Original Article

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Determination of Total Protein and Free Amino Acid Content of *Artemisia Abrotanum L.* in the Blooming and Pre-Blooming Period

Artemisia abrotanum L. Bitkisinin Çiçekleme ve Çiçeklenme Öncesi Dönemdeki Toplam Protein ve Serbest Amino Asit İçeriğinin Belirlenmesi

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ABSTRACT

Objective: The aim of this study is to determine the total protein and free amino acid content of *Artemisia abrotanum L.* in two different periods, the pre-blooming and blooming periods.

Methods: The Dumas and Kjeldahl methods were applied comparatively to determine the total protein amount. In addition, after the sample was extracted with water:methanol (80:20) containing 0.1% formic acid, free amino acids were determined in liquid chromatography-mass spectrometer.

Results: By using the Dumas method, the % nitrogen content was found to be 2.10 and 2.20, in the pre-blooming and blooming period samples. The results obtained by applying the Kjeldahl method are 2.16 and 2.25, respectively. The total protein content was calculated from the nitrogen content of the plant by using a nitrogen conversion factor of 6.25. The total free amino acid content in the pre-blooming and blooming period was found to 453.41 and 606.18 mg/100 g dried plant respectively.

Conclusion: Since the total protein and free amino acid content of *Artemisia abrotanum L.* is higher during the blooming period, it should be preferred to be harvested during this period.

Keywords: *Artemisia abrotanum*, Kjeldahl method, Dumas method, protein, free amino acid, LC-MS/MS

ÖZ

Amaç: Bu çalışmanın amacı *Artemisia abrotanum L.* bitkisinin çiçeklenme öncesi ve çiçeklenme dönemi olmak üzere iki farklı dönemde toplam protein ve serbest amino asit içeriğinin belirlenmesidir.

Yöntemler: Toplam protein miktarının belirlenmesinde Dumas ve Kjeldahl metodu karşılaştırmalı olarak uygulanmıştır. Ayrıca numune %0,1 formik asit içeren su:metanol (80:20) ile ekstrakte edildikten sonra serbest amino asitler sıvı kromatografisi-kütle spektrometresi de tayin edilmiştir.

Bulgular: Çiçeklenme öncesi ve çiçeklenme dönemi numunelerde Dumas metodu kullanılarak % azot miktarı sırası ile 2,10 ve 2,20 olarak bulunmuştur. Kjeldahl metodu uygulayarak elde edilen sonuçlar ise sırası ile 2,16 ve 2,25'tir. Toplam protein içeriği 6,25 azot dönüşüm faktörü kullanılarak % azot miktarından hesaplanmıştır. Çiçeklenme öncesi ve çiçeklenme dönemindeki toplam serbest amino asit içeriği ise sırasıyla 453,41 ve 606,18 mg/100 g kurutulmuş bitki olarak bulunmuştur.

Sonuç: *Artemisia abrotanum L.* bitkisinin toplam protein ve serbest amino asit içeriği çiçeklenme döneminde daha yüksek olduğu için bu dönemde hasat edilmesi tercih edilmelidir.

Anahtar Sözcükler: *Artemisia abrotanum*, Kjeldahl metot, Dumas metot, protein, serbest amino asit, LC-MS/MS

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Introduction

One of the largest and widely distributed members of the *Asteraceae* family is the *Artemisia* genus. It has more than 500 species distributed throughout Europe, Asia, and North America (1). The ethanol extracts of fresh young flower leaves of *Artemisia abrotanum* L. have been used in particular in homeophatic treatment. In traditional medicine, the leaves of the plant are used as a peptic and appetising agent (2). Today, it is mostly used for flavoring and cosmetic purposes (3).

People have to consume proteins for growth, cell reparation, and also for a healthy life. A high protein diet is very popular for its effectiveness for losing weight, preserving muscle mass, and increasing strength. Although generally animal products are preferred for protein intake, recently plant proteins have been recommended to be consumed as an alternative to those proteins. Animal protein presents growing costs and can be dangerous for human health due to causing cardiovascular diseases and others (4).

Determination of protein content in food is based on the nitrogen content analysis done by the Kjeldahl and Dumas methods. In the Kjeldahl method measures the total nitrogen content of a food, which is then used to estimate the crude protein content by applying a conversion factor to the result (5). The Dumas method is an alternative to the Kjeldahl method with some advantages such as only requiring the use of small quantities of dry chemicals (6).

