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Research Article

Optimization of Protein Extraction from Mallow Leaves: Enhancing Yield Through Various Extraction Methods

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Abstract

This study focuses on optimizing protein extraction from mallow leaves as a potential protein source. Various extraction methods were evaluated to improve the efficiency of recovering the existing protein. Firstly, frozen, vacuum-dried, and freeze-dried mallow leaves were compared in terms of protein content and the freezing (42.33%) has the highest protein content on a dry basis. In order to increase protein yield, as the second step, the extraction process was carried out at different pH values (9 and 10) and the highest protein concentration of 1.535 mg/mL was detected at pH 10. Step 3 was the extraction temperature, which is performed at 55, 60 and 65℃ and the protein content of protein concentrates were 50.07%, 45.31% and 36.96%, respectively. In the next step, 10, 15 and 20 mL/g solvent/solid ratios were tried, and 10 mL/g had the highest protein content (53.07%). As the last step, the optimum ultrasound-assisted extraction parameters were determined as 20 min at 100% amplitude. Finally, the protein yield reached 60% with all these process steps.

Keywords: Mallow leaf, plant protein, optimization, ultrasound assisted extraction, enzyme assisted extraction

Ebegümeci yapraklarından protein ekstraksiyonunun optimizasyonu: Çeşitli ekstraksiyon yöntemleriyle verimin artırılması

Öz

Bu çalışma, potansiyel protein kaynağı olarak belirlenen ebegümeci yapraklarından çeşitli ekstraksiyon yöntemleri yardımı ile protein verimini arttırmayı amaçlamaktadır. İlk olarak dondurulmuş, vakumla kurutulmuş ve dondurularak kurutulmuş ebegümeci yaprakları protein içeriği açısından karşılaştırıldığında, kuru bazda en yüksek protein içeriğinin dondurularak (%42,33) elde edildiği görülmüştür. Protein verimini arttırmak amacıyla ikinci adım olarak farklı pH değerlerinde (9 ve 10) ekstraksiyon işlemi gerçekleştirilmiş ve en yüksek protein konsantrasyonu 1.535 mg/mL ile pH 10'da tespit edilmiştir. 3. adım ekstraksiyon sıcaklığıdır, 55, 60 ve 65℃'de gerçekleştirilen çalışmada protein konsantrelerinin protein içeriği sırasıyla %50,07, %45,31 ve %36,96 olmuştur. Bir sonraki aşamada 10, 15 ve 20 mL/g solvent/katı oranları denenmiş ve en yüksek protein içeriği (%53,07) 10 mL/g'da elde edilmiştir. Son adım olarak optimum ultrason destekli ekstraksiyon parametreleri %100 genlikte 20 dk olarak belirlenmiştir. Sonunda tüm bu işlem adımları ile protein verimi %60'a ulaşmıştır.

Anahtar Kelimeler: Ebegümeci yaprağı, bitkisel protein, optimizasyon, ultrases destekli ekstraksiyon, enzim destekli ekstraksiyon

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1. Introduction

Food-sourced proteins are divided into two: animal and plant-based protein sources. While animal sources are rich in nutritional properties, especially essential amino acids, plant proteins are rich in antioxidants and fiber, positive for health and sustainable sources. Leaf proteins are the most abundant in nature, economical and renewable proteins compared to animal and legume proteins [1]. In recent years, studies on leaf proteins such as cassava leaves [2], sugar beet leaves [3], olive leaf [4] and tea leaf [5] is becoming increasingly important. Mallow is the general name of biennial or perennial herbaceous plants with 1500 species of the same genus in the Malvaceae family. Found mostly in Europe and Asia, 8 species of mallow grow in Turkey, the most important of which is *Malva sylvestris* (Great mallow) [6,7]. Mallow is a plant that is widely found in Turkey, grows on its own with the seeds it sheds, and contains a high amount of protein and does not require special care. Mallow leaves were chosen due to their nutritional profile, including their notable protein content, as well as their availability and underutilization in current food and industrial applications [8].

