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Effect of Planetary Ball Milling on Particle Size, Physicochemical Characteristics and Bioactive Properties of Ulva lactuca

Niken Dharmayanti
Jakarta Technical University of Fisheries
Ni Putu Tantri Miranti
Jakarta Technical University of Fisheries
Tatty Yuniarti
Jakarta Technical University of Fisheries
Aef Permadi
Jakarta Technical University of Fisheries
Aris Widagdo
Jakarta Technical University of Fisheries
I Ketut Sumandiarsa
Jembrana Marine and Fisheries Polytechnic
Muhammad Miftah Jauhar
Nano Center Indonesia
Suwarti Suwarti
National Research and Innovation Agency
Fera Roswita Dewi
National Research and Inovation Agency
Sri Sugiwati
National Research and Innovation Agency
A'liyatur Rosyidah
aliyatur.rosyidah@brin.go.id

National Research and Innovation Agency

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Abstract

The aim of this study was to evaluate the effect of Planetary Ball Milling (PBM) on particle size, physicochemical characteristics, and bioactive properties of powder (ULP) and nanopowder (ULNP). is a green seaweed rich in bioactive compounds, with promising applications in functional foods and nutraceuticals. However, its utilization remains limited due to relatively large particle size and complex cellular structure, which restrict the release and bioavailability of bioactive compounds. Dried was ground into powder and further processed into nanopowder using PBM approach. Characterization included particle size distribution, morphology, mineral composition (Zn, Fe, Cu, and Se), total phenolic content (TPC), antioxidant activity (DPPH and FRAP assays), and α-amylase inhibitory activity. The results demonstrated that PBM effectively reduced particle size from the micrometer range to submicron-nanometer scale, with a Z-average of approximately 1001 nm for ULNP. Morphological changes were confirmed by Raman microscopy. Milling influenced mineral distribution, with reductions observed in Zn, Fe, and Cu, while Se remained relatively stable. ULNP exhibited significantly higher TPC (4.017 ± 0.195 mg GAE/g) compared to ULP (2.014 ± 0.054 mg GAE/g). Antioxidant activity increased markedly in ULNP, as indicated by both DPPH and FRAP assays. Furthermore, ULNP showed substantially stronger α-amylase inhibition, with an IC_{50} value of 52.29 µg/mL compared to 667.55 µg/mL for ULP. Overall, planetary ball milling is a simple and effective strategy to enhance the physicochemical properties and bioactivity of , supporting its potential development as a functional food ingredient and nutraceutical material.

Introduction

Sea lettuce, or *Ulva lactuca*, is a form of green algae that grows naturally in water and widely distributed throughout the Indonesian coast. Sulfated polysaccharides (ulvans), phenolics, flavonoids, micro-minerals, and bioactive pigments are only a few of the bioactive substances found in this green algae.. According to Ouahabi et al. (2024), U. *lactuca* possesses substantial antioxidant activity and other biological potentials, such as antidiabetic action through the suppression of carbohydrate digestion enzymes of α-amylase. This potential makes *U. lactuca* as an important raw material candidate in the development of functional foods, nutraceuticals, and pharmaceutical applications based on marine natural ingredients (Tong et al., 2020; Shalaby and Amin, 2019). However, *U. lactuca* has several drawbacks, such as the requirement for intricate procedures to optimize its bioactive activity. Therefore, in order to get the benefits from its bioactive ingredients, a simple processing must be employed.

Particle size is a key factor that influences the physicochemical characteristics, specific surface area, solubility, stability, and bioavailability of bioactive compounds in a material. Piras et al., (2019) found that reducing size to nano may enhance specific surface area, accelerate solvent diffusion, and enable the release of bioactive chemicals previously held in the cellular matrix. Mohamed et al., (2023) also found that reducing the particle size of marine biomass *Turbinaria triquetra*, increased antioxidant capacity, functional characteristics, and biological activity. Thus, particle size engineering is a key tool for increasing the functional value of natural substances, including seaweed.

Planetary ball milling (PBM) is a top-down nanoprocessing technology that employs high-energy mechanical impact. Compared to bottom-up approaches such as chemical precipitation, enzymatic hydrolysis, or solvent-based nanoparticle synthesis, top-down techniques like PBM are considered more environmentally friendly, solvent-free, and scalable for industrial applications. Top-down milling avoids the use of chemical reagents that may alter sensitive bioactive compounds and allows direct modification of dry biomass without extensive pretreatment, thereby preserving structural integrity and reducing processing complexity (Reshma *et al*., 2025). Moreover, mechanical size reduction through PBM enables simultaneous particle refinement and cell wall disruption, which enhances mass transfer and bioactive compound accessibility in marine biomass systems.This methods is not only reduces particle size but also affects the materials shape, surface structure, and physicochemical properties (Ke et al., 2024). Asmara et al., (2024) showed how PBM of *Sargassum polycystum* flour affected size reduction, physicochemical property changes, extraction yields, and the release of phenolic compounds from the matrix, which improved the antioxidant activity of the extract. Sakr et al., (2024) found that particle reduction with ball milling might result in a specific surface area, which boosts water holding capacity.