In plants, amino acids have so many functions such as being used both in protein biosynthesis, and for building blocks for several other biosynthesis pathways (7). In the human diet, proteins consist of amino acids linked by peptide bonds, and the amino acids are vital for maintaining the function of all organs. Protein quality is related to amino acids and the amount of nitrogen (8). Each amino acid has a different and important role in the functioning of the organism. The non-essential amino acids alanine, arginine, aspartic acid, cysteine (Cys), glutamic acid, glycine, proline (Pro), serine (Ser), and tyrosine (Tyr) can be synthesized in the human body (9), while the essential amino acids histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), threonine (Thr), tryptophan (Trp), and valine (Val) cannot be synthesized. They must be provided in the diet (8,10).

The aim of the study is to evaluate the nutritional value of the pre-blooming and blooming periods of *Artemisia abrotanum L.* by determining the protein and free amino acids amounts. The comparison of Kjeldahl and Dumas will enable the selection of the most appropriate method for the protein determination. The determination of the amino acids of the plant will give information about the protein quality.

Methods

Samples

Artemisia abrotanum L. samples were harvested from Lisinia Nature Project Area near the village of Karakent in Burdur (Turkey). The material collection process was carried out at two different times: pre-blooming and the blooming periods. After harvesting the plants were dried at room temperature for one week in the shade, ground and stored in sealed plastic containers until the analysis time. The herbarium of the plants was done by Prof. Dr. M. Zeki Haznedaroğlu from İzmir Katip Çelebi University.

Chemicals

The free amino acid (Lysine, Cystine, Histidine, Arginine, Aspartic acid, Serine, Threonine, Glutamic acid, Alanine, Glutamine, Pro, Valine, Methionine, Tyrosine, Isoleucine, Leucine, Phenylalanine) analytical standard mixtures were purchased from Sigma-Aldrich. All the other reagents (H_2SO_4, $CuSO_4$, K_2SO_4, NaOH, HC_l) were of analytical grade purity and purchased from Merck. The ultra-pure water was obtained from the water purification system (Human Power I, Human Corporation, KR).

Total Protein by Dumas Method: The elemental analyzer system (2400 Series II, Perkin Elmer, US) was used to determine the nitrogen amount. The dried samples (2-3 mg) were weighed directly into tin capsules by using ultra-micro balance (AD 6000, Perkin Elmer, US) capable of weighing samples to a resolution of 0.1 μg and placed the auto sampler of the instrument. A cystine analytical standard (29.99% C, 5.03% H, 11.66% N, 26.69% S) was used as a reference standard. Each sample was analyzed in three replicates. The instrument parameters were shown in Table 1.

Total Protein by Kjeldahl Method: In addition to the nitrogen determination by the elemental analyzer, the Kjeldahl method was also applied by using a distillation system (Vapodest-50, Gerhardt, DE). The dried samples (≈1.0 g) were digested in 30 mL H_2SO_4 in the presence of the catalyst 1 g of $CuSO_4$ and 10g K_2SO_4, after digestion, NaOH was added followed by steam distillation, and the distillate was collected in 20 mL 4% boric acid. Then, the nitrogen content was determined by using titration with 0.01 N HCl.

Free Amino Acid Profile by UPLC-MS/MS: The dried samples (≈0.5 g) were extracted with 10 mL of 0.1% (v/v) formic acid in water: methanol (80:20) (v/v). The mixture was vortexed for 5 min and then centrifuged at 4,000 rpm at 4 °C for 15 min. The

Table 1. Instrument parameters (Dumas method) for nitrogen detection

Parameters	Value
Combustion temperature	925 °C
Reduction temperature	500 °C
Thermal conductivity detector (TCD) temperature	82.2 °C
Separation column	GC column SS - 2 m 6x5 mm (CHNS)
Carrier gas (helium) purity	99.999%
Combustion gas (oxygen) purity	99.999%
Pneumatic gas (air) purity	99.995%

upper phase obtained after centrifugation was passed through a 0.2 µm PTFE membrane filter and injected to the UPLC-MS/MS (Dionex Ultimate 3,000 - TSQ Fortis, Thermo Fisher Scientific Inc. US) (11).

The chromatographic separation was achieved using a gradient program. The analysis began with 100% mobile phase A and was held 2 min at this composition. After 2 min, the mobile phase A percentage was linearly decreased to 0% in 1.5 minutes. The mobile phase A percentage was held at 0% for 3 min. Then, the gradient was changed to a 100% mobile phase A and re-equilibration time took 0.5 min. The flow rate was 0.4 mL/min and the run time of the analysis was 7 min (12). The other chromatographic and mass spectrometric conditions are shown in Table 2. The MS-MS optimization for each free amino acid was performed by using a single analytical standard in order to determine the ion transitions and collision energies (Table 3). An appropriate amount of dilutions was done from the stock mixed solution to generate the calibration curve. Different calibration points in the range of 0-15 mg/L were established for each free amino acid.

