



Valorization of cauliflower (*Brassica oleracea* L. var. botrytis) and beetroot (*Beta vulgaris* L.) leaves into leaf protein concentrates: techno-functional properties and effects on cell viability and glucose uptake

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Abstract

To meet the increasing demand for protein, search for alternative protein sources has increased, where upcycling waste leaves is a promising approach in terms of nutrition and sustainability. Leaf protein concentrates (LPCs) were extracted from beetroot and cauliflower leaves, and precipitated via heat treatment. Their potential as upcycled ingredients compared to commercial plant-based protein powders, soy and pea, was evaluated. LPCs exhibited significantly higher water and oil holding capacities than commercial proteins. Although pea protein had the highest foaming capacity, the foams formed by LPCs had higher stability over time. Protein solubility varied between 13 and 43% for beetroot LPC and 8–49% for cauliflower LPC. Despite variations in emulsion activity index, LPCs showed better emulsifying performance than commercial proteins, particularly above pH 4, according to droplet size data. *In vitro* bioaccessibility of LPCs was low, potentially due to impurities or phenolic compounds, and further analysis of antinutrients is needed. Cytotoxicity assay using Caco-2 cells showed low toxicity at all tested protein concentrations. The highest concentration (100 µg BSA/mL) resulted in 71% cell viability for beetroot and 86% for cauliflower LPC, with no significant differences at lower concentrations. Notably, cauliflower LPC selectively promoted proliferation in healthy HEK293T cells without stimulating HepG2 cancer cells. It did not influence glucose uptake in HEK293T cells, suggesting its effects are not linked to glucose metabolism. In contrast, beetroot LPC enhanced glucose uptake. Overall, LPCs show promising techno-functional properties, in some cases outperforming commercial proteins, and their selective bioactivity suggests potential use in plant-based or functional food applications.

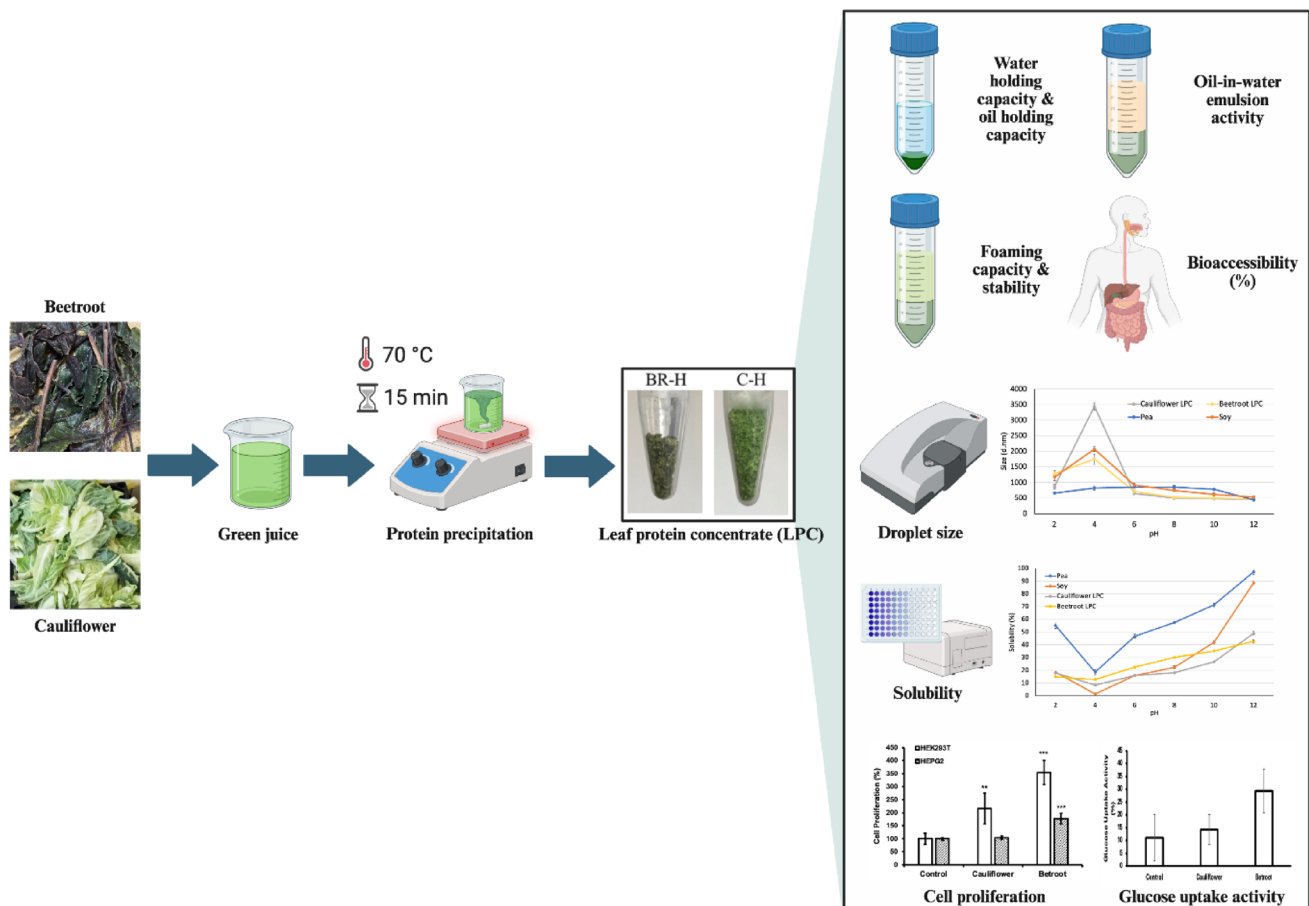
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Graphical abstract



Keywords Leaf protein concentrate · Plant protein · Techno-functional properties · Upcycling · Cytotoxicity · Glucose metabolism

Introduction

Solid waste is expected to accumulate up to 249 million tons by 2030 and 340 million tons by 2050 with an increase of 36.5% [1]. The transformation of waste into high-value products presents an effective and innovative solution to the global energy challenge. Food production leads to waste streams that are rich in protein, carbohydrates, minerals, vitamins and bioactive compounds, such as pigments, dietary fiber, essential oils [2]. The rise in the global population, which is projected to reach 9.7 billion by 2050, has resulted in an increased demand for proteins in the human diet [3]. Alternative protein sources from agro-industrial vegetable waste are becoming increasingly popular among people who are looking for ways to reduce their meat consumption or follow a vegetarian or vegan diet [4]. This demand has led food technologists to search and unlock untapped potential, particularly from plant-based sources [5].

In addition to the accumulated literature regarding plant-based proteins, there has been an increase in studies focusing on protein extraction from food waste sources such as tomato, apple and watermelon peels [6], quince seed [7], sesame cake [8], hazelnut meal [9], and rice bran [10]. Green leaves represent underutilized yet cost-effective and sustainable source of plant-based protein, which also contributes to climate change mitigation by capturing carbon dioxide from the atmosphere [3]. Due to their nutritional quality and abundance in agricultural waste, green leaves are recognized as a valuable source of high-quality protein for use in conventional foods and as functional ingredients [11]. Apart from the sustainable and nutritional view, the use of plant proteins in the food industry is still challenging due to their poor water solubility [12]. Almost half of the leaf proteins are insoluble [13], however, the most abundant soluble protein in leaves, RuBisCO (Ribulose-1,5-bisphosphate carboxylase/oxygenase), has been shown to have

good functional properties compared to other proteins [14]. Since the nutritional and techno-functional properties of leaf proteins vary among leaf type [15, 16], precipitation method [17], and even the mechanical pre-treatments [18], each leaf protein concentrate (LPC) should be evaluated individually prior industrial use.