The extraction is an important step in protein isolation that affects protein yield. Solvent/solid ratio, pH value, temperature, time and solvent type are the parameters that affect the extraction [9]. By optimizing these parameters that affect the extraction, high yield protein can be obtained. Various alternative extraction methods are used to make the extraction step more effective. Ultrasound and enzyme-assisted extraction are extraction methods that help increase protein yield. With these methods, the proteins inside the cell can be transferred to the solvent by breaking down the cell wall, which is the extra barrier in plant cells [10]. In addition to obtaining protein in the desired yield and quality, the moderate extraction conditions they offer make these methods one step forward [11]. In this study, in order to obtain high yield of protein from mallow leaf, which was determined as an alternative protein source, the form of the raw material (frozen, vacuum dried and freeze dried), pH value (9 and 10), temperature (55, 60 and 65℃), solvent/solid ratio (10, 15 and 20 mL/g) and ultrasound-assisted extraction (amplitude and time) were optimized.

2. Material and Methods

2.1. Materials

The mallow leaf (*Malva sylvestris* L.) was collected from the garden in Bayındır district of Izmir in November 2023 (Figure 1). To remove soil and mud, they were washed under the water, and they were divided into 3 groups. First group leaf was frozen (-18℃), second group was vacuum dried (65℃), and the rest of them was freeze-dried (-48℃). Pectinex UF and Alcalase L were supplied from Novozymes (Denmark). For Bradford protein method, Bovine Gamma Globulin (2 mg/mL) from Thermo Fisher Scientific (USA) was provided.

Figure 1. The mallow leaf used in the study

2.2. Methods

2.2.1. Chemical properties of mallow leaves

The total dry matter and protein content analysis were performed both mallow leaves and protein powders.

Total Dry Matter Content

Total dry matter content of samples were detected according to AOAC [12]. 5 grams of mallow leaves were taken into petri. After that, until they reached a constant weight, the sample were dried in a vacuum oven at 65 °C. Total dry matter (%) was calculated as below (Eq. 1).

$$Total\ dry\ matter\% = \frac{m_3 - m_1}{m_2 - m_1} \times 100 \quad (1)$$

m_1: Tare of empty petri (g)

m_2: Sample + petri (g)

m_3: Constant weight of sample + petri (g)

Protein Content

The protein content of samples were detected according to Kjeldahl [12]. 13-14 mL 95-96% H_2SO_4 were added to 1 gram mallow leaves. After the combustion at 425℃ for 4 h, the distillation was done using 40% NaOH. Then, the consumption was recorded by titration with 0.1 N HCl. The nitrogen content was calculated using the equations below (Eq. 2). The nitrogen-to-protein conversion factor was 6.25 (Eq. 3).

$$N\% = \frac{(C-B)\times 0.014 \times N}{m} \times 100 \quad (2)$$

$$Protein\% = N\% \times 6.25 \quad (3)$$

C: Consumption volume of 0.1 N HCl in sample (mL)

B: Consumption volume of 0.1 N HCl in blank trial (mL)

N: Normality of HCl (0.1)

m: Sample weight (g)

2.2.2. Isoelectric Precipitation

The buffer solution was added to mallow leaves and the mixture was homogenized with using homogenizer (Daihan, HG-A5A). Then, the mixture was arranged in water bath at a certain temperature for 30 min to extract proteins into the solvent. After that, the mixture was filtered with cheesecloth and then centrifuged (6000 rpm, 20 min) to remove fiber part. The pH value of supernatant was adjusted to 3.5 and stirred for 30 min and then centrifuged (6000 rpm, 30 min). The pellet was freeze-dried (Liyolife) at -48℃ for 15 h. In ultrasound and enzyme assisted extraction, the ultrasound and enzyme was used before the extraction process, and it continues in the same way afterwards [13,14].

