ±0.03^{b}	5.63 ±0.07^{abc}	7.32 ±0.03^{a}	8.12 ±0.03^{cd}	0.5739 ±0.0011^{e}
CR	Control	3.99 ±0.02^{b}	6.01 ±0.02^{ab}	7.12 ±0.03^{a}	10.42 ±0.03^{b}	0.7853 ±0.0011^{a}
CL	1st	4.65 ±0.04^{a}	6.52 ±0.03^{a}	6.99 ±0.02^{a}	9.01 ±0.02^{bc}	0.4597 ±0.0005^{h}
CR	1st	4.93 ±0.02^{a}	6.46 ±0.01^{a}	7.84 ±0.05^{a}	9.39 ±0.02^{bc}	0.4715 ±0.0005 g
CL	2nd	3.75 ±0.07^{b}	5.34 ±0.04^{bcd}	6.05 ±0.06^{ab}	7.42 ±0.03^{d}	0.4771 ±0.0010^{f}
CR	2nd	3.83 ±0.03^{b}	4.34 ±0.04^{cd}	5.70 ±0.03^{ab}	8.15 ±0.01^{cd}	0.6974 ±0.0004^{c}
CL	3rd	3.02 ±0.03^{c}	5.07 ±0.06^{bcd}	5.83 ±0.05^{b}	7.27 ±0.04^{d}	0.6192 ±0.0017^{d}
CR	3rd	3.17 ±0.03^{c}	3.97 ±0.05^{d}	3.90 ±0.02^{b}	7.11 ±0.02^{d}	0.7591 ±0.0011^{b}
Trolox	-	-	-	-	97.22 ±0.13^{a}	0.025 ±0.0020^{i}

Note: CL = *C. lentillifera*; CR = *C. racemosa* var. *corynephora*. Control = single step water extraction; 1st = hexane extraction; 2nd = ethyl acetate extraction; 3rd = 70% ethanol extraction. Values are mean ± standard deviation (n = 3). Different superscript letters within the same column indicate significant differences (Duncan's multiple range test, $p < 0.05$). EC$_{50}$ = Effective concentration at 50% of maximum inhibition (E$_{max}$); E$_{max}$ = Maximum percent inhibition at 3.0 mg/mL. EC$_{50}$ (rather than IC$_{50}$) was used for seaweed extracts due to weak antioxidant activity (E$_{max}$ < 50%), while Trolox IC$_{50}$ is from literature (Brand-Williams method).

The time-dependent kinetics of DPPH radical scavenging revealed a distinctive three-phase reaction pattern characteristic of free radical-antioxidant interactions (Figs. 1 and 2). A rapid reaction phase occurred within the first 5 min (green zone). During this phase, extraction methods reached approximately 70% of their maximum inhibitory capacity, indicating rapid access to radical-binding sites. This rapid phase directly correlates with the extraction method potency ranking observed in Table 4. Hexane extracts (EC$_{50}$ = 0.4597–0.4715 mg/mL) entered this rapid phase most steeply, followed by ethyl acetate (EC$_{50}$ = 0.4771–0.6974 mg/mL). In contrast, 70% ethanol extracts (EC$_{50}$ = 0.6192–0.7591 mg/mL) demonstrated the slowest entry rate.

The rapid phase was followed by a deceleration phase (5–10 min, yellow zone) with continued, but slower, inhibition, reflecting partially saturated antioxidant-radical interactions (Suhag et al., 2025). Finally, a plateau phase (10–40 min, blue zone) characterized most extraction methods, during which inhibition remained relatively constant, indicating saturation of available radical-binding sites or completion of the antioxidant reaction (Angeli et al., 2023; Suhag et al., 2025). Notably, the timing of the plateau phase varied across extraction methods. Non-polar extracts rapidly reached a plateau by 10 min. Polar extracts continued to show gradual increases, reflecting their different potency profiles.