Cauliflower, with global production of approximately 25 million tons, has leaves up to 35–40% of its weight which are mostly discarded as waste, but rich in high quality proteins, fiber and phenolic compounds [19]. Beetroot leaves and stems, on the other hand, have been shown to be a suitable raw material for obtaining high-value compounds due to their significantly higher protein content than in the bulb, and their rich structure in polyphenols and betalains [20]. LPCs previously precipitated from four different agricultural waste leaves using two different precipitation methods (acid and heat) were compared, and their physicochemical properties, amino acid profiles, and heat stability were characterized by our group [17]. Among these leaves, LPC production from cauliflower and beetroot leaves via thermal precipitation holds promise in terms of protein purity, yield, characteristics, and industrial adaptability. Therefore, we aimed to investigate the technofunctional and bioactive properties of these LPCs to uncover their potential for use as upcycled food ingredients.

Plant proteins are incorporated into foods as additives not only to enhance their nutritional value, but also due to their techno-functional properties, which are primarily solubility, hydrophobicity, water and oil holding, foaming, emulsifying and gelling [7]. Protein solubility is one of the essential properties due to the direct effect on other techno-functional properties, stability and appearance of foods [3]. It depends on pH, as electrostatic repulsion is weakest at the isoelectric point and increases as the pH moves away from it [21]. Therefore, solubility and other solubility-related functional properties should be investigated in a wide pH range. Water and oil holding capacities directly influence the sensory properties and texture of foods by affecting viscosity, palatability, flavor retention, and other related attributes [22, 23]. Recent research has focused on plant-derived proteins, such as those from pea, soy, and rice, as sustainable and consumer-friendly alternatives to synthetic, inorganic, or animal-based emulsifiers, due to their natural origin, functional versatility, and compatibility with environmentally conscious production methods [24]. It should be evaluated in each case since considerable differences in emulsifying activity have been observed between studies, even for the same plant protein [25].

Beyond the techno-functional properties, bioactivity of LPCs is also essential when using them as food additives. The protein bioaccessibility is critical to see the nutritional value of LPCs since plant proteins may be resistant

to enzymatic hydrolysis [18] and contain antinutritional components that reduce the nutrient uptake [26]. To ensure consumer safety, the cytotoxicity of LPCs must be evaluated. Caco-2 cells, derived from human colon carcinoma, are commonly used to evaluate *in vitro* cytotoxicity and model intestinal epithelium permeability for predicting *in vivo* bioactive absorption [27]. Although extensive research exists on medicinal plants used in health industry, studies on the cytotoxicity of proteins derived from food industry by-product leaves remain highly limited. Comparing the proliferation responses of healthy human embryonic kidney (HEK293T) and human hepatocellular carcinoma (HepG2) cells to LPC exposure is crucial for determining whether the effect is cancer-selective.

The objectives of this study were to investigate the techno-functional properties of LPCs extracted from cauliflower and beetroot leaves, together with their effects on different cell lines and their glucose metabolism. The techno-functional properties were compared with commercial protein powders. The literature lacks in comprehensive examination of green leaves addressing their techno-functional, nutritional, and bioactive properties. The effect of the precipitation method on physicochemical properties of LPCs was previously investigated, and further analyses were carried out to two selected LPCs. To the best of our knowledge, this study is the first to investigate the functional and health-related effects of LPCs at the same time.

Materials and methods

Materials

Fresh leaves of beetroot and cauliflower were collected from the Mediterranean region of Türkiye in winter season, and the proximate composition of leaves were determined previously [17]. Leaves were rinsed with tap water, stored at -20 °C and thawed before processing. Commercial protein powders of pea (81.7% protein) and soy (70% protein) were purchased from Vegrano (Türkiye) for the comparison of protein properties. All the chemicals were of analytical grade.

Leaf protein concentrate (LPC) production

Leaf protein concentrates (LPCs) were produced as described previously [17]. Leaves were cold-pressed by using a twin-screw extruder (GSE-5000-F, Tribest, USA), obtaining green juice and pulp fractions. Leaf protein was precipitated from the green juice by heat treatment at 70 °C for 15 min with continuous stirring. The precipitates were collected by centrifugation at 10,414 g for 15 min. The

pellet was freeze-dried and stored at 4 °C for further analyses. The crude protein concentration of LPCs were determined as 44.98% and 25.68%, respectively, via the standard Kjeldahl method ($P\% = N\% \times 6.25$).

Techno-functional properties of LPCs

Water and oil holding capacity

Water and oil holding capacity (WHC and OHC) of LPCs were determined gravimetrically. LPCs were mixed with distilled water and corn oil (Sirma, Türkiye) with the ratios of 1:40 and 1:60 (w/v), respectively. After 2 min of vortex, the mixture was centrifuged by 3,000 g for 30 min. The pellet and the supernatant were weighed, and the amount of absorbed water or oil by LPC was calculated.

Foaming capacity and stability

The LPC solution with a concentration of 20 mg/mL was prepared by distilled water. The solution was homogenized at 20,000 rpm for 2 min by using UltraTurrax (T25 Digital, IKA, Germany). The volume of the solution before homogenization (V_0), right after the homogenization (V_1), 30 and 60 min after the homogenization (V_2) were recorded. The foaming capacity and the stability were calculated by following equations:

$$\text{Foaming capacity (\%)} = \frac{V_1 - V_0}{V_0} \times 100 \quad (1)$$

$$\text{Foaming stability (\%)} = \frac{V_2 - V_0}{V_1 - V_0} \times 100 \quad (2)$$

Solubility

The solubility of LPCs was investigated in the pH range of 2–12. LPC solutions were prepared with distilled water at a concentration of 1% (w/v). The pH of solutions was adjusted by using 1 M HCl or NaOH in adequate amounts, and preserving the protein concentration in the solution. The protein solutions were mixed at 150 rpm for 30 min at room temperature, and centrifuged at 10,414 g for 10 min. The protein content of the supernatant was determined spectrophotometrically by using the Bradford method. In each well, 50 μ L of the sample was mixed with 150 μ L of Bradford reagent (B6916, Sigma-Aldrich, Germany). For control, 50 μ L distilled water was mixed instead of the protein solution.

After storing in dark for 10 min, the absorbance of samples was measured at 595 nm by a microplate reader (Epoch 2, BioTek Instruments, USA). The calibration curve was constructed using bovine serum albumin (BSA) and the results were expressed as mg BSA/g LPC. The solubility of LPCs were calculated by the following equation:

$$\text{Solubility (\%)} = \frac{\text{Soluble protein content in different pH solutions}}{\text{Soluble protein content in 1 M NaOH solution}} \times 100 \quad (3)$$

Emulsifying activity

1% (w/v) LPC solutions were prepared and the pH of the solutions were adjusted by using 1 M HCl or 1 M NaOH. The protein solutions were mixed at 150 rpm for 30 min at room temperature, and centrifuged at 10,414 g for 10 min. Corn oil (Sirma, Türkiye) was added to the LPC containing supernatant with a concentration of 10% (v/v). The emulsion was homogenized at 20,000 rpm for 3 min by UltraTurrax.