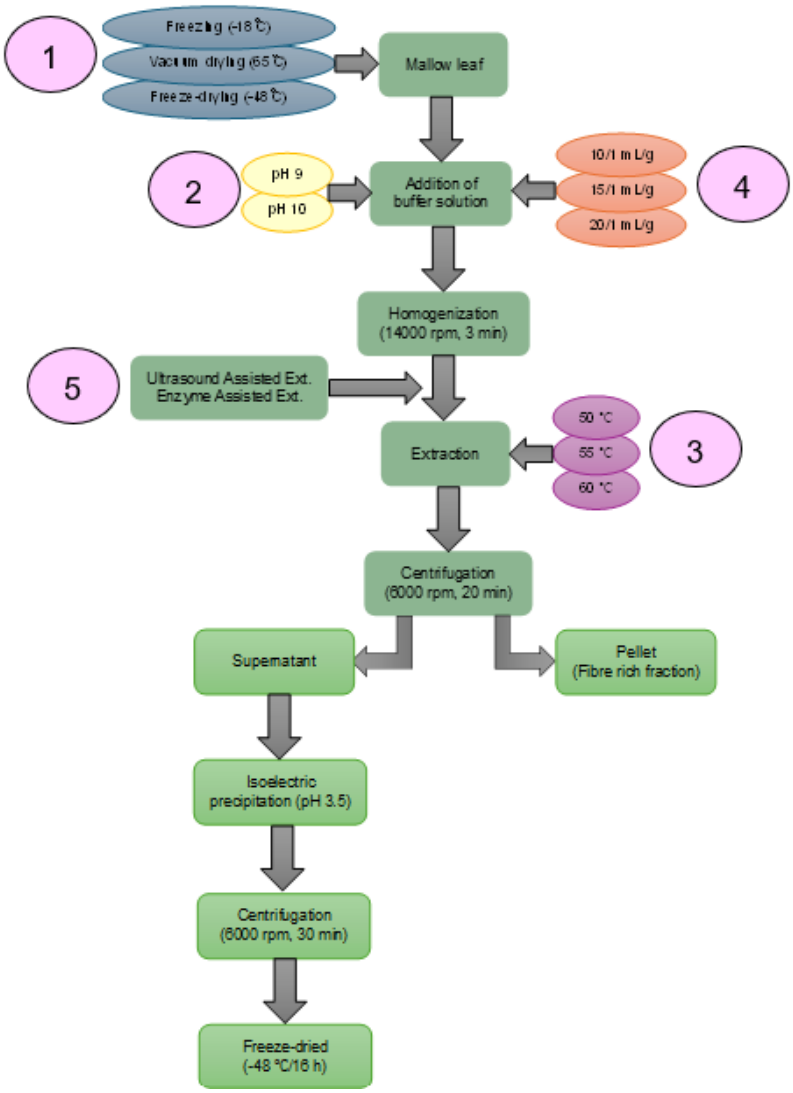

Figure 2. The flowchart of the protein isolation

2.2.3. Optimization of process parameters

The process steps were evaluated one by one in order to obtain protein from mallow leaves at maximum efficiency. The flow chart of the protein isolation was given in Figure 2. The first step (1) was to choose the preservation method from 3 options: freezing, vacuum drying and freeze-drying. The second step (2) was to find the pH value of extraction medium. Then, the temperature (3) of the extraction was determined. After that, the solvent/solid ratio (4) was investigated for maximum yield. Furthermore, to enhance the protein yield, the ultrasound and enzyme assisted extraction methods were applied. In ultrasound assisted extraction, amplitude and time were evaluated and in enzyme assisted extraction (5), it has been investigated which enzyme is more effective.

Preservation method

The first step was to select the preservation method in between freezing, vacuum drying and freeze-drying in terms of protein content of mallow leaves. The mallow leaves were divided into 3 groups. First group (F) leaf was frozen (-18℃), second group (V) was vacuum dried (65℃), and the rest of them (L) was lyophilized (-48℃). After that, the protein content of F, V, and L in dry basis was determined by Kjeldahl method [12].

pH value of extraction

The sugar beet leaves were homogenized with buffer solution with 10/1 solvent/solid ratio. The pH 9 and 10 value of buffer solution were compared in terms of protein concentration. The selection of pH 9 and pH 10 buffer solutions for comparison in terms of protein concentration was based on the well-established principle that protein solubility generally reaches its maximum at alkaline pH levels. For plant-based proteins such as those in *Malva sylvestris*, increasing the pH above the isoelectric point enhances protein solubilization and extraction efficiency. The extraction was carried out at 50℃ for 30 min and then the mixture was filtered by cheesecloth and centrifuged (6000 rpm, 20 min). The protein concentration of supernatant was determined by Bradford method [15].

2 mL Coomassie Blue solution was added to 100 µL of the sample and the protein concentration (mg/mL) was measured by spectrophotometry at 595 nm. Bovine gamma globulin was used for the calibration curve.

Temperature of extraction

After determining the pH value, sugar beet leaves were homogenized with a buffer solution at this pH value with a solvent:solid ratio of 10:1. Then, the extraction was performed at 50℃, 55℃, and 60℃. Afterwards, protein powder was obtained as shown in the flow chart (Figure 2) and the protein contents were determined by the Kjeldahl method.