The extraction method profoundly influenced reaction kinetics. Hexane extracts of both species showed the steepest initial reaction slopes and the highest final inhibition values (9.39±0.10%), consistent with their superior EC$_{50}$ rankings. Ethyl acetate extracts showed intermediate kinetic profiles (8.15±0.08% inhibition). 70% ethanol extracts exhibited the slowest reaction rates and the lowest final inhibition (7.11 ±0.13%), which directly aligns with their weakest EC$_{50}$. The extraction method kinetic ordering (Hexane > Ethyl acetate > 70% Ethanol) perfectly mirrors the EC$_{50}$ potency ranking. The validation of reaction rate is the fundamental driver of potency differences observed in the 40 min EC$_{50}$ measurement.

Species-specific kinetic differences were apparent and mechanistically revealing. *C. racemosa* var. *corynephora* water control demonstrated continuous reaction kinetics throughout the 40 min incubation (reaching ∼10.42±0.09% inhibition), never fully entering the plateau phase. The continuous kinetics pattern suggests enrichment in slowly reacting glycosidic compounds (Hu et al., 2025), in which sugar moieties block phenolic groups available for radical scavenging. Conversely, *C. lentillifera* water control plateaued earlier (8.12±0.03%), consistent with the abundance of rapidly reacting free phenolic compounds that are characterized by higher reactivity *in vitro* DPPH assays (Hu et al., 2025).

The differential cellular compositions between species, particularly regarding glycosidic *vs.* aglycone profiles (Zhou et al., 2025), provide a mechanistic basis for understanding their distinct kinetic profiles. Consequently, the compositional differences explain their differential extraction efficiency with organic solvents (Tahar et al., 2025). These kinetic profiles mechanistically explain the extraction method's selectivity. Non-polar solvents (hexane) preferentially recover lipophilic aglycones, which are structurally simple with exposed hydroxyl groups and undergo rapid bimolecular reactions with DPPH radicals (Shojaee et al., 2022). The compound selectivity thereby yields fast reaction kinetics and high potency. Semi-polar solvents (ethyl acetate) show intermediate selectivity for both aglycones and some glycosides, producing intermediate kinetic rates and intermediate potency. Polar solvents (70% ethanol) preferentially recover glycosidic compounds, which are structurally complex with glycosyl moieties that must undergo acid/enzyme-catalyzed hydrolysis before DPPH interaction (Li

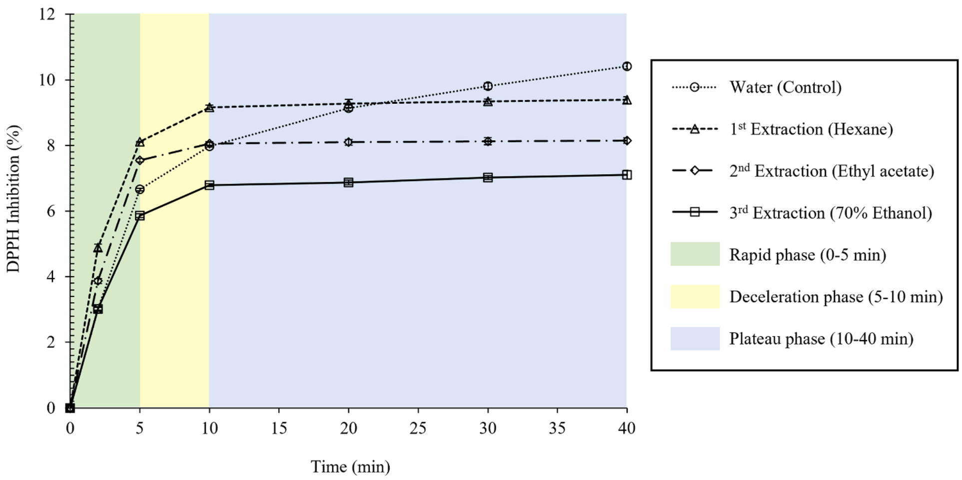

Fig. 1. Time-dependent DPPH radical scavenging kinetics of *C. racemosa* var. *corynephora* extracts (3.0 mg/mL). Shaded regions indicate kinetic phases: rapid (0–5 min, green), deceleration (5–10 min, yellow), and plateau (10–40 min, blue). Four extraction methods tested: water control (O), hexane (△), ethyl acetate (⋄), and 70% ethanol (□) at seven timepoints (0, 2, 5, 10, 20, 30, 40 min).