Nevertheless, there are still relatively few scientific studies that explicitly assess how PBM affects *U. lactuca*. The majority of research on *U. lactuca* has concentrated on the biological extraction, ignoring the impact of raw material physical modification and particle size on their bioactive qualities (Gazali et al., 2024). Additionally, there is a substantial knowledge gap because there hasn't been much research done on the systematic relationship between *U. lactuca*'s reduced particle size, altered physicochemical properties, and improved bioactive activity in powder and nanopowder forms.

The purpose of this study was to determine the effect of PBM processing on the *U. lactuca*'s particle size and examine their physicochemical changes and bioactive qualities through antioxidant and α-amylase inhibition activity. Scientifically, the significance of this research was to providing the information on the relationship between marine biomass physical structure and its biological activity. The resulted *U. lactuca* nanopowder is projected to be firstly developed as a high-value added functional raw material for pharmaceutical products, health supplements, and functional food applications.

Materials and Methods

Preparation of U.lactuca powder and nanopowder

Ulva lactuca samples were obtained from costal waters of Sekotong Barat, West Lombok, West Nusa Tenggara, Province, Indonesia (8.7342ºS, 115.9711ºE). All the samples were washed using sea water to remove any dirt or sand. The samples were then cleaned with tap water, sun-dried for a day, and then dried for 48 hours at 35ºC in a Getra low-pressure gas oven (NFC-8Q) (Moreira et al., 2017; Fithriani et al., 2017).

The dried *U. lactuca* was ground using a grinder (BRANDT, France) and then sieved using a 40-mesh sieve to produce *U. lactuca* powder (ULP). To prepare the *U. lactuca* nanopowder (ULNP), particle size

reduction was carried out using a Planetary Ball Mill (DECO-PBM-V-60L brand) via a top-down approach for 12 h, operated in intermittent cycles of 10–15 min on and 10–15 minoff at milling speed of 400–700 rpm. The milling parameters, including speed and duration, were optimized according to the material characteristics. The process flow for making *U. lactuca* nanopowder is presented in Fig. 1.

Characterization of ULP and ULNP

Particle size characterization was carried out using a Particle Size Analyzer (PSA) (Zetasizer Pro, Malvern). The ULP and ULNP sample was dispersed in the aquadest with 2% sodium polyphosphate and sonicated at room temperature for 1 min. The morpholody of ULP and ULNP was carried out using raman microscope (inVia Raman Microscope, Gloucestershire, United Kingdom).

Mineral composition analysis

Mineral composition analysis of ULP and ULNP was performed using Atomic Absorption Spectroscopy (AAS) (Shimadzu AA-700, Japan). The parameters evaluated included zinc (Zn), iron (Fe), copper (Cu), and selenium (Se). The Limit of Detection (LOD) for the instrument was 0.06 ppm for Fe, 0.02 ppm for Zn, 0.01 ppm for Cu, and 0.2 ppm for Se.

Preparation of methanolic extracts for antioxidant activities

ULP and ULNP sample was extracted using Ultrasound-Assisted Extraction (UAE) method according to Mohandoss *et al*., (2023) and Sumandiarsa *et al*., (2022) with some modifications. The sample was mixed with methanol as the extraction solvent at a ratio of 1:5 (w/v). The extraction was carried out using bath ultrasonicator (Elma Ultrasonic LC 60 H, Germany) for 1 h. In order to maintain constant temperature conditions at ≤ 15°C and avoid heat-induced instability during the processing, the sample was immersed in an ice bath. Extracted sample was centrifuged a 10.000 rpm, 4°C for 30 min. The remaining solvent in the supernatant was evaporated using rotary evaporator (Heizbad HB contr, Germany) for 120 min at 40ºC.

Determination of Total Phenolic Content (TPC)

Total Phenolic Content (TPC) of ULP and ULNP was assessed using Folin-Ciocalteau method with gallic acid (Sigma-Aldrich Co., G7384) as a standard. A 0.5 mL of standard solution or sample was mixed with 2.5 mL aquadest and 0.5 mL ethanol. The mixtures were then homogenized and 2.5 mL of the Folin-Ciocalteau reagent (Merck KGaA, Darmstadt, 1.09001.0500) was added. The mixture was incubated for 5 minutes in the dark at room temperature, followed by addition of 0.5 mL of 35% Na_2CO_3 solution and incubated again in the dark for 1 hour. The absorbance of each sample was measured at a wavelength of 725 nm using UV-vis spectrophotometer (UV-1800, Shimadzu, Japan) (Ningsih et al., 2017).