Solvent/solid ratio

The sugar beet leaves, and the buffer solution were mixed with solvent/solid ratio of 10, 15, and 20 mL/g. Then, the extraction was performed at the determined temperature for 30 min. Afterwards, protein powder was obtained as shown in the flow chart (Figure 2) and the protein contents were determined by the Kjeldahl method.

Ultrasound and enzyme assisted extraction

Ultrasound assisted extraction

As seen in Figure 2, the ultrasound was applied before the extraction process. The parameters of ultrasound application that are amplitude, time, and batch/continuous were determined according to protein concentration. After ultrasound, the extraction was carried out and then the mixture was filtered by cheesecloth and centrifuged (6000 rpm, 20 min). The protein concentration of supernatant at each condition was determined by Bradford method [15].

Firstly, 60%, 80%, and 100% amplitude were applied, keeping other parameters constant. Then, the processing time at the determined amplitude was selected among 10, 15, and 20 min applications. Finally, it was decided whether ultrasound would be applied batch or continuously.

Enzyme assisted extraction

The enzyme assisted extraction was carried out before extraction. The application conditions of the enzymes selected for enzyme-assisted extraction are as follows.

Pectinex UF: pH 7 value, 50 ˚C, 30 min, 7% enzyme concentration [8]

Alcalase L: pH 8 value, 50 ˚C, 30 min, 1% enzyme concentration [8]

After enzyme pretreatment, the extraction was carried out and then the mixture was filtered by cheesecloth and centrifuged (6000 rpm, 20 min). The protein concentration of supernatant for both enzymes was determined by Bradford method [15].

2.2.4. Statistical Analysis

The analyses were performed in triplicate, and the results are expressed as mean ± standard deviation. The difference between the protein contents and protein concentrations were determined by performing t-test and Duncan test 95% confidence level in SPSS program (SPSS Inc., USA).

3. Results and Discussion

3.1. Physicochemical properties of mallow leaves

Total dry matter content of mallow leaf was 21.52±0.03% and the protein content of mallow leaf in dry basis was found 44.18±0.23%. When compared to the literature, the results are generally consistent, except for the higher protein content observed. This high protein content

could be attributed to the fact that mallow grows across diverse regions encompassing 1500 species within the same genus. The chemical composition of mallow varies depending on factors like harvest time, climate, and soil characteristics [7].

3.2. Impact of Preservation Method on Protein Content

The preservation methods of freezing, vacuum drying, and freeze-drying were chosen based on their proven efficacy in maintaining the nutritional and functional properties of plant-based proteins. Freezing is widely used to preserve biological activity and prevent oxidative degradation by halting enzymatic processes at low temperatures, as demonstrated by Cui et al. [5] in protein extraction from tea residues. Vacuum drying effectively removes moisture under low temperatures, minimizing thermal degradation while retaining key nutrients, as highlighted by Tabaraki et al. [7] in the processing of *Malva sylvestris* leaves. Freeze-drying, considered one of the most effective preservation methods, maintains structural and functional integrity by sublimating water under low pressure and temperature. Vergara-Barberán et al. [4] reported its superior ability to preserve functional properties in protein extraction from olive leaves. These methods were selected to ensure the maximum retention of protein content, bioactivity, and functionality in *Malva sylvestris* leaf protein, supported by findings from the aforementioned studies. The preservation method significantly influenced the protein content of mallow leaves. The protein content of frozen (F) mallow leaves exhibited the highest protein content at 42.33%. In comparison, vacuum-dried (V) and freeze-dried (L) leaves yielded lower protein contents, measured at 36.62% and 38.51%, respectively. These findings suggest that the freezing process is the most effective method for preserving protein content in mallow leaves. The studies have shown that protein isolation from dried samples is low in terms of protein yield, protein recovery and protein content. This is thought to be due to the changing microstructure during drying. It has been stated that the decrease in intracellular fluid and low rehydration make the extraction of intracellular proteins difficult [16].