Emulsifying activity index (EAI) and emulsion stability index (ESI) were determined by the method described by Yang et al. [28], with some modifications. 50 μ L of homogenized sample was collected at 0 and 30 min after the emulsion formation, and was mixed with 5 mL of 0.1% sodium dodecyl sulfate solution (SDS). The absorbance of samples was measured at 500 nm by a microplate reader (Epoch 2, BioTek Instruments, USA), and the EAI and ESI were calculated by the following equations:

$$\text{EAI (m}^2\text{/g)} = \frac{2 \times 2.303 \times A_0 \times d}{C \times \Phi \times 10^4} \quad (4)$$

$$\text{ESI (min)} = \frac{A_0}{A_{30}} \times 100 \quad (5)$$

where A_0 is the absorbance at 0 min, A_{30} is the absorbance at 30 min, d is the dilution factor, C is the initial protein concentration (g/mL), and Φ is the oil fraction of the emulsion (v/v).

Droplet size (diameter) of the emulsion samples were measured by dynamic light scattering (DLS) equipment (Malvern Zetasizer NanoZS, Worcestershire, UK), and expressed in nanometer. Refractive index of the corn oil and water were used as 1.473 and 1.330 in the calculations.

Bioactivity of LPCs

In vitro protein bioaccessibility

The *in vitro* digestion protocol based on the simulation of sequential gastric and intestinal digestion was conducted [29] with slight modifications. All simulated fluids were freshly prepared prior the analysis. First of all, 0.3 g sample was mixed with 4 mL of salivary fluid, 25 μ L of 0.3 mol/L CaCl_2 , and 975 μ L of distilled water, and the pH was adjusted to 7. The mixture was shaken for 2 min at 37°C. After that, 7.5 mL of gastric fluid, 1.6 mL of pepsin solution (25,000 U/mL), and 5 μ L of 0.3 mol/L CaCl_2 were added to the mixture for the gastric digestion, and the pH was adjusted to 3. The mixture was again shaken for 2 h at 37°C. For the analyses of gastric phase, aliquots of 5 mL were collected for each sample before the intestinal digestion. Lastly, 8.25 mL of intestinal fluid, 3.75 mL of pancreatin (800 U/mL), 1.875 mL of bile (0.16 mol/L), and 30 μ L of 0.3 mol/L CaCl_2 were added on the remaining mixture for the simulation of intestinal digestion, and the pH was adjusted to 7. The mixture was again shaken for 2 h at 37°C. For the analyses of intestinal phase, aliquots of 5 mL were collected for each sample. The blank was used to adjust for any interference caused by digestive fluids or enzymes. All samples from gastric and intestinal phases were centrifuged at 12,850 g for 30 min. The soluble protein contents of supernatant of gastric or intestinal phase were determined by using the Bradford method described at Sect. “Solubility”. The initial protein content of LPCs were determined by the standard Kjeldahl method. The *in vitro* bioaccessibility was calculated using the following equation:

$$\text{Bioaccessibility (\%)} = \frac{\text{Protein content of the intestinal phase}}{\text{Protein content of the undigested LPC}} \times 100 \quad (6)$$

Cytotoxicity assay (CCK-8)

For the cytotoxicity analysis of LPCs, Caco-2 cells were cultured at 37°C and 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) including 4.5 g/L glucose and stable glutamine supplemented with 20% fetal bovine serum (FBS), 1% NEAAs, 1% penicillin/streptomycin. Cells were routinely subcultured when reaching to confluency of 70–80%.

The Caco-2 cell viability was determined by CCK-8 assay [30]. Caco-2 cells were seeded in 96-well plates at a concentration of 10,000 cells in 50 μ L of growing medium per well. After 24 h, cells were treated with LPC samples at different concentrations ranging from 0.0625 to 1% (v/v) in serum-free medium to avoid interference with protein components. After 24 h of exposure, 10 μ L of CCK-8 reagent

was added to each well. After standing 3 h in the dark conditions, the optical density was recorded at 450 nm using a microplate reader. Results were stated as percentages (%) of cell viability relative to the negative control (cells cultured in medium alone). The cells used in CCK-8 assay had passage numbers between 39 and 44. Cellular assays were performed at least in triplicate.

Cell proliferation assay (MTS)

HEK293T cells (passage 26) were cultured in DMEM (Gibco 11965092) supplemented with 10% FBS (Gibco A5670801), penicillin/streptomycin (P/S, 1% Gibco 15140122). Hepatocellular carcinoma cells, HepG2 (passage 20) were cultured in Eagle Minimal Essential Medium (EMEM, Gibco M4655-500mL) supplemented with FBS (10%) and P/S (1%). Cells were cultured under appropriate conditions in a heated and humidified incubator (Nuve EC160, 5% CO_2 , 37 °C). Phosphate-buffered saline (DPBS, 1X Gibco 141920144) and Trypsin-EDTA (0.25% Gibco 25200056) were employed to rinse and detach the cells from the plates at 37 °C for 5 min. Later, the corresponding amounts of LPCs were applied to these cells, and subsequent responses were recorded under an inverted microscope (Leica DMi1).

Cultured cells in a 96-well plate (10×10^4) were treated with LPCs (S5, 10 μ L / 100 μ L v/v) for 24 h. The viability of cells was measured with MTS (Promega CellTiter 96® Aqueous One Solution, G3581). After PBS rinsing, 100 μ L of 10% MTS solution was added, and the colorimetric changes were monitored by a microplate spectrophotometer (Biotek Epoch, 490 nm).

Glucose uptake assay

The glucose uptake capacity of the HEK293T cells (passage 26) was measured spectrophotometrically based on color change from the glucose uptake assay kit (Invitrogen, EIAGLUC). Briefly, cells in a 96-well plate were cultured with low glucose serum-free DMEM for 24 h. Next, the LPCs were applied in the same media and this was collected into microcentrifuge tubes after 24 h for the assay. These samples were diluted at a ratio of 1:10 with the Assay Buffer supplied within the kit and then transferred into another 96-well plate. HRP solution (1X), glucose oxidase (1X), and the substrate solution were added into each well at a ratio of 1:1:1 following the manufacturer's protocol. Control indicates HEK293T cells without treatment and following calculations followed the standard provided by the manufacturer. After 30 min of incubation, the absorbance values were recorded by the microplate spectrophotometer (Biotek

Epoch, 570 nm). The glucose uptake activity (%) was calculated using the following formula:

$$\text{Glucose Uptake Activity (\%)} = \frac{(\text{Control Glucose} - \text{Sample Glucose})}{\text{Control Glucose}} \times 100 \quad (7)$$

In this equation, “Control Glucose” refers to a 4.2 mg/mL solution of D(+)-glucose (Biofroxx, 1191KG001), which was prepared and used as the baseline glucose concentration in the assay. “Sample Glucose” represents the glucose concentration in the medium of cells treated with LPCs. The difference between these values reflects the amount of glucose consumed by the cells. This value is then normalized to the control condition and expressed as a percentage. A higher glucose uptake percentage indicates increased glucose consumption by the cells, whereas a lower percentage suggests reduced glucose uptake. This calculation provides a quantitative measure of the effect of LPC treatments on cellular glucose metabolism.