DPPH free radical scavenging activity

Comparison of the antioxidant activity of ULP and ULNP was determined using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method according to Theafelicia and Wulan (2023) with some modifications. 60 µM DPPH solution (Himedia, 1898-66-4) was prepared in methanol and protected from light. Trolox solution (Sigma-Aldrich, 238813-1G) in methanol with a concentration range of 5–25 µg mL^{-1} was prepared as a standard. For the assay, 2.85 mL of the DPPH solution and 0.15 mL of the sample solution or Trolox standard were combined, homogenized with a vortex, and allowed to sit at room temperature in the dark for 30 min. UV–Vis spectrophotometer (UV-1800, Shimadzu, Japan) was used to detect absorbance at a wavelength of 517 nm. The antioxidant activity was measured using a Trolox standard curve and reported as mg Trolox equivalents per milliliter of extract (mg TE mL^{-1}). The measurement was carried out three times.

Ferric Reducing Activity Power (FRAP) analysis

FRAP assay was also used to assess antioxidant activity of ULP and ULNP according to Benzie and Strain (1996) method modified by Saerang *et al*. (2025). The FRAP reagent was made by combining 10 mM TPTZ solution (Sigma-Aldrich, T1253) in 40 mM HCl, 20 mM $FeCl_3$ solution in a 10:1:1 (v/v) ratio, and 300 mM acetate buffer (pH 3.6). A standard Trolox solution (Sigma-Aldrich, 238813-1G) was prepared in methanol with a concentration of 5–25 µg mL^{-1}. 2600 µL of the FRAP reagent and 400 µL of the sample solution or Trolox standard were combined, homogenized with a vortex, and then incubated for 30 minutes at 37°C. A UV-Vis spectrophotometer was used to detect absorbance at a wavelength of 594 nm. The FRAP antioxidant activity was determined using a Trolox standard curve and a linear regression equation. The results were reported as mg Trolox equivalents per milliliter of extract (mg TE mL^{-1}). The measurement was carried out three times.

α-Amylase inhibitory activity

A 0.1 g sample of ULP and ULNP was diluted in 10 mL of 0.02 M phosphate buffer solution (pH 6.9), and vortexed for one to two minutes. A series of concentrations of 100, 200, 300, 400, and 500 ppm were prepared and 0.2 mL of α-amylase enzyme solution was added to each concentration followed by incubation at 37°C for 10 min in a water bath. Afterwards, each test tube was filled with 0.2 mL of the 1% starch solution and incubated for 10 min more at 37°C in a water bath. To stop the reaction, 0.6 mL of DNS reagent was added and boiled for 5 min. The absorbance of each sample was measured at 540 nm using UV-vis spectrophotometer. The percent of inhibition was calculated using:

$$\text{Inhibition (\%)} = \frac{(Abs\ control - Abs\ blank\ control) - (Abs\ sample - Abs\ blank\ sample)}{(Abs\ control - Abs\ blank\ control)} \times 100$$

The IC_{50} was calculated using a non-linier regression analysis from the inhibition activity. The test was conducted in two repetition.

Statistical analysis

The comparison results are presented in the form of mean ± standard deviation (SD). The data were analyzed using an independent sample t-test withIBM SPSS Statistics version 25 (IBM, USA).

Results

Particle Size Determination

This research focused on the effect of PBM on the physicochemical and biological activities of *U. lactuca*, by comparing ULP and ULNP. The PSA equipment was used to determine the particle sizes of both samples. According to the findings, the hydrodynamic particle size of the ULNP was 700 nm, whereas the ULP particle size was greater than 10 µm. The polydispersity index (PDI) value of ULNP was 0.4041, indicating that natural biomass materials that undergo demanding mechanical operations, such as PBM, often have a fairly heterogeneous particle size distribution.

According to the study's findings, the particle distribution data D10, D50, and D90 display particle sizes of 71.69 nm, 91.01 nm, and 122.7 nm, respectively. These numbers show the particle diameter at a particular size that corresponds to 10%, 50%, or 90% of the total population.

Morphological Observation Using Raman Microscope

Observations of the morphology of U. lactuca particles after the PBM process were carried out using optical imaging mode on a Raman microscope. This approach provided qualitative insights into particle shape, distribution, and aggregation behavior rather than precise particle size determination, thereby complementing the quantitative measurements obtained from PSA analysis (Fig. 2).

Optical scans demonstrated that ULP exhibited large irregular particle forms and heterogeneous fragment sizes. Based on direct observation of the optical images, the particle size of ULP ranged from tens to hundreds of micrometers, with an average value of approximately 114.76 ± 65 µm. This indicates the characteristics of marine biomass that has not undergone extensive mechanical treatment. In contrast, ULNP samples exhibited notable morphological alterations after the grinding process. Compared to the ULP sample, the particles observed in the Raman microscope optical images were considerably smaller, more uniform, and finer. Measurements obtained from the Raman microscope showed that the average particle size of the milled sample was 1.765 ± 620.8 nm, with particle diameters ranging from 494 nm to 2966 nm. These changes indicate that the macro-particle structure can be effectively broken down into micro- and submicro-scale fractions through high-energy mechanical forces during the milling process.