3.3. Optimization of Alkaline Extraction Process

The protein concentrations at extraction pH 9 and pH 10 were 1.318 mg/mL and 1.535 mg/mL, respectively (Table 1). Therefore, pH value of extraction was determined as pH 10. In many studies, it has been found that proteins have high solubility and high extractability in high alkaline environments. In the literature, pH 9 and pH 10 values have been determined as the optimum extraction conditions in protein isolation from various plant sources [17,18]. Secondly, the temperature of the extraction was determined as 55 ˚C since the highest protein content (50.07%) was detected at this temperature. The protein content gradually decreased with increasing temperature (Table 1). It is thought that the reason for this is protein denaturation due to the increase in temperature [19]. The temperatures of 50 °C, 55 °C, and 60 °C were selected based on their relevance to protein extraction efficiency and functionality preservation. Moderate temperatures in this range are known to enhance protein solubility and extraction yield by promoting the release of proteins from plant matrices while minimizing thermal denaturation. According to Cui et al. [5], higher extraction efficiencies for plant-based proteins can be achieved at temperatures within 50–60 °C without significant loss of functional properties. Akyüz and Ersus [8] emphasized that maintaining moderate temperatures during

protein extraction from *Malva sylvestris* leaves preserved the bioactivity of proteins. Furthermore, Akyüz et al. [14] demonstrated the importance of optimizing temperature parameters, such as 55 °C, for maximizing protein recovery from sugar beet leaves. Based on these findings, the temperatures of 50 °C, 55 °C, and 60 °C were studied to determine the optimal balance between extraction efficiency and protein functionality in *Malva sylvestris*. The protein contents of protein concentrations at 10, 15, and 20 mL/g solvent/solid ratio were 53.07%, 45.49%, and 43.16%, respectively (Table 1). 10 mL/g solvent/solid ratio was determined for the protein extraction from mallow leaves. The solvent/solid ratio is very important for the production capacity of large-scale protein concentrates. It varies according to whether the raw material is dry or fresh and the other components it contains. While higher solvent addition is required for dry samples, the presence of components known for their water retention properties such as cellulose, hemicellulose and pectin will also require higher solvent addition [8].

Table 1. The extraction parameters and optimum point according to protein content/protein concentration

Conditions	**Parameters**	**Result**	**Optimum parameter**
Preservation Method		Protein content (dry basis)	Frozen Duckweed
	Frozen Mallow Leaf (F)	42.33±0.11^{a} %	
	Vacuum-Dried Mallow Leaf (V)	36.62±0.38^{c} %	
	Freeze-Dried Mallow Leaf (L)	38.51±0.26^{b} %	
pH value		Protein concentration	pH 10
	pH 9	1.318±0.18^{b} mg/mL	
	pH 10	1.535±0.47^{a} mg/mL	
Temperature		Protein content (dry basis)	55 ˚C
	55 ˚C	50.07±0.17^{a} %	
	60 ˚C	45.31±0.02^{b} %	
	65 ˚C	36.96±0.19^{c} %	
Solvent/solid ratio		Protein content (dry basis)	10 mL/g
	10 mL/g	53.07±0.11^{a} %	
	15 mL/g	45.49±0.24^{b} %	
	20 mL/g	43.16±0.02^{b} %	
Ultrasound	Amplitude	Protein concentration	100%
	60%	0.64±0.02^{b} mg/mL	
	80%	0.66±0.01^{b} mg/mL	
	100%	0.79±0.15^{a} mg/mL	
	Time	Protein concentration	20 min
	10 min	1.04±0.03^{c} mg/mL	
	15 min	1.15±0.02^{b} mg/mL	
	20 min	1.22±0.04^{a} mg/mL	
	Application method	Protein concentration	Continuous

	Batch	0.85±0.11[b] mg/mL	
	Continuous	0.96±0.27[a] mg/mL	
	Enzyme	Protein concentration	
Enzyme	Pectinex UF	1.07±0.12[b] mg/mL	Alcalase L
	Alcalase L	1.39±0.18[a] mg/mL	

*Differences between values within the same column denoted by different lowercase letters are significant at $P<0.05$.

3.4. Ultrasound-Assisted Extraction Parameters

In the ultrasound process, as the first step, 60%, 80% and 100% amplitude experiments were performed and protein concentrations were found to be 0.64±0.02 mg/mL, 0.66±0.01 mg/mL, and 0.79±0.15 mg/mL, respectively. In the second step, protein concentrations at 10, 15 and 20 min application times were 1.04±0.03 mg/mL, 1.15±0.02 mg/mL, and 1.22±0.04 mg/mL, respectively. Finally, it was decided that continuous application instead of batch application was the most suitable application method in terms of protein yield (Table 1). Therefore, the ultrasound process were carried out at 100% amplitude for 20 min, continuously.