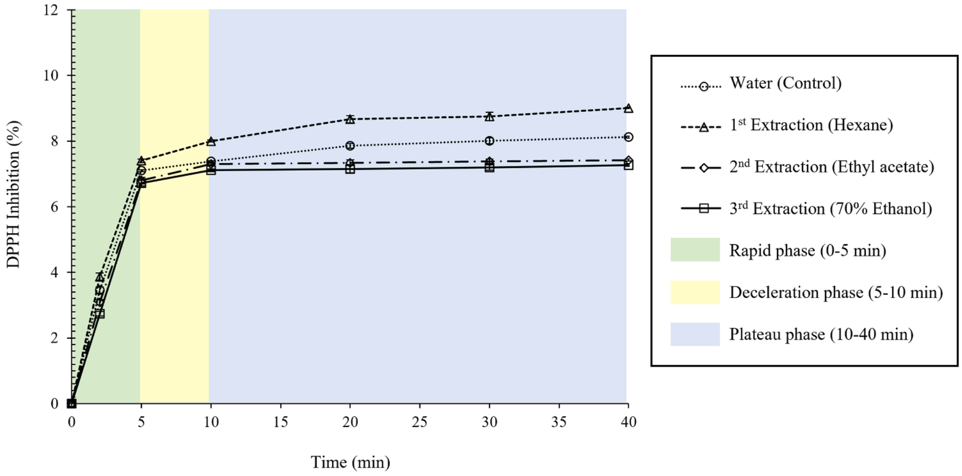

Fig. 2. Time-dependent DPPH radical scavenging kinetics of *C. lentillifera* extracts (3.0 mg/mL). Shaded regions indicate kinetic phases: rapid (0–5 min, green), deceleration (5–10 min, yellow), and plateau (10–40 min, blue). Four extraction methods tested: water control (O), hexane (△), ethyl acetate (⋄), and 70% ethanol (□) at seven timepoints (0, 2, 5, 10, 20, 30, 40 min).

et al., 2025). The hydrolysis requirement results in slower reaction kinetics and reduced potency. The remarkable correlation between kinetic slopes (Figs. 1 and 2) and EC$_{50}$ rankings (Table 4) validates both datasets. This correlation confirms that the 40 min EC$_{50}$ endpoint captures the kinetic differences in potency between extraction methods.

3.4. FTIR spectroscopic analysis

FTIR spectra of *C. lentillifera* and *C. racemosa* var. *corynephora* extracts from sequential solvent extraction are presented in Fig. 3A, 3B, and Table 5, respectively, validating the distinct chemical profiles of each fraction.

FTIR spectroscopic analysis revealed characteristic functional groups associated with phenolic and flavonoid compounds across all extraction treatments (Fig. 3, Table 5). Dried seaweed powder exhibited broad O—H stretching at 3354 cm^{-1} (*C. lentillifera*) and 3398 cm^{-1} (*C. racemosa* var. *corynephora*), indicating hydroxyl groups from polysaccharides and phenolic compounds. Characteristic functional groups were observed, including aliphatic C—H stretching (2921–2852 cm^{-1}), C = O stretching at 1733 cm^{-1}, and C—O stretching in the fingerprint region (1500–900 cm^{-1}). These features confirmed the presence of complex carbohydrate structures consistent with those of seaweed polysaccharides (Vandanjon et al., 2023). Water extracts exhibited similar patterns, with enhanced O—H stretching, reflecting the extraction of polar compounds and species-specific compositional differences in the 1600–1000 cm^{-1} region. The hydroxyl to aliphatic C—H stretching intensity ratio (I_{3398}/I_{2921}) progressively decreased from water (2.8 ± 0.2) through ethanol (1.5 ± 0.1) and ethyl acetate (0.8 ± 0.1) to hexane extracts (0.3 ± 0.05), quantitatively confirming the polarity gradient achieved through sequential extraction. The FTIR ratio-based approach for assessing polarity has been validated in various organic matrices (Dai et al., 2023) and seaweed polysaccharides (Vandanjon et al., 2023).