Statistical analysis

All experiments were analyzed statistically via Minitab (Version 18.1, USA). Each experiment was conducted as three biological replicates (independent extractions) versus three technical replicates (repeated measurements), and the data were presented as mean $\pm$ standard deviation. The differences between groups were compared via one-way analysis of variance (ANOVA), followed by Tukey’s multiple range test. For MTS and glucose uptake assays, statistical significance was determined using a one-tailed Student’s *t*-test with two samples assuming unequal variance (Welch’s *t*-test). A *p*-value less than 0.05 was considered statistically significant.

Results and discussion

LPCs were extracted from green leaves accumulated in agricultural fields, in an attempt to contribute to the Sustainable Development Goals (SDGs) by upcycling of food wastes.

Table 1 Water (WHC) and oil holding capacities (OHC) of LPCs and commercial protein powders

Protein	WHC (g water/g protein)	OHC (g oil/g protein)
Leaf protein concentrate		
Beetroot	9.57 $\pm$ 0.38 ^B	5.85 $\pm$ 0.52 ^B
Cauliflower	13.55 $\pm$ 0.99 ^A	7.54 $\pm$ 0.54 ^A
Commercial protein powder		
Pea	9.60 $\pm$ 0.11 ^B	3.61 $\pm$ 0.64 ^C
Soy	5.39 $\pm$ 0.49 ^C	3.39 $\pm$ 0.14 ^C

Differences between values with different letters within the same column are statistically significant (*p* < 0.05)

The crude protein content of cauliflower and beetroot LPCs were determined as 44.98% and 25.68%, and the protein recovery from leaf to LPCs (%) were 6.31% and 12.91%, respectively. The physicochemical and structural properties of LPCs, together with two others from broccoli and black carrot, were previously investigated in detail [17]. The protein quality and stability of cauliflower and beetroot LPCs were promising; therefore, the techno-functional properties and the bioactivity were further investigated to ensure the possible use of selected LPCs as upcycled food ingredients.

Water and oil holding capacity

WHC and OHC of cauliflower and beetroot LPCs as well as commercial protein powders were given in Table 1. Cauliflower LPC had the highest WHC and OHC holding capacity (*p* < 0.05). Beetroot LPC had similar or superior capacity compared to commercial proteins (*p* < 0.05).

The amount of water or oil that proteins can hold per unit mass is determined by their water and oil holding capacities (WHC and OHC), respectively [31]. Sensory properties and texture of the food products are significantly influenced by these features. While WHC is linked to the suitability of protein extracts for product development and is essential for the consistency of viscous foods, OHC contributes to palatability, mouth feel, flavor retention and emulsifying activity [22, 23]. A similar study reported that cauliflower leaf protein exhibited a higher WHC compared to broccoli, cabbage, and beetroot leaf proteins; however, in contrast to our findings, beetroot leaf protein demonstrated the highest OHC [32]. OHC tends to rise with greater surface hydrophobicity [18], and the higher OHC observed in cauliflower LPC compared to beetroot LPC in our study may be attributed to its greater concentration of hydrophobic amino acids [17, 33]. Previous review have indicated that leaf proteins exhibit superior WHC and OHC compared to various commercial proteins [13], which is in agreement with the findings of the present study. The presence of non-protein components within the matrix affect these properties [23], mostly polysaccharides [34], which may explain the higher holding capacities of LPCs than commercial proteins. In support of this, a study reported higher WHC in beetroot stems than leaves due to higher dietary fiber content [35]. It may also be the reason of high WHC of LPCs in this study as the green juice, starting material for protein precipitation, was extracted from both leaves and stems without prior separation [17]. Additionally, the differences in WHC and OHC may also be related to factors including protein content, balance of hydrophilic and hydrophobic regions, amino acid composition, protein conformation, and interfacial properties [36]. The WHC and OHC values reported in this study for commercial soy and pea protein are slightly higher than those in the literature,

which has reported varying values for these proteins [25]. This may be due to the reasons mentioned above. Although WHC is an essential functionality in bakery products for enhancing the texture and shelf stability [34], plant proteins exhibiting strong WHC and OHC are good candidates for meat extenders or plant-based meat alternatives, as they help retain moisture and fat within the product, thereby reducing cooking loss and providing porosity [25]. A study reported that the texturized vegetable proteins had significantly higher WHC compared to their OHC, along with reduced protein solubility following extrusion [37].

Foaming capacity and stability

The foaming capacity and stability of LPCs were comparable with commercial protein powders (Table 2). While pea protein had the highest foaming capacity, the foam stability of soy protein was the highest ($p < 0.05$). Both LPCs maintained more than 50% of their foam even after 60 min.

Plant proteins have the ability to stabilize foams by forming a protective layer at the air-water interface, which is crucial for achieving desirable textures in aerated food products such as cakes, whipped cream, ice cream, aerated chocolates and beverages [25, 38]. Foaming properties of proteins are determined by two characteristics, foaming capacity and stability, which are the ability of a protein solution to generate foam under agitation, and the duration over which the formed foam remains intact before collapsing, respectively. Sedlar et al. [32] reported 86% and 90% foaming capacity for beetroot and cauliflower LPCs, respectively. These values were higher than those found in this study; however, the greater foaming ability of cauliflower LPC was in consistency. Sugar beet, kale and lucerne LPCs had $> 50\%$ foam stability after 15 min [16]. Cauliflower and beetroot LPCs reached this value after 60 min, with even higher values

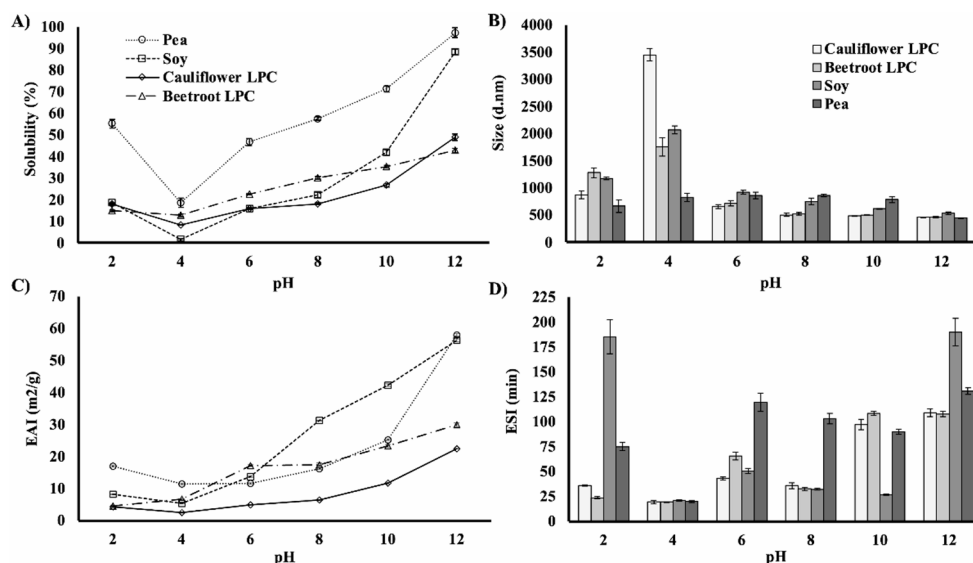
Table 2 Foaming capacity and foam stability of LPCs and commercial protein powders