Mineral Content Analysis

The mineral composition of samples before and after milling was determined by analyzing the levels of Zn, Fe, Cu, and Se, which are categorized as microminerals (trace elements). As presented in Table 1, the

Zn, Fe, and Cu contents in ULP were higher than those in ULNP. In contrast, the Se content remained relatively stable and even tended to increase after the ball milling process.

Table 1
Mineral content of ULP and ULNP measured by AAS Instrument

Mineral	Composition (ppm)	
	ULP	ULNP
Zn	199.5 ± 33.2[a]	93.6 ± 12[a]
Fe	859 ± 155.6[a]	481.5 ± 2.1[a]
Cu	12.7 ± 2.5[a]	4.6 ± 0.2[b]
Se	183 ± 11.3[a]	195.5 ± 2.1[a]
Data are presented as mean ± standard deviation (SD) of three technical replicates.		
Numbers with different superscript letters (a,b) are significantly different ($p < 0.05$)		

The mineral profile shown in Table 1 indicates that particle size reduction by PBM influenced the measured levels of several microminerals. Although the Zn and Fe contents tended to decrease after milling, the differences were not statistically significant ($p > 0.05$). The reductions corresponded to approximately 53.1% for Zn and 43.9% for Fe compared with the ULP sample. In contrast, Cu content showed a statistically significant decrease ($p < 0.05$) following PBM treatment, corresponding to an approximate 63.8% reduction relative to ULP. Unlike the other minerals, Se content remained stable and did not change significantly between ULP and ULNP. However, ULNP showed a slightly higher Se content (195.5 ± 2.1 ppm) compared with ULP (183 ± 11.3 ppm).

Total Phenolic Content

The availability of phenolic compounds may also be influenced by the particle size reduction process using PBM due to modifications in particle size and the cell matrix structure. The total phenolic content (TPC) of ULP and ULNP was determined using a colorimetric method with the Folin–Ciocalteu reagent, as described by Ningsih et al. (2017). The TPC values of ULP and ULNP are presented in Table 2.

Table 2
Total phenolic content of ULP and ULNP

Sampel	Total Phenolic Content (mgGAE g^{-1})
ULP	2.014 ± 0.054^{a}
ULNP	4.017 ± 0.195^{b}
Data presented as mean ± standard deviation (SD) of three technical replicates.	
Different superscript letters (a,b) indicate significant differences at $p < 0.05$	

Phenolic compounds present in ULP and ULNP form a blue green complex with the Folin–Ciocalteu reagent that absorbs radiation, allowing absorbance to be measured (Ramadhani et al., 2022). The statistical analysis results (Table 2) indicated a significant difference ($p < 0.05$) in total phenolic content as affected by particle size reduction. PBM treatment resulted in an approximately 99.5% increase in TPC in ULNP compared with ULP. This difference was confirmed to be extremely statistically significant by the independent t-test, which yielded $p = 0.000$.

Previous studies reported that the TPC of U. lactuca collected from the same coastal area, Seriwe in East Lombok, was 2.675 ± 0.5 mg GAE g^{-1} when extracted using ethanol (Prasedya et al., 2019). In another study, Gazali et al. (2024) reported that crude U. lactuca extract obtained using polar solvents had a lower total phenolic content than the n-hexane fraction (5.48 ± 0.16 mg GAE g^{-1}).

Antioxidants Activity

To assess the effect of particle size on the antioxidant capacity of ULP and ULNP, antioxidant activity tests were conducted using two methods, DPPH and FRAP, to evaluate radical scavenging activity and the ability to reduce Fe^{3+} ions to Fe^{2+}. The antioxidant activity results are expressed as µmol Trolox g^{-1} extract and are presented in Table 3.

Table 3
DPPH and FRAP antioxidant activity results

Sample	DPPH *scavenging activity* (µmol troloks/g extract)	FRAP *non scavenging activity*
ULP	31.70 ± 0.334^{a}	66.76 ± 4.119^{a}
ULNP	72.43 ± 1.270^{b}	93.49 ± 3.456^{b}
Data presented as mean ± standard deviation (SD) of three technical replicates.		
Different superscript letters (a,b) within the same column indicate significant differences ($p < 0.05$)		

The results indicated that particle size reduction to nanoparticles (ULNP) exhibited significantly higher radical-scavenging activity and reducing power compared with ULP. Using the DPPH method, the scavenging activity increased from 31.70 ± 0.334 to 72.43 ± 1.270 µmol Trolox g^{-1} extract. Similarly, the FRAP results showed an increase in reducing capacity from 66.76 ± 4.119 to 93.49 ± 3.456 µmol Trolox g^{-1} extract. These findings indicate that the reduction of particle size to the nanoscale increases the measurable antioxidant capacity of the extract.

α-amylase Inhibitory Activity

U. lactuca, a green seaweed, contains bioactive polysaccharides that can inhibit α-amylase activity in the gut and blood plasma (Aunurrahman et al., 2024). This study evaluated the α-amylase inhibitory activity of *U. lactuca* based on particle size, specifically powder (ULP) and nanopowder (ULNP).