Statistical Analysis

The results were evaluated using the t-test function available in MS Office Excel.

Results and Discussion

The protein content of *Artemisia abrotanum* L. was determined as the amount of the total N by Kjeldahl and Dumas methods. The amount of nitrogen content of the plant was calculated by using a nitrogen conversion factor of 6.25. The results showed that there was no significant difference between the amount of total protein in the pre-blooming and blooming periods of the plant ($p>0.05$) (Table 4). The accuracy of the Dumas method was checked by using cystine and the relative error was found to be 1.66. The precison of both methods was calculated and the RSD results were given in Table 4. In both methods, the results of the N contents of the plants were quite close to each other although the precision was better in the Kjeldahl method ($p>0.05$).

There are not so many studies on the protein content of the Artemisia species. Ochkur et al. (13) determined the N content of *Artemisia abrotanum* L. level, which was grown in Ukraine, as 26.9% by using the Dumas method. In our study, the protein content of the plant was found to be 13.1% and 13.54% in pre-blooming and blooming periods of the plant, respectively by Dumas method.

Table 2. Chromatographic and MS conditions

UPLC	
Mobile phase A	4 mM ammonium formate, 0.1% formic acid (95:5, H_2O: MeOH)
Mobile phase B	4 mM ammonium formate, 0.1% formic acid (95:5, MeOH: H_2O)
Column	HYPERSIL GOLD C18 (50 x 2.1 mm, 1.9 µm)
Column oven temp	40 °C
Injection volume	10 µL
MS/MS	
Ionization type	ESI (Electrospray ionization)
Spray voltage	+3,500 V
Sheath gas	50 Arb
Aux gas	20 Arb
Capillary temp.	270 °C
Vaporizer temp.	50 °C
Detection mode	MRM (multiple reaction monitoring)

MS: Multiple sclerosis

Table 3. Retention time and MRM method parameters of free amino acids using UPLC-MS/MS

Amino acid	RT (min)[a]	Quantification transition (m/z)	Confirmatory transition (m/z)	CE (V)[b]
Lysine	0.74	147.0	84, 130.1	20
Cysteine	0.77	241.3	120, 152	15
Histidine	0.76	156.1	93.1, 110.2	10
Arginine	0.76	175.2	70, 116	15
Aspartic acid	0.78	134.1	88, 116	15
Serine	0.78	106.0	60, 88	15
Threonine	0.78	120.1	56.1, 102.1	15
Glutamic-acid	0.79	148.1	102.1, 130.2	20
Alanine	0.78	90.0	57.1, 71	15
Glutamine	0.74	147.1	84.1, 130.1	15
Proline	0.81	116.1	43.3, 70.1	15
Valine	0.86	118.1	55, 72	20
Methionine	0.94	150.2	104.1, 133.2	15
Tyrosine	1.01	182.2	136.1, 165.1	20
Isoleucine	1.28	132.2	69.2, 86.1	15
Leucine	1.27	132.1	68, 86	15
Phenylalanine	1.99	166.2	103.1, 120	15

[a] RT: Retention time, [b] CE: Collision energies, MRM: Multiple reaction monitoring, UPLC-MS/MS

Pereira et al. (14) and Pérez et al. (15) compared the two methods for the protein analysis in their studies. In both studies, a small difference was found between the values of the two methods. The results of this study supported those results. In another study published in 2001, it was concluded that the Dumas method was superior to the Kjeldahl method in determining the total N concentration of many agricultural samples analyzed in a routine analytical laboratory (6).

In some previous studies, the total amount of protein in the leaves of some plants was 15.00% in *Tribulus terrestris* L., 15.14% in *Zygophyllum simplex* L., 13.20% in *Fagonia cretica* L., 11.15% in *Peganum harmala* L. (16), 8.32% in *Cassia sophera* Linn (17), 17.9% in *Cynodon dactylon*, 29.8% in *Dactylis glomerata*, 18.8% in *Ehrtharta erecta*, 26.5% in *Lolium multiporum*, 16.7% in *Paspalum dilatatum*, and 14.7% in *Pennisetum clandestinum* (18). The amount of nitrogen found in this study was approximately 13%. Total protein content of *Artemisia abrotanum* L. grown in Turkey showed similar values with other plants.