Protein	Foaming Capacity (%)	Foam Stability (%)	
		30 min	60 min
Leaf protein concentrate			
Beetroot	68.33±10.10 ^B	62.92±5.05 ^{AB}	54.22±7.02 ^B
Cauliflower	72.50±9.01 ^B	53.33±5.77 ^B	52.29±6.86 ^B
Commercial protein powder			
Pea	199.17±11.27 ^A	47.54±7.50 ^B	31.31±5.58 ^C
Soy	78.33±5.77 ^B	74.51±8.38 ^A	73.53±8.71 ^A

Differences between values with different letters within the same column are statistically significant ($p < 0.05$)

observed at 30 min, indicating the formation of more stable foams. Additionally, except for commercial pea protein, the foam stabilities (%) at 30 and 60 min did not differ statistically according to paired t-test ($p > 0.05$), indicating that the stability of these proteins is not time-dependent. On the other hand, LPCs had lower foaming capacity than commercial protein powders, especially pea protein. The limited foaming capacity observed may be attributed to the insufficient diffusion of proteins to the air-water interface, hindering effective foam formation [39]. It is generally correlated to the solubility of the protein [18], and this may be the underlying reason of almost 3-fold higher foaming capacity of pea protein, which also had the highest protein solubility (Fig. 1). Higher protein solubility enhances water-protein interactions and facilitates the unfolding of the protein structure, which improves air encapsulation [7]. In contrast to the lower foaming capacity, LPCs showed significantly higher foaming stability than pea protein ($p < 0.05$). Rawiwan and Quek [15] explained this phenomenon as follows: Although broccoli, cabbage, and radish LPCs exhibited low solubility, the random formation of high-molecular-weight aggregates near their isoelectric point may have contributed to the development of a dense and robust network at the air-water

Fig. 1 A The solubility of LPCs and commercial protein powders in pH 2–12 range; B Droplet size; C Emulsifying activity index; and D Emulsion stability index of the o/w emulsions prepared by protein samples



interface, thereby enhancing foam stability and preventing collapse. Foaming ability of proteins are also affected by the precipitation method, botanical species, protein concentration, other constituents in the matrix, pH [31], conformation and surface properties [8].

In the literature, as a result of various formulation trials, it has been shown that soy, wheat, potato [40] and pea proteins [41] can be used successfully as egg substitutes in eggless cakes. Replacing 50–100% of egg/milk proteins with lentil protein in angel cakes and muffins did not significantly impact product volume [42]. Notably, muffins with 100% lentil protein were rated more favorably by consumers, indicating lentil protein is a viable substitute for foam and emulsion stabilization in cakes. Hydrolyzed pea protein concentrate exhibited the lowest foaming capacity (%), whereas hydrolyzed pea protein isolate demonstrated nearly twice the foaming capacity of whole eggs [43]. These findings highlight the importance of protein concentration in foam formation, and suggest that cauliflower and beetroot LPCs could also be purified depending on the intended application.

Solubility

Solubility of LPCs and commercial protein powders were determined at different pHs, in the range of 2–12 (Fig. 1A). The highest solubility at each pH belonged to pea protein ($p < 0.05$). However, at pH 4–8, LPCs had comparable or higher solubility than soy protein. All samples had an isoelectric point near 4, where their solubility was the lowest.

Protein solubility plays a crucial role in determining its functional properties and potential applications within the food industry. For instance, emulsification and foaming abilities of plant proteins are particularly impacted by their protein solubility, which affects their migration to the oil-water or air-water interface [25]. Due to the poor water solubility of plant proteins, their use in industry is still challenging [12].

Where the proteins exhibit no net electrical charge, as the positive and negative charges are balanced, is called as the isoelectric point, and the reduction in electrostatic repulsion facilitates protein aggregation and precipitation via hydrophobic interactions [39]. Many leaf proteins, such as alfalfa, beetroot, broccoli, and carrot, had an isoelectric point in the pH range of 3.5–4.5 [44]. According to the solubility profile in Fig. 1A, all protein samples had an isoelectric point near pH 4. Similarly, the lowest solubility, thus, the isoelectric point, of beetroot LPC [16] and cauliflower LPC [45] was reported to be at pH 4. The soluble proteins in plants are mainly consisted of RuBisCO, which has an isoelectric point around pH 4.4–5.5 [13]. The isoelectric point near pH 4 was likely influenced by the elevated content of aspartic

and glutamic acids in the LPCs [17], due to their side chain pKa values of 3.5 and 4.2, respectively [15].

Different from the leguminous plants and oil seeds, the solubility of leaf proteins tend to increase in alkaline conditions than acidic conditions, rather than the characteristic U-shape solubility profile [32]. As the pH shifts from the isoelectric point, the protein surface acquires a net charge, becoming more positive at lower pH values and more negative at higher pH values. This increase in electrostatic repulsion helps prevent protein aggregation, enhances protein–water interactions, and consequently leads to improved solubility [19, 36, 44]. In addition to the solubility in alkaline environments, beetroot and cauliflower LPCs also showed notable solubility under neutral conditions. Challenges in maintaining an alkaline environment, along with the fact that plant-based protein beverages generally have a pH range of 6.8–7.0, highlight the importance of protein solubility under neutral conditions [23].

The LPCs have relatively low solubility than other plant proteins since approximately the 50% of total protein in leaves are insoluble [13]. The similar or lower solubility of commercial soy protein than beetroot and cauliflower LPCs was in consistence with a previous study which reported that the lowest solubility belonged to commercial soy protein isolate compared to *Brassicaceae* LPCs, however, LPCs had more than 2-fold higher solubility than our results [15]. Protein solubility is majorly influenced by the amino acid composition and the protein conformation [33], together with the protein purity, since the strong association between the polyphenolic compounds and the leaf proteins cause a reduction in the solubility [16]. The relatively low solubility of LPCs in this study may be explained by their green color and high chlorophyll content [17]. By the help of enzymes [33, 45] or sieve filtration for the uniform size distribution [19], the solubility of protein concentrates can be improved. Previous studies also reported that ultrasonication enhanced the solubility of mulberry leaf protein [46] and pea protein [47] by reducing particle size, which in turn increased the surface area and facilitated protein–water interactions.

As mentioned above, solubility is important not only for the functional properties of proteins but also for the effect they exert on the food product to which they are added. Hazelnut meal proteins with higher solubility compared to LPCs in this study did not cause an increased turbidity compared to the reference, when added to the beverage [9]. However, protein solubility may decrease during storage or due to denaturation during heat treatment, causing protein aggregates, hence, turbidity in beverages [48]. Instead, it is worth noting that incorporating both soluble and insoluble proteins in plant-based meat substitutes might help them achieve the desired texture and overall quality [25].

Emulsifying activity

Emulsifying activity of protein samples was investigated in terms of droplet size, EAI and ESI (Fig. 1B–D). At alkaline conditions (pH 10–12), droplet size of LPCs decreased significantly, and at all pHs except the isoelectric point, the smallest droplet size belonged to cauliflower LPC ($p < 0.05$). The ESI of commercial protein samples were higher in acidic conditions; however, when the pH shifts to the alkaline conditions up to 10, LPCs showed similar or higher stability compared to commercial ones ($p < 0.05$).

Plant proteins exhibit emulsifying properties due to their amphiphilic nature, allowing them to adsorb at the oil-water interface, reduce interfacial tension, and form a stabilizing layer that prevents droplet coalescence. This adsorption typically occurs in two stages: initial migration and binding to the oil-water interface, followed by stabilization driven by hydrophobic interactions [49]. Emulsifying properties are commonly assessed by measuring emulsion turbidity for the EAI and by evaluating the ESI for stability [31].