Table 4 shows that *U. lactuca* exhibited inhibitory activity against the α-amylase enzyme. The inhibitory activity differed between ULP and ULNP forms at concentrations ranging from 100 to 500 ppm. The ULNP sample showed higher inhibition values that increased with concentration. In contrast, the ULP form only demonstrated moderate inhibition at lower concentrations and tended to decrease at higher concentrations. The inhibition values also differed substantially between the two particle sizes. The ULNP sample exhibited an IC_{50} value of 52.29 µg mL^{-1}, whereas the ULP required 667.55 µg/mL to achieve the same inhibitory effect.

Table 4
αamilase inhibition activity

Concentration (µg/mL)	ULP inhibition (%)	IC_{50} (µg/mL)	ULNP inhibition (%)	IC_{50} (µg/mL)
100	1.31 ± 0.35[a]	667.55[a]	88.18 ± 0.31[b]	52.29[b]
200	14.80 ± 9.48[a]		98.64 ± 3.12[b]	
300	26.33 ± 6.36[a]		111.69 ± 0.41[b]	
400	37.69 ± 6.24[a]		121.64 ± 0.96[b]	
500	43.83 ± 0.12[a]		131.87 ± 22.04[b]	
Data presented as mean ± standard deviation (SD) of three technical replicate				
Different superscript letters (a,b) within the same row indicate significant differences ($p < 0.05$)				

Discussion

Particle Size Determination

According to Stetefeld et al., (2016), because the light scattering intensity is directly related to the sixth power of the particle diameter, light scattering-based methods like Dynamic Light Scattering (DLS) are

highly sensitive to the presence of big particles or agglomerates. As a result, the average value of the measured particle size might be greatly impacted by a small number of big particles or agglomerates in suspension. According to the study's findings, the particle distribution data D10, D50, and D90 each display particle sizes of 71.69 nm, 91.01 nm, and 122.7 nm, respectively. These data highlight the possibility of particle agglomeration within each threshold and provide statistical distribution information across a broad range of particle sizes (Maguire et al., 2018). These numbers show the particle diameter at a particular size that corresponds to 10%, 50%, or 90% of the total population. Xu et al., (2021) included D50 as the average particle size, D10 as the equivalent diameter at 10% cumulative volume, and D90 as the equivalent diameter at 90% cumulative volume. These findings lend credence to the theory that the process of re-agglomeration frequently affects the particle size distribution in colloidal systems or milled biomass, particularly in materials rich in polysaccharides and hydrophilic bioactive chemicals (Hantke et al., 2018).

Morphological Observation Using Raman Microscope

These findings are consistent with several previous studies that have demonstrated the effectiveness of planetary ball milling in reducing the particle size of natural fibrous materials and marine biomass. Mohamed et al. (2023) reported that the ball milling process of algal biomass caused morphological alterations accompanied by a significant reduction in particle size. Furthermore, Kumayanjati et al. (2024) explained that the breakage of intermolecular bonds during the milling process of spirulina resulted in powder with very small particles and irregular surface structures.

According to Mascolo et al. (2019), variations in particle size observed using Raman microscope optical imaging are qualitative to semi-quantitative and are not intended to provide precise measurements of particle size. These observations complement PSA data, which measures the hydrodynamic diameter of particles in dispersed systems (Stetefeld et al., 2016). The differences in particle size values obtained from the two techniques may arise from their different measurement principles. Optical imaging reflects the geometric size of dry particles, whereas PSA considers the effects of agglomeration and solvation layers in dispersed systems (Hantke et al., 2018). Overall, the morphological observations obtained using the Raman microscope visually confirm the effectiveness of the PBM process in reducing the particle size of *U. lactuca*.

Mineral Content Analysis

The observed reductions in Zn and Fe suggest that these minerals may be affected by intense mechanical treatment during the PBM process. In seaweeds, Zn is typically associated with carboxylate ($–COO^-$) and sulfate ($–OSO_3^-$) groups present in cell wall polysaccharides, whereas Fe is often bound within complexes with proteins and structural polysaccharides. The ball milling process can disrupt the cell wall structure and the polysaccharide matrix, potentially leading to the release or redistribution of Zn and Fe from their binding sites, thereby affecting their detectability in total mineral composition analysis (Andrade et al., 2004).

Among the analyzed microminerals, Cu appeared to be the most susceptible to mechanical treatment. According to Komari and Saufari (2025), Cu exhibits a strong binding affinity to proteins and enzymatic systems. High-energy mechanical forces generated during PBM may induce protein denaturation and matrix disruption, which could promote the displacement of Cu from its native binding sites. These structural changes may explain the significant decrease in measurable Cu content in ULNP compared with ULP.