Mallow leaves contain high levels of mucilage. Mucilage is a polysaccharide with a complex structure that holds water [20]. This structure is a significant difficulty in extracting protein from the cell by the solvent. Ultrasound application helps to break down this structure and extract proteins. At the same time, cavitation of the cells increases efficiency [14].

3.5. Enzyme-Assisted Extraction Parameters

The protein concentrations obtained from Pectinex UF and Alcalase L assisted extractions were 1.07 mg/mL and 1.39 mg/mL, respectively. A single pH value, temperature, time, and enzyme concentration were selected for Pectinex UF and Alcalase L based on their optimal conditions reported in the literature and preliminary experiments conducted in our study. Enzyme-assisted extraction studies often utilize established optimal parameters to maximize efficiency and ensure reproducibility. In literature, it emphasizes the importance of focusing on specific conditions that are most effective for enzyme activity and substrate compatibility [4, 11]. Alcalase L and Pectinex UF have well-documented optimal operating ranges, and the selected parameters in this study align with those reported by Cui et al. [5] and Akyüz and Ersus [8], who demonstrated high protein yields and preserved bioactivity using these enzymes. While Pectinex UF helps release proteins by breaking down the cell wall, Alcalase L helps protein extraction by breaking down proteins into smaller building blocks [14]. The effective enzyme type varies depending on the structure of the raw material and the other components it contains [21,14]. However, despite the high protein concentration with the extraction of Alcalase L, it was determined that the amount of protein powder obtained during the precipitation stage was quite low. As a result of that, it was decided to use ultrasound instead of enzyme as an assisted extraction method.

3.6. Protein Isolation at Optimum Point and Comparison of Protein Yield

After all, the alkaline extraction parameters were pH 10 value, 55 ˚C, 30 min and 10 mL/g. At this extraction condition, the protein yield was found as 15.22%. The ultrasound pretreatment

was carried out at 100% amplitude for 20 min, continuously. With helping ultrasound, the protein yield reached to 41.15%. Finally, when all isolation steps were examined, it was determined that mechanical disintegration before extraction was critical, considering the structure of the mallow leaf. For this reason, after adding buffer (pH 10) at a solvent/solid ratio of 10 mL/g, the mixture was heated until it reached the extraction temperature (55 ˚C), and the homogenization time was extended, and the mixture was disintegrated in Ultra-Turrax for 5 min. Following this process, ultrasound-assisted extraction and alkaline extraction processes were applied, and protein precipitation was performed. With the applied pre-heating and extended mechanical disintegration time, the protein yield reached 60.04%.

4. Conclusion

This study optimized protein extraction from mallow leaves, identifying freezing (F) as the best preservation method, yielding a protein content of 42.33%. The extraction process was most effective at pH 10 and 55°C, with a solvent/solid ratio of 10 mL/g. The combination of pre-heating and ultrasound-assisted extraction significantly enhanced protein yield, reaching up to 60%. Additionally, enzyme-assisted extraction with Alcalase improved protein concentration, though it was most effective when combined with mechanical or thermal methods. These optimized techniques provide a promising approach for sustainable protein extraction from plant sources, with potential applications in the food industry.

Ethics in Publishing

There are no ethical issues regarding the publication of this study.

Author Contributions

Zülal AKSOY: Investigation; Validation; Writing-original draft.

Ayça AKYÜZ: Funding acquisition; Resources; Supervision; Writing-review & editing.

Seda ERSUS: Supervision; Writing-review & editing.

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References

[1] Sun, C., Wu, W., Ma, Y., Min, T., Lai, F., & Wu, H. (2017) Physicochemical, functional properties, and antioxidant activities of protein fractions obtained from mulberry (morus atropurpurea roxb.) leaf, International journal of food properties, 20(3), S3311-S3325.

[2] Fasuyi, A. O., & Aletor, V. A. (2005) Varietal composition and functional properties of cassava (Manihot esculenta, Crantz) leaf meal and leaf protein concentrates, Pakistan Journal of Nutrition, 4(1), 43-49.

[3] Akyüz, A., & Ersus, S. (2021) Optimization of enzyme assisted extraction of protein from the sugar beet (*Beta vulgaris* L.) leaves for alternative plant protein concentrate production, Food Chemistry, 335, 127673.

[4] Vergara-Barberán, M., Lerma-García, M. J., Herrero-Martínez, J. M., & Simó-Alfonso, E. F. (2015) Use of an enzyme-assisted method to improve protein extraction from olive leaves, Food chemistry, 169, 28-33.