Emulsifying activity of LPCs and commercial protein powders showed a similar trend to solubility: lowest at the isoelectric point and increased by the increased pH ($p < 0.05$). Studies with similar results attributed this trend to enhanced electrostatic repulsion and protein solubility [7, 15, 23]. It is not surprising that beetroot LPC had higher EAI than cauliflower LPC, since it also had higher solubility (Fig. 1C). An increase in solubility implies a higher concentration of proteins in the solution that can contribute to emulsion formation. When there is no enough protein in the environment, it also lacks in electrostatic repulsion that normally prevents oil droplet aggregation [39]. The EAI of LPCs and pea protein were comparable; however, after pH 6, soy protein showed significantly higher emulsifying ability than both LPCs ($p < 0.05$). In contrast to our study, a previous work recorded better emulsion formation by broccoli and cabbage LPCs compared to commercial soy protein [15]. When the commercial proteins are compared to each other, soy protein exhibited higher EAI under alkaline conditions. Although soluble pea protein is about 1.5 times more hydrophobic than whey protein, its limited surface hydrophobicity may hinder the formation of a stable interfacial film, whereas the amphiphilic nature of soy proteins

enables them to act as effective emulsifiers in stabilizing emulsions [50].

The reduced ESI likely results from inadequate solubility, limited hydration, and weak electrostatic repulsion, all of which failed to effectively inhibit oil droplet aggregation [7]. Lentil protein's higher hydrophobicity led to rapid interfacial adsorption but limited emulsion stability due to low solubility, whereas pea and fava bean proteins, with greater solubility, formed more stable emulsions by creating densely packed interfacial films [49]. This may be the reason for soy protein generally having higher ESI than LPCs (Fig. 1D), even its EAI was lower. By enzymatic hydrolysis, the ESI can be enhanced. A study improved the jackfruit LPC's ESI from approximately 30% to almost 90%, by 180 min of hydrolysis with pepsin [11].

Although the EAI and ESI methods are widely used, the emulsion turbidity does not directly correlate with particle size and can vary unpredictably. Stability, appearance, texture, and sensory attributes of protein-stabilized emulsions are largely influenced by droplet size. Thus, emulsion stability is best measured using a particle size analyzer [50]. The droplet sizes of LPCs (Fig. 1B) in the neutral and alkaline conditions were significantly lower than commercial soy and pea protein ($p < 0.05$), which indicates a better emulsifying activity. RuBisCO exhibits good emulsifying activity either in its isolated form or as a mixed leaf extract, and this functionality can be influenced by extraction methods including moderate heating [13]. Also, as in WHC and OHC, impurities may improve the emulsifying activity. Bocarando-Guzmán et al. [39] examined both flour and protein isolate from *Moringa oleifera* Lam. leaves. They found that the leaf flour had significantly better emulsifying activity than leaf protein isolate, and linked it to the non-protein components. Proteins with such good emulsifying properties are ideal for use in emulsified foods like mayonnaise, creams, and salad dressings [31].

In vitro protein bioaccessibility

Protein bioaccessibility (%) of LPCs was calculated by the ratio of soluble protein concentration in the intestinal fluid to the total protein concentration in the initial stage, and the results were given in Table 3. Even though cauliflower LPC had a higher (44.98%) protein content than beetroot LPC (25.68%), there was no significant difference between their *in vitro* bioaccessibility (%) ($p > 0.05$).

In order to determine the nutritional properties of the LPCs, it is essential to determine the bioaccessibility or digestibility. The nutritional value of plant proteins is typically low since they show resistance to enzymatic hydrolysis inherently [18]. There is limited *in vitro* bioaccessibility studies related to LPCs, especially for beetroot

Table 3 *In vitro* protein bioaccessibility (%) of LPCs

LPC	Initial	Stomach	Intestine	Bioaccessibility (%)
Beetroot	25.68 ± 7.93 ^{Ab}	0.23 ± 0.03 ^{Bb}	1.79 ± 0.03 ^{Bb}	6.95 ± 0.12 ^a
Cauliflower	44.98 ± 1.85 ^{Aa}	0.44 ± 0.02 ^{Ca}	3.16 ± 0.09 ^{Ba}	7.03 ± 0.20 ^a

Differences between values with different uppercase letters within the same row are statistically significant ($p < 0.05$)

Differences between values with different lowercase letters within the same column are statistically significant ($p < 0.05$)

and cauliflower LPCs. While mulberry LPC had up to 67% digestibility [51], the hull-less pumpkin LPC demonstrated an *in vitro* digestibility higher than 80% [33]. Alpha-amylase assisted extraction of radish leaves ended up with a LPC with 92% digestibility [52]. Benhammouche et al. [26] reported an almost complete protein digestibility (99.8%) of enzymatically (by Viscozyme L, multicomponent carbohydrase) extracted *Moringa oleifera* LPC, while it was 64.7% in defatted leaves. These values are approximately 10-fold higher than our results. The main differences are the source of LPC, extraction and the analysis methods. Even for the same source, minor differences in *in vitro* protein digestibility can take place due to the variations in sample composition, particularly protein quality, purification methods, or processing conditions [33]. For alfalfa LPC, there are studies both argue that the processing history does not affect the digestibility [53], or, the digestibility is decreased by even pressing the leaves more than twice [54]. In a study where soybean LPC with high digestibility was produced by both acid and heat precipitation, a greater decrease in N availability during storage was observed after papain digestion of proteins precipitated by heat treatment [55]. This study suggests that, even though the initial effect of the precipitation method may not be significant, it can lead to differences during storage. Also, β -turns and particle size also have an impact on the protein digestibility, since the presence of hydrophilic amino acids and the fragility of β -turns in the protein structure make it prone to degradation by proteases [51].

Another possible reason for the low bioaccessibility of beetroot and cauliflower LPCs is the presence of impurities, which were also proved by the FTIR spectra of LPCs [17]. Although this is less relevant for isolated proteins like RuBisCO, the presence of antinutrients in plants can reduce the protein utilization, bioaccessibility and bioavailability [13, 26]. These compounds, including phenolic compounds, are considered antinutrients as they can impair nutrient

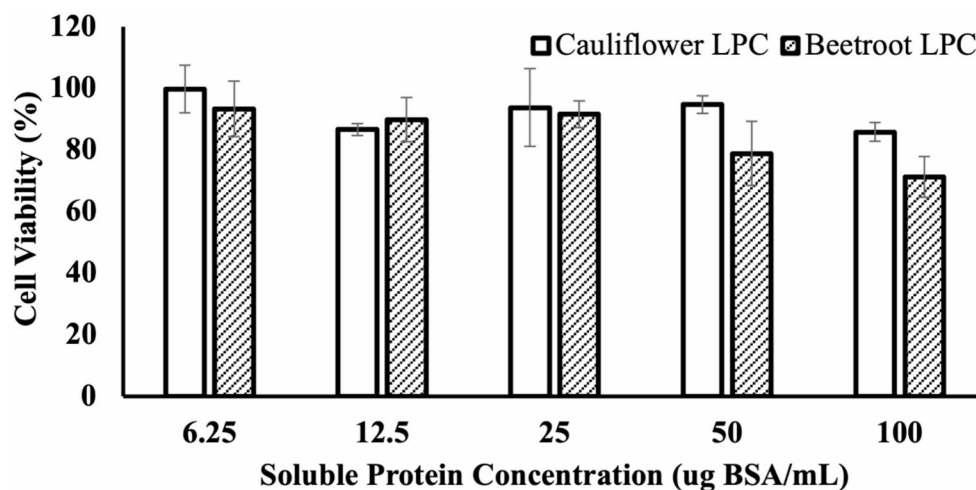
assimilation and reduce protein digestibility at high concentrations, ultimately lowering nutritional value [14]. Although the total phenolic compounds of LPCs were not analyzed, this possibility is strong to explain their low bioaccessibility since procedure does not contain any phenolic removal step, final product has a green color and chlorophyll content, and protein purity is found to be lower than 50% [17]. Studies show that washing LPCs with acetone or buffer increased the *in vitro* digestion and nutrient absorption by removing the antinutrients like tannins or phenolics, respectively [56]. For the enhancement of the protein bioaccessibility, novel extraction technologies can be implemented. In the study of Ortega et al. [22], *in vitro* protein digestibility of olive leaf proteins, in which antinutrients form complexes that make the proteins resistant to enzymatic hydrolysis, was increased from 50% to 62% by ultrasound-assisted alkaline extraction. They attributed the improved results to cavitation-induced changes in protein conformation, which enhanced accessibility to hydrolytic enzymes.