In contrast, Se content remained relatively stable after PBM treatment. Seaweed generally contains Se in the form of organoselenium compounds, particularly selenoamino acids such as selenomethionine and selenocysteine. Chemically, selenomethionine is structurally similar to methionine, with the sulfur atom in the thioether group (–S–) replaced by a selenium atom (–Se–). The covalent C–Se bond in this structure is relatively stable even under mechanical stresses such as shear and impact forces generated during PBM (Brigelius-Flohé and Maiorino, 2013).

The relatively stable or slightly increased Se content observed after milling may therefore be attributed to this structural stability. According to Navarro-Alarcón and Cabrera-Vique (2008), such an apparent increase is not due to the formation of new Se but rather to improved analytical detection efficiency resulting from the disruption of the cellular matrix and the increase in specific surface area following particle size reduction.

Total Phenolic Content

The observed increase in TPC in ULNP suggests that particle size reduction enhanced the overall reducing capability of the sample. Mechanistically, decreasing particle size weakens the cell wall structure and increases the specific surface area, thereby facilitating the diffusion and dissolution of secondary metabolites into the extraction solvent. Consequently, the ball milling process can enhance the extraction yield and promote the release of phenolic compounds from the cellular matrix, leading to increased antioxidant potential (Asmara et al., 2024).

The comparison with previous studies also indicates that TPC values may vary considerably depending on the physical structure of the sample and the composition of dissolved compounds resulting from the extraction process. Differences in solvent polarity, extraction conditions, and sample structure can therefore influence the measured phenolic content.

However, TPC values should be interpreted with caution because the Folin–Ciocalteu reagent reacts with all reducing substances, including reducing sugars, ascorbic acid, aromatic amino acids, pigments, and other non-phenolic compounds. As explained by Perez et al. (2023), the Folin–Ciocalteu assay measures the total reducing capacity rather than phenolic compounds in a strictly chemical sense. Therefore, the increase in TPC observed in ULNP may originate either from improved accessibility of phenolic compounds or from the presence of additional non-phenolic reducing substances released due to cell structure disruption during the milling process. Further studies using chromatographic techniques such as HPLC are required to confirm whether the increase in TPC in the nanopowder is specifically

attributable to phenolic compounds through the identification and quantification of individual phenolic constituents.

Antioxidants Activity

The observed increase in antioxidant activity suggests that particle size reduction enhances the availability of antioxidant compounds in the extract, particularly phenolics and other bioactive substances that function as radical scavengers or reducing agents. Mohamed et al. (2023) applied a similar size-reduction technique to the seaweed Turbinaria triquetra and reported that nanosizing significantly improved phenolic extractability and DPPH radical scavenging activity. Furthermore, Asmara et al. (2024) reported that the ball milling process can reduce particle size and modify the chemical composition of powder samples, thereby increasing extraction yield and facilitating the release of phenolic compounds from the matrix, which ultimately enhances antioxidant activity.

According to Piszcz et al. (2014), the DPPH assay measures the ability of a sample to donate hydrogen atoms or electrons to neutralize organic radicals, making this method particularly sensitive to small phenolic compounds such as phenolic acids and flavonols (e.g., quercetin) that rapidly react with radicals. In contrast, the FRAP assay measures the reducing power of a sample by evaluating the electron transfer capability that converts the Fe^{3+} complex to Fe^{2+}. This method reflects the contribution of various reducing agents, including phenolics, organic acids, reducing sugars, and some degraded polysaccharides, but is generally less sensitive to compounds that act primarily through hydrogen atom transfer mechanisms (Kiss et al., 2025).

These differences in assay principles help explain why particle size reduction may lead to increased antioxidant activity. The smaller particle size facilitates the release of small phenolic molecules and other active compounds that were previously trapped within the cellular structure of *U. lactuca*. These molecules become more soluble in the extraction solvent and are able to react more rapidly in antioxidant assays.

α-amylase Inhibitory Activity

The results indicate that particle size reduction through ball milling enhances the bioactive potential of *U. lactuca* against carbohydrate-digesting enzymes. This finding is consistent with previous studies showing that particle size reduction increases reactive surface area, disrupts cell walls, and accelerates the release of active compounds that were previously bound within the cellular matrix (Mohamed et al., 2023).

This observation is also consistent with the findings of Chau et al. (2007), who demonstrated that mechanical size reduction can convert insoluble fiber fractions into more soluble forms. In the context of the present study, such structural transformations likely increased the solubility and bioaccessibility of polysaccharides and phenolic compounds in ULNP. Consequently, the enhanced α-amylase inhibitory

activity observed in ULNP may be attributed not only to reduced particle size but also to the increased availability of water-soluble bioactive fractions capable of interacting directly with the enzyme.