Sequential extraction demonstrated distinct spectral profiles corresponding to this polarity gradient (Table 5). Hexane extracts displayed intense C—H stretching (2955–2852 cm^{-1}) and sharp C = O ester peaks (1741–1712 cm^{-1}), characteristic of lipid functional groups. Similar FTIR patterns have been observed for lipid extracts from oleaginous microorganisms (Forfang et al., 2017), confirming the presence of long-chain fatty acids, lipids, and triglycerides. Minimal O—H stretching and fingerprint region peaks (1528–969 cm^{-1}) indicated the presence of lipophilic compounds with low hydroxylated content, characteristic of

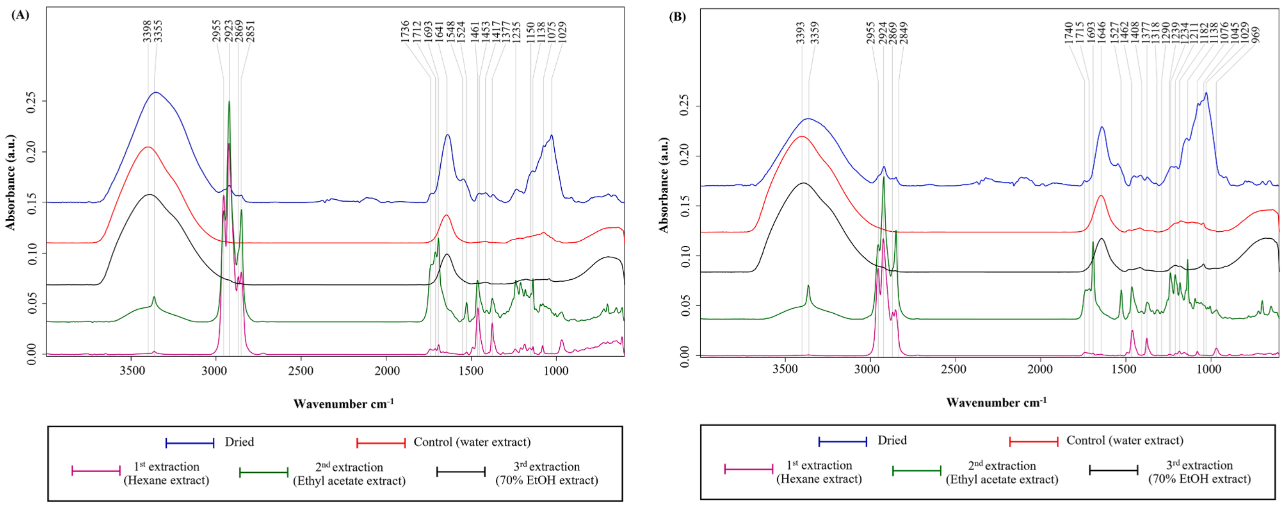

Fig. 3. FTIR spectra of sequential extraction fractions from *Caulerpa* species. (A) *C. lentillifera* and (B) *C. racemosa* var. *corynephora* extracted with different solvents: dried powder (blue), water extract/control (red), 1st extraction - hexane (purple), 2nd extraction - ethyl acetate (green), and 3rd extraction - 70% ethanol (black). Peak assignments indicate characteristic functional groups at specific wavenumbers (cm^{-1}).