Cytotoxicity assay (CCK-8)

Cytotoxic effect of LPCs on Caco-2 cells was shown in Fig. 2. Although the cell viability was decreased by the increasing soluble protein concentration, the effect was not significant ($p > 0.05$), which can be interpreted as the LPCs did not show cytotoxicity on the Caco-2 cell line.

In order for proteins to be used as food ingredients, their cytotoxicity against human cells must be checked so that they do not present a threat to consumer health. The literature is advanced with research on medical plants used in the healthcare industry. However, cytotoxicity studies of green leaves that are waste or by-products of the food industry, especially the proteins derived from these leaves, are extremely limited. A study examined the cytotoxicity of extracts from different parts (floret, leaf and seed) of broccoli [57]. They showed cytotoxicity against lung carcinoma

Fig. 2 Cytotoxic effect of LPCs on the Caco-2 cells



(A549), colorectal adenocarcinoma (Caco-2) and hepatocellular carcinoma cells (HepG2), but not for the normal liver cells (FL83B) [57]. Blackcurrant, blueberry, wild strawberry and blackberry leaves' extracts had concentration-dependent cytotoxicity towards keratinocyte cells (HaCaT), where the elevated concentrations caused inhibition of the viability and metabolic activity of cell lines [58]. In another study, even though cell viability decreased with increasing concentration, no significant cytotoxic effect of jackfruit LPC on HaCaT cells was found within the tested range, suggesting its suitability for wound healing and anti-aging applications [59]. The increase in effect on the cell viability with increasing concentration is similar to our findings. Soluble leaf protein hydrolysate of cauliflower by-products showed no significant impact on HepG2 cell viability within the 1–500 µg/mL concentration range [60]. Avilés-Gaxiola et al. [61] examined the effect of 200–1000 µg/mL of moringa LPC and moringa LPC hydrolysate against human colon fibroblasts (CCD18-Co) cells. While no cytotoxic effects of LPCs were observed, the hydrolysates caused a significant decrease in cell viability, resulting in 75.63% cell mortality at the highest concentration.

For the cytotoxicity analysis, the Caco-2 cell line was selected since it is widely used as an *in vitro* model to evaluate the permeability of compounds, as it mimics the small intestinal epithelium and expresses key digestive enzymes and transporters, therefore provides a reliable prediction of the *in vivo* absorption of bioactive compounds [27, 62]. Although neither beetroot nor cauliflower LPCs were found to be cytotoxic to Caco-2 cells, beetroot LPCs had a more negative effect on cell viability, especially at increasing concentrations. The underlying reason for this may be related to

the impurity of LPCs, as discussed in the techno-functional properties and bioaccessibility results above, since the crude protein content was 25.68% and 44.98% for beetroot and cauliflower, respectively. The results of a study investigating the cytotoxicity of *Moringa oleifera* leaf extract and its protein-rich fraction towards human peripheral blood mononuclear cells (PBMC) supported the influence of protein purity [63]. Both fractions did not have cytotoxicity, and the difference between them was not significant. However, the protein-rich fraction affected cell viability to a lesser extent.

Cell proliferation assay (MTS)

The MTS assay was used to evaluate the proliferative effects of cauliflower and beetroot LPCs on healthy human embryonic kidney (HEK293T) and human hepatocellular carcinoma (HepG2) cells (Fig. 3). The results demonstrate that cauliflower LPC significantly enhanced the proliferation of HEK293T cells, while having no notable impact on the proliferation of HepG2 cells. This selective proliferative effect on healthy cells indicates that cauliflower LPC may possess bioactive compounds that promote normal cell growth without stimulating the proliferation of cancerous cells. Such a profile highlights its potential as a safe, health-supportive agent with low risk of oncogenic stimulation. In contrast, beetroot LPC led to a 170% increase in cell proliferation compared to the control. This suggests that beetroot LPC contains bioactive molecules capable of broadly stimulating cellular metabolism and growth, regardless of the cell type. While this metabolic stimulation may be beneficial in non-malignant settings, the fact that beetroot LPC also enhances proliferation in carcinoma cells is a concern.

Fig. 3 Effect of LPCs on the proliferation of HEK293T and HepG2 cells. (** $p < 0.01$, *** $p < 0.001$)