The inhibitory activity observed in ULP and ULNP was also influenced by the sample preparation procedure used in this study. The materials were dissolved in phosphate buffer and vortexed for approximately 1–2 minutes without prior solvent extraction. Under these conditions, the physical accessibility and release of bioactive compounds from the structural matrix of *U. lactuca* played an important role in determining the amount of dissolved compounds. In the powder form (ULP), most of the cell wall structure, cellulose complexity, and polysaccharide aggregates likely remained intact (Chau et al., 2007), allowing only a limited fraction of free phenolics and flavonoids to disperse in aqueous solvents. In contrast, the ball milling process used to produce ULNP caused cell wall disruption, increased surface porosity, and improved dispersion stability. These changes likely enhanced mass transfer and enzyme–substrate interaction during the inhibition assay (Zhao et al., 2024). Such improved physicochemical accessibility may promote stronger binding of inhibitory compounds to the active site of α-amylase, explaining the substantially lower IC_{50} value observed for ULNP compared with ULP.

These findings are consistent with previous studies indicating that the antidiabetic activity of seaweeds is strongly associated with the presence of water-soluble phenolic compounds that can interact directly with digestive enzymes (Ouahabi et al., 2024). In comparison with previous studies on *U. lactuca* that used solvent extraction, it is evident that the preparation method plays a significant role in determining biological activity.

For instance, Shannon and Hayes (2025) reported that water extracts of *U. lactuca* exhibited strong antidiabetic and α-amylase inhibitory activities due to their high content of phenolic compounds and sulfated polysaccharides. In nanopowder form, the inhibition of α-amylase may occur through interactions between free phenolics and sulfated polysaccharide fragments released during cell disruption. These compounds can interact with the enzyme through hydrogen bonding, hydrophobic interactions, or competition with the substrate at the active site (Dobson et al., 2019).

Conclusion

The Planetary Ball Milling technique successfully reduced *Ulva lactuca* particle size from ± 114.76 µm to submicro-nano scale, with an average hydrodynamic size of 1001 nm (D50 = 91.01 nm; PDI = 0.4041). This reduction in particle size was accompanied by significant morphological changes and an increase in specific surface area, which had a direct impact on the availability of bioactive chemicals. ULNP significantly increased total phenolics compared to powder, from 2.014 ± 0.054 to 4.017 ± 0.195 mg GAE g^{-1} ($p < 0.05$). Antioxidant activity rose dramatically in ULNP, with DPPH values of 72.43 ± 1.27 µmol Trolox g^{-1} extract and FRAP values of 93.49 ± 3.46 µmol Trolox g^{-1} extract, which were greater than those of the ULP (31.70 ± 0.33 and 66.76 ± 4.12 µmol Trolox g^{-1} extract). Moreover, the ULNP displayed much higher α-amylase inhibitory action (IC_{50} = 52.29 µg mL^{-1}) than the ULP (IC_{50} = 667.55 µg mL^{-1}).

These findings demonstrate that reducing particle size via planetary ball milling improves *U. lactuca's* physicochemical characteristics and bioactive potential.

Declarations

Conflict of interest

The authors declare no conflict of interest

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Author Contribution

Niken Dharmayanti and I Ketut Sumandiarsa contributed to the conceptualization and design of the study. Methodology development was carried out by Niken Dharmayanti , Ni Putu Tantri Miranti , Tatty Yuniarti , Aef Permadi , I Ketut Sumandiarsa and Sri Sugiwati . Investigation, validation, and formal analysis were performed by Niken Dharmayanti , Ni Putu Tantri Miranti , Tatty Yuniarti , Aef Permadi , Aris Widagdo , Suwarti , Fera R. Dewi and A'liyatur Rosyidah . Software development, data curation, and visualization were conducted by I Ketut Sumandiarsa and Muhammad Miftah Jauhar . The original draft of the manuscript was prepared by Niken Dharmayanti and Ni Putu Tantri Miranti , while all authors contributed to reviewing and editing the manuscript. Supervision and project administration were carried out by Niken Dharmayanti , Ni Putu Tantri Miranti , I Ketut Sumandiarsa , and Tatty Yuniarti . All authors have read and approved the final version of the manuscript.