Table 5
FTIR peak assignments for sequential extraction fractions.

Wavenumber (cm^{-1})	Functional group	Dried	Water	Hexane	ethyl acetate	70% Ethanol
3398–3354	O-H stretching	+++	+++	+	++	+++
2955–2921	C—H stretching	++	+	+++	+++	++
1741–1735	C = O ester	+	-	+++	++	+
1527–1465	C = C aromatic	+	++	+	+++	++
1479–1044	C-O-C glycosidic	++	+++	-	+	+++

Note: Intensity: (+++) strong, (++) medium, (+) weak, (-) absent.

non-polar lipid extracts (Forfang et al., 2017). Progressive removal of non-polar components enhanced subsequent solvent access to polar compounds, demonstrating a key advantage of sequential extraction. Ethyl acetate extracts exhibited intermediate polarity characteristics with enhanced C—H stretching (2954–2849 cm^{-1}), sharp C = O stretching (1711–1697 cm^{-1}), aromatic C = C stretching (1527–1465 cm^{-1}), and complex fingerprint patterns (1318–970 cm^{-1}), confirming flavonoid aglycones and phenolic esters with methoxyl and hydroxyl substituents (Abbas et al., 2017). Ethanol extracts displayed intense O—H stretching at 3389 cm^{-1}, aromatic C = C stretching at 1643 cm^{-1}, and C—O-C glycosidic linkages (1479–1044 cm^{-1}), indicating phenolic glycosides and carbohydrate-flavonoid conjugates (Thummajitsakul et al., 2023). These smooth spectra confirmed the structural similarity of polar compounds, consistent with high TPC and TFC values. Notable spectral differences among the extraction fractions (Table 5) confirmed the successful isolation of compounds with distinct polarities and chemical structures. This differentiation provides molecular-level validation of the quantitative phytochemical data and supports the efficacy of sequential extraction for developing specialized seaweed-based products.

Principal component analysis of the FTIR spectra (Fig. 4) further validated these spectroscopic findings, demonstrating a clear separation among the extraction treatments based on their distinct chemical fingerprints. For *C. lentillifera* (Fig. 4A), PC-1 and PC-2 explained 61% and 13% of total spectral variance, respectively, while for *C. racemosa* var. *corynephora* (Fig. 4B), these components accounted for 72% and 7% of variance. The primary separation along PC-1 distinguished polar extracts (water and 70% ethanol) in the negative region from non-polar materials (dried powder, hexane, and ethyl acetate extracts) in the positive region. Dried powder samples formed distinct clusters positioned in the positive PC-1 region (approximately +0.05), confirming their unique spectral profiles, which are dominated by native polysaccharides and structural components. Water extracts clustered at the