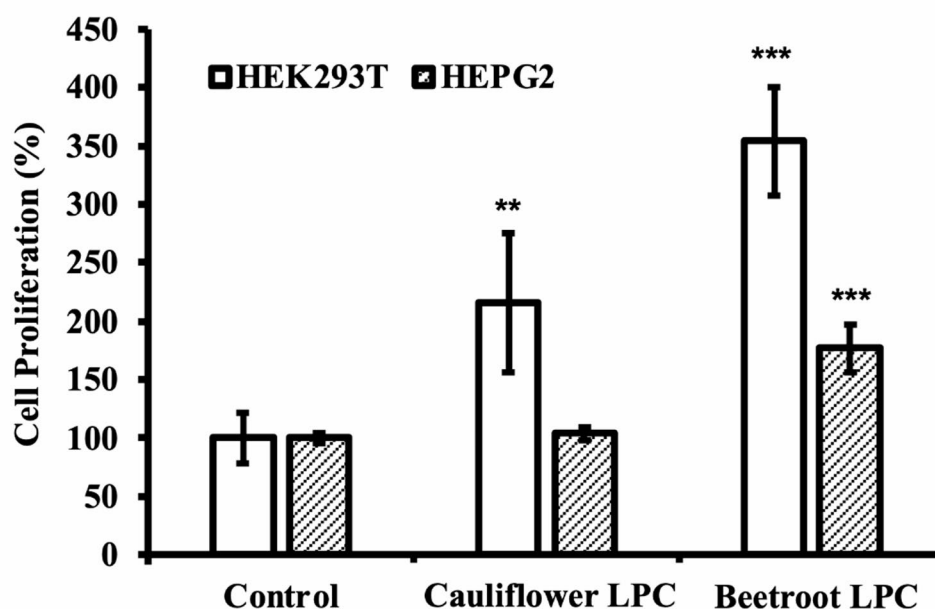
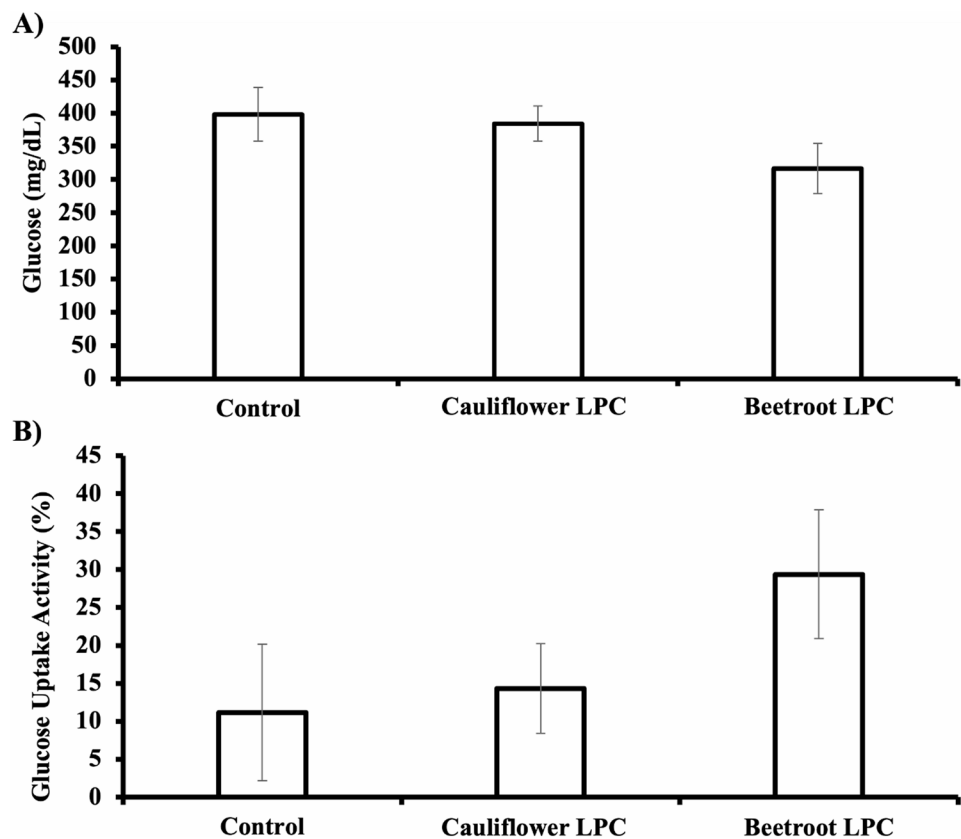


Fig. 4 Measurement of the effects of LPCs on glucose uptake in HEK293T. **A** remaining glucose in the culture medium; **B** glucose uptake capacity. * indicates $p < 0.05$



In cancer cells, increased proliferation may be associated with enhanced survival signaling and metabolic reprogramming, characteristics typical of tumor progression [64]. The differential response of HEK293T cells and HepG2 cells highlights cell line-specific metabolic and signaling profiles. HEK293T cells are likely more responsive to cauliflower LPC's bioactive compounds, such as glucosinolates, which are metabolized into isothiocyanates (ITCs) upon ingestion. ITCs are known to modulate signaling pathways that support cell survival and proliferation in healthy cells. For example, studies on cruciferous vegetables suggest that ITCs can activate protective pathways like Nrf2-mediated antioxidant responses, which may support cell growth in normal cells [65]. Research shows that ITCs from cruciferous vegetables, including cauliflower, can inhibit proliferation in cancer cells through apoptosis and autophagic cell death [66]; the lack of significant effect in HepG2 cells suggests that the specific composition of cauliflower LPC may not be potent enough to elicit a strong response. This aligns with our studies showing that cauliflower LPC can have protective effect in healthy cells but limited impact on cancer cell proliferation.

Glucose uptake assay

The glucose uptake assay in HEK293T cells was conducted to assess whether the metabolic effects observed in the MTS assay were linked to changes in glucose metabolism (Fig. 4). The results revealed a clear distinction between the two LPCs. Beetroot LPC treatment significantly enhanced glucose uptake in healthy HEK293T cells, which suggests that beetroot leaf proteins or associated phytochemicals activate cellular pathways that increase glucose transport, likely through upregulation of glucose transporters such as GLUT1 or GLUT4 or stimulation of glycolytic flux [67]. These results support the MTS data, where increased cell proliferation may be explained in part by the elevated glucose uptake and corresponding energy availability. Additionally, beetroot juice supplementation has been associated with increased expression of 5' adenosine monophosphate-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α), leading to enhanced mitochondrial content and oxidative metabolism in myocyte [68]. Dietary nitrates in beetroot LPC can enhance the efficiency of mitochondria, leading to increased production of ATP, the cell's energy source, and promoting cell growth. These nitrates also activate AMPK, a protein that improves the cell's ability to take in glucose from the bloodstream [69, 70]. These effects are consistent with

the substantial increase in cell proliferation observed in HEK293T cells, indicating that the cells enter an energy-rich state that supports their survival and proliferation. In contrast, cauliflower LPC had no significant impact on glucose uptake in HEK293T cells. This indicates that its proliferative effect in healthy cells is unlikely to be mediated by glucose metabolism. Instead, the mechanism may involve other cellular processes such as modulation of growth factor signaling, antioxidant defense, or changes in protein synthesis pathways [65]. Taken together, the data indicates that while cauliflower LPC supports healthy cell proliferation without affecting glucose uptake or cancer cell growth, beetroot LPC simultaneously promotes proliferation and enhances glucose uptake across both healthy and cancerous cell types. This dual effect of beetroot LPC may be beneficial in energy-demanding physiological contexts but could be detrimental in oncogenic settings, as increased glucose uptake is a hallmark of cancer cell metabolism.

Conclusion

This study demonstrates the potential of beetroot and cauliflower leaf protein concentrates (LPCs) as sustainable, plant-based alternatives to conventional commercial proteins. LPCs exhibited favorable techno-functional properties such as high water and oil holding capacities, comparable or superior foaming and emulsifying ability. Although their *in vitro* bioaccessibility was relatively low, the overall cytotoxicity remained minimal, supporting their safety at the tested concentrations. Importantly, cauliflower LPC selectively promoted the proliferation of healthy human embryonic kidney cells without enhancing human hepatocellular carcinoma cell proliferation, and did not affect glucose uptake, indicating a different mechanism for its bioactivity, such as modulation of growth factor signaling or antioxidant defense. These findings highlight the value of LPCs, both as functional food ingredients and as contributors to circular food systems by valorizing agricultural waste. Overall, our findings suggest that upcycled leaf protein concentrates have the potential to serve as thickeners, emulsifiers, and heat-stable ingredients in various processed food applications. Further studies are needed to improve their bioavailability and to fully understand their nutritional and therapeutic potential. Low *in vitro* bioaccessibility can be improved by investigating its underlying causes and analyzing and/or removing antinutritional factors. Additionally, bioavailability assessments using cell culture models can provide further insights about the nutritional quality. Although this study examined the behavior of proteins in water or oil-in-water emulsions, evaluating their properties within selected model food matrices would enhance the applicability and

industrial relevance of the findings. Focusing on process and product development approaches that will facilitate the adaptation of LPCs to different food formulations on an industrial scale will better reveal the functional and commercial values of these ingredients.

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Data availability Data is available from the corresponding author upon request.

Declarations

Conflict of interest The authors declare no competing interests.

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