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Data Availability

Data will be made available upon reasonable request

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Figures

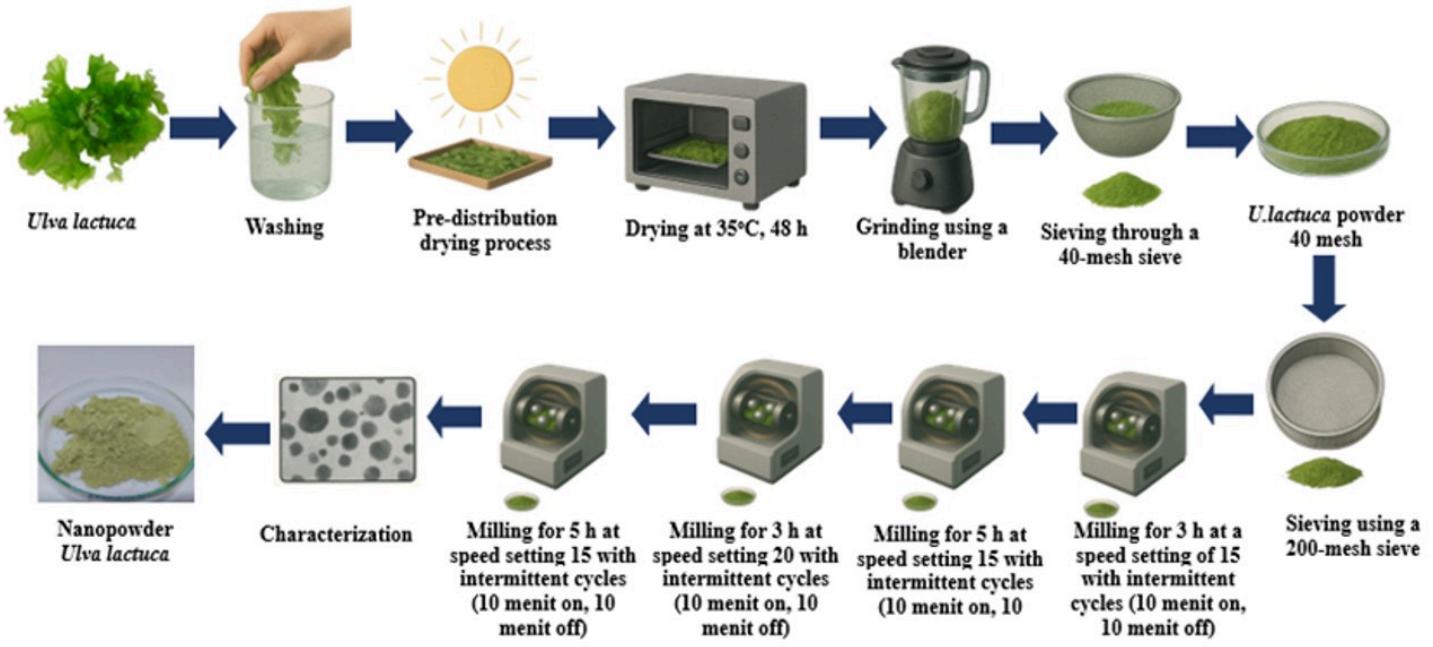


Figure 1

Diagram of *U. lactuca* powder and nanopowder preparation.

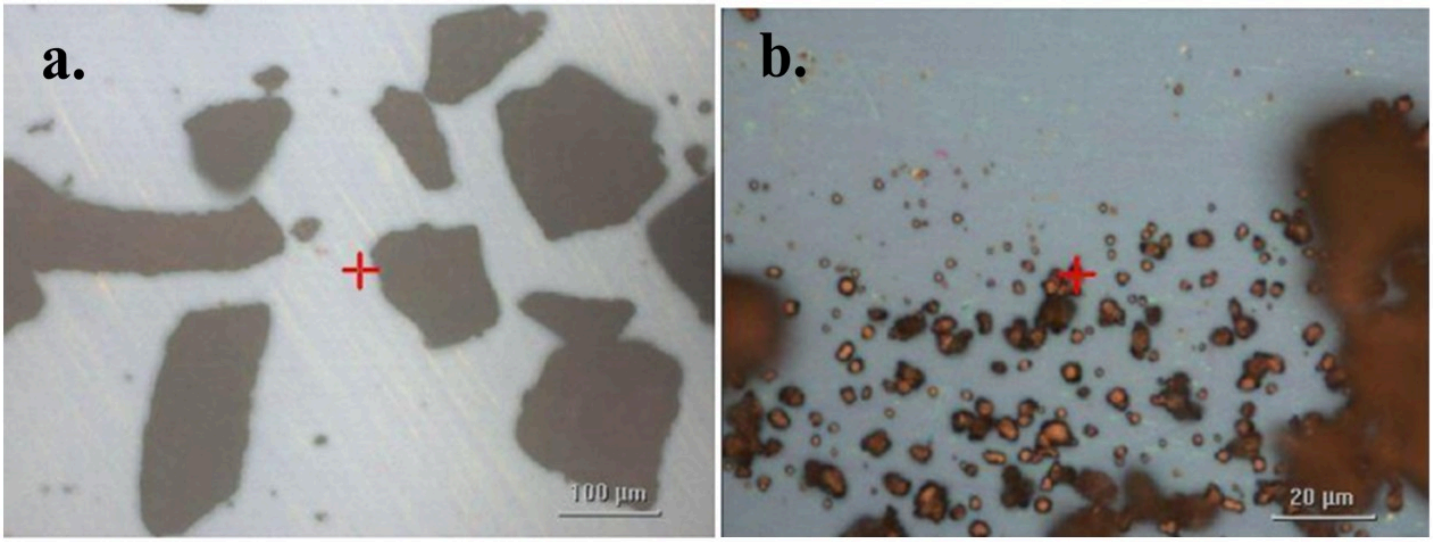


Figure 2

Optical micrographs of (a) ULP (scale bar: 100 µm) and (b) ULNP (scale bar: 20 µm) observed using the optical mode of a Raman microscope.

Supplementary Files

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