[5] Cui, Q., Ni, X., Zeng, L., Tu, Z., Li, J., Sun, K., Chen, X. ve Li, X. (2017) Optimization of Protein Extraction and Decoloration Conditions for Tea Residues, Horticultural Plant Journal, 3(4), 172–176.

[6] Keskin, F. E. R. A. Y., Cihanalp, C., Külcü, R., & Yilmaz, D. (2015) Ebegümeci Bitkisinin Bazı Fiziko Mekanik Özelliklerinin Belirlenmesi, 29. Ulusal Tarımsal Mekanizasyon ve Enerji Kongresi, 2-5.

[7] Tabaraki, R., Yosefi, Z. ve Asadi, G. H. A. (2012) Chemical Composition and Antioxidant Properties of *Malva sylvestris* L., Journal of Research in Agricultural Science, 8(1), 59-68.

[8] Ersus, S., & Akyüz, A. (2023) Enzyme assisted extraction of protein from mallow leaf (*Malva sylvestris* L.) for production of alternative protein concentrate, Journal of Food Measurement and Characterization, 17(4), 3283-3294.

[9] Meshkani, S. M., Mortazavi, S. A., Rad, A. H. E., & Beigbabaei, A. (2016) Optimization of protein extraction and evaluation of functional properties of tomato waste and seeds from tomato paste plants, Biosciences Biotechnology Research Asia, 13(4), 2387-2401.a

[10] De Almeida, N. M., de Moura Bell, J. M. L. N., & Johnson, L. A. (2014) Properties of soy protein produced by countercurrent, two-stage, enzyme-assisted aqueous extraction, Journal of the American Oil Chemists' Society, 91(6), 1077-1085.

[11] Marathe, S. J., Jadhav, S. B., Bankar, S. B., & Singhal, R. S. (2017) Enzyme-assisted extraction of bioactives, In Food Bioactives (pp. 171-201). Springer, Cham.

[12] AOAC. (1990) Official Methods of Analysis of the AOAC, Volume 2 (No. Ed. 15), Association of Offical Analytical Chemists Inc.

[13] Aksoy, Z., & Ersus, S. (2023) The comparative studies on the physicochemical properties of mung bean protein isolate–polysaccharide conjugates prepared by ultrasonic or controlled heating treatment, Biocatalysis and Agricultural Biotechnology, 50, 102690.

[14] Akyüz, A., Tekin, İ., Aksoy, Z., & Ersus, S. (2024) Determination of process parameters and precipitation methods for potential large-scale production of sugar beet leaf protein concentrate, Journal of the Science of Food and Agriculture, 104(6), 3235-3245.

[15] Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Analytical biochemistry, 72(1-2), 248-254.

[16] Nitiwuttithorn, C., Wongsasulak, S., Vongsawasdi, P., & Yongsawatdigul, J. (2024) Effects of alkaline and ultrasonication on duckweed (*Wolffia arrhiza*) protein extracts' physicochemical and techno-functional properties, Frontiers in Sustainable Food Systems, 8, 1343615.

[17] Zhang, Y., Huang, X., Zeng, X., Li, L., & Jiang, Y. (2023) Preparation, functional properties, and nutritional evaluation of chickpea protein concentrate, Cereal Chemistry, 100(2), 310-320.

[18] Pasrija, D., & Sogi, D. S. (2022) Extraction optimization and functional properties of muskmelon seed protein concentrate, Journal of Food Measurement and Characterization, 16(5), 4137-4150.

[19] Sethi, S., Yadav, D. N., Snigdha, S., & Gupta, A. (2021) Optimization of process parameters for extraction of protein isolates from Khesari dhal (*Lathyrus sativus* L.), LWT, 137, 110368.

[20] Gasparetto, J. C., Martins, C. A. F., Hayashi, S. S., Otuky, M. F., & Pontarolo, R. (2012) Ethnobotanical and scientific aspects of *Malva sylvestris* L.: a millennial herbal medicine, Journal of Pharmacy and Pharmacology, 64(2), 172-189.

[21] Naseri, A., Marinho, G. S., Holdt, S. L., Bartela, J. M., & Jacobsen, C. (2020) Enzyme-assisted extraction and characterization of protein from red seaweed Palmaria palmata, Algal Research, 47, 101849.