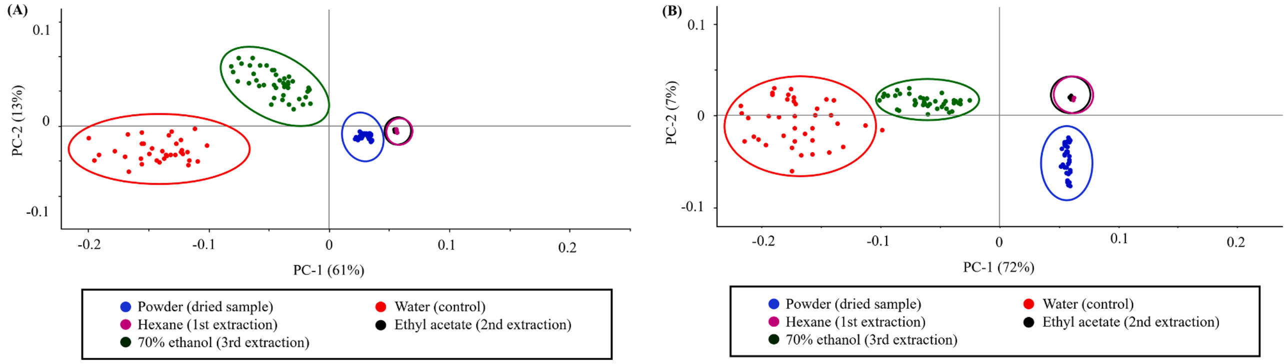

Fig. 4. Principal component analysis (PCA) of FTIR spectra demonstrating clear differentiation between extraction methods for *C. lentillifera* (A) and *C. racemosa* var. *corynephora* (B). Distinct clustering patterns indicate unique chemical fingerprints for each treatment: blue circles = dried powder; red circles = water extract (control); pink circles = hexane (1st extraction); black circles = ethyl acetate (2nd extraction); green circles = 70% ethanol (3rd extraction). PC-1 and PC-2 collectively explain the majority of spectral variance, confirming successful isolation of chemically distinct fractions through sequential extraction.

most negative PC-1 values (approximately −0.18) in both species, indicating their high content of polar hydroxylated compounds and glycosylated phenolics. The 70% ethanol extracts occupied intermediate negative positions (approximately −0.08 to −0.10), reflecting their mixed profile of polar glycosides and moderately polar phenolic compounds. Remarkably, substantial overlap was observed between hexane and ethyl acetate extracts in both seaweed species, with both fractions co-clustering in the positive PC-1 region. The overlap pattern indicates that both fractions share similar spectral characteristics, particularly in terms of their low hydroxylation and absence of glycosidic linkages (Table 5). While hexane extracts primarily contained non-polar lipids and fatty acids, ethyl acetate fractions extracted semi-polar phenolic aglycones and lipophilic flavonoids that retained similar C—H stretching (2955–2849 cm^{-1}) and C = O vibrations (1741–1697 cm^{-1}) characteristic of ester and carbonyl groups (Abbas et al., 2017; Forfang et al., 2017). The primary driver of separation along PC-1 was the presence or absence of polar functional groups (hydroxyl groups and glycosidic C—O-C linkages) (Table 5) rather than subtle differences in the degree of lipophilicity. Consequently, hexane and ethyl acetate fractions clustered together as 'non-glycosylated' compounds, distinct from the highly hydroxylated and glycosylated polar fractions (water and 70% ethanol extracts). The aromatic signatures observed in ethyl acetate FTIR spectra (aromatic C = C stretching at 1527–1465 cm^{-1}) confirmed the presence of phenolic aglycones (Abbas et al., 2017). Nonetheless, the overall spectral variance was dominated by the similarity in their non-glycosylated nature rather than differences in aromatic content. This pattern suggests that while ethyl acetate successfully extracted phenolic aglycones distinct from hexane-extractable lipids, the PCA primarily discriminated based on glycosylation status rather than the nature of the compounds as lipids or phenolics. The clustering of hexane and ethyl acetate extracts as non-glycosylated compounds correlates with their superior flavonoid stability during digestion (Table 2), suggesting that glycosylation status is a key determinant of digestive stability. These findings demonstrate that sequential extraction successfully isolated chemically distinct fractions, with glycosylation status serving as the primary discriminating factor and determinant of digestive stability.

Recent advances in *Caulerpa* extraction research have highlighted several unresolved gaps. Previous investigations have predominantly employed single-solvent methanol or ethanol extractions (Dissanayake et al., 2022; Nurkolis et al., 2023), which cannot resolve glycosylated from non-glycosylated compound classes. Sulfated polysaccharides in seaweed are bioactive constituents known for their immunomodulatory and antioxidant properties (Ciancia et al., 2020; Nagahawatta et al., 2023). Aqueous extraction and chromatographic purification of *C. racemosa* yielded ulvans and sulfated galactans with antioxidant and anticoagulant activities (Premarathna et al., 2024), providing valuable structural data but without evaluating compound stability during gastrointestinal digestion. Similarly, recent *Caulerpa* studies focused on carotenoid and nutrient profiles (Kurniawan et al., 2025; Zhou et al., 2025) or employed alkaline extraction to solubilize cell wall-bound bioactive compounds (Tahar et al., 2025), without addressing bioaccessibility.

The identified gaps were addressed in the present study through the integration of sequential solvent fractionation of two compositionally distinct *Caulerpa* species, time-dependent DPPH kinetic analysis, and standardized *in vitro* gastrointestinal digestion (INFOGEST protocol) for bioaccessibility assessment. Such a combined approach has not been previously applied to *Caulerpa* species, despite its established use in red and brown algae (Gonçalves et al., 2025; Suhag et al., 2025; Ummat et al., 2024). Hexane and ethyl acetate fractions demonstrated significantly greater flavonoid stability during digestion than polar fractions. FTIR-PCA further confirmed that glycosylation status, rather than lipophilicity, was the primary determinant of fraction differentiation (Hu et al., 2025). Collectively, the findings provide a comprehensive framework for structure-dependent bioactivity evaluation directly applicable to functional food and nutraceutical development.

4. Conclusions

This study demonstrates that sequential solvent extraction effectively isolates chemically distinct bioactive fractions from *Caulerpa* species with differential stability and bioaccessibility profiles. *In vitro* gastrointestinal digestion revealed contrasting behaviors between phenolic and flavonoid compounds, with phenolics showing enhanced release while flavonoids in hexane and ethyl acetate extracts demonstrated superior stability compared to polar extracts. Antioxidant responses varied substantially by assay methodology, emphasizing the importance of employing multiple analytical approaches for comprehensive evaluation. FTIR-PCA analysis confirmed that fractionation was primarily driven by glycosylation status, successfully separating non-glycosylated lipophilic compounds from polar glycosides. These findings indicate that sequential extraction represents a promising strategy for developing seaweed-based functional ingredients with targeted phytochemical profiles and enhanced nutritional value. However, *in vivo* studies are warranted to validate bioaccessibility findings and determine actual bioavailability in physiological conditions.

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Declaration of generative AI and AI-Assisted technologies in the manuscript preparation process

During the preparation of this work, the author(s) used Grammarly for grammar checking and Claude (Anthropic) for improving language clarity and readability. All scientific content, experimental design, data analysis, and interpretations represent the authors' original work. The author(s) reviewed and edited all AI-assisted content and take(s) full responsibility for the accuracy and integrity of the published article.

Ethical approval status

Not Applicable.

This study utilized only plant materials (seaweed) and *in vitro* analytical methods. No human subjects or animal subjects were involved. Ethical approval was not required.

Data availability

Data are contained within the article.

CRediT authorship contribution statement

Natta Kachenpukdee: Writing – original draft, Visualization, Investigation, Funding acquisition, Data curation, Conceptualization. **Tanayt Sinprachim:** Resources, Investigation, Data curation. **Supraewpan Lohalaksanadech:** Resources, Investigation, Data curation. **Watcharaporn Toommuangpak:** Visualization, Methodology, Formal analysis. **Kanokkarn Rabpairee:** Formal analysis, Data curation. **Ekarat Vasupen:** Writing – review & editing, Supervision, Resources, Project administration. **Siriwan Nawong:** Writing – review & editing, Writing – original draft, Validation, Supervision, Software, Methodology, Conceptualization. **Numphon Thaiwong:** Writing – original draft, Visualization, Validation, Supervision, Software, Methodology, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Numphon Thaiwong reports was provided by Rajamangala University of Technology Isan. Numphon Thaiwong reports a relationship with Rajamangala University of Technology Isan that includes:. 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. None of the authors have any additional relationships or activities that could be perceived as a conflict of interest. If there are other authors, they 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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