The amino-acids content of *Artemisia abrotanum* L. is given in Table 5. The amino-acids levels were relatively high in the blooming period. When the total amount of the free amino acid concentration was compared, there were significant differences between the pre-blooming period and the blooming period of the plant ($p<0.05$). The total free amino acid concentrations in pre-blooming and blooming periods were 453.41±9.62 and 606.18±8.23, respectively. This result overlapped the knowledge of the usage of the flowers of this plant in homeopathy (2).

The nutritional quality of a protein is evaluated with its content of essential amino acids. "Lys is essential for body nitrogen balance, Val assist in motor coordination, Met + Cys is related to the immune system, and Ile + Leu are the building blocks present in most proteins" (8). Ochkur et al. (13) determined in their study the amino acid content of *Artemisia abrotanum* collected from Ukranie. The lysine concentration in *Artemisia abrotanum* L. collected from Turkey was found to be lower than those collected from Ukranie (7.39 mg/100 g <19.9 mg/100 g). There was information on the N concentration of the plant; therefore, it was impossible to evaluate the lysine and protein contents of the plant. The valine concentration was relatively high (120.44 mg/100 g >36.9 mg/100 g). In this study, the amount of Met + Cys (sulfur amino acids) and Ile + Leu were

Table 4. Amount of total protein in *Artemisia abrotanum* L. by using two different methods

	Pre-blooming period			Blooming period		
	Nitrogen % mean ± SD	RSD	Total protein %	Nitrogen % mean ± SD	RSD	Total protein %
Dumas method	2.10±0.06	3.07	13.10	2.20±0.07	3.22	13.77
Kjeldahl	2.16±0.04	1.94	13.53	2.25±0.01	0.50	14.09

SD: Standard deviation, RSD: Relative standard deviation

Table 5. Amount and type of free amino acids in two different periods of *Artemisia abrotanum* L. Results are expressed as mg of free amino acid per hundred grams of dried samples and standard deviation is given (n=6)

Amino acid	Abb.	Type	*Artemisia abrotanum* L. (mg/100 g ± SD)					
			Pre-bloom period			Bloom period		
Alanine	Ala	Non-essential	43.17	±	2.40	50.72	±	2.21
Arginine	Arg	Non-essential	16.19	±	0.45	20.19	±	0.91
Aspartic_acid	Asp	Non-essential	5.63	±	0.28	7.67	±	0.39
Cystine	Cys	Non-essential	4.42	±	0.18	5.11	±	0.32
Glutamic_acid	Glu	Non-essential	23.90	±	1.02	31.07	±	1.80
Glutamine	Gln	Non-essential	1.58	±	0.19	1.77	±	0.16
Proline	Pro	Non-essential	66.04	±	1.68	111.60	±	5.66
Serine	Ser	Non-essential	35.67	±	1.09	42.91	±	1.75
Tyrosine	Tyr	Non-essential	6.11	±	0.24	8.15	±	0.55
$\Sigma_{\text{non-essentials}}$			202.71±51.84			279.19±61.43		
Histidine	His	Essential	3.91	±	0.38	5.81	±	0.36
Isoleucine	Ile	Essential	43.27	±	0.77	53.33	±	0.79
Leucine	Leu	Essential	37.03	±	1.30	40.30	±	1.46
Lysine	Lys	Essential	4.73	±	0.29	7.39	±	0.31
Methionine	Met	Essential	3.62	±	0.26	4.43	±	0.38
Phenylalanine	Phe	Essential	64.17	±	2.01	75.24	±	1.43
Threonine	Thre	Essential	14.49	±	0.51	20.05	±	0.98
Valine	Val	Essential	79.48	±	1.96	120.44	±	6.06
$\Sigma_{\text{essentials}}$			250.70±48.35			326.99±57.87		

SD: Standard deviation, Abb: Abbreviations

found to be 9.54 and 93.63 mg/100 g and also in the study of Ochkur et al. (13) they were found to be 33.4 and 22.1 mg/100 g, respectively. The other essential amino acid Phe concentration was higher in the Turkish *Artemisia abrotanum* L. (75.24 mg/100 g >32.6 mg/100 g).

In general, for both the essential and non-essential, the amount of each amino acid differs, in some, higher in the Ukranian species, and in some higher in those collected from Turkey. It was observed that the comparison of some medicinal plant free amino acid contents gave similar results (13,16).

Conclusion

It has become important to know more about the structure, growing conditions, and harvest time of *Artemisia abrotanum* L. and similar medical plants with the start of cultivation of these species . In this study, the protein and free amino acid content of the *Artemisia abrotanum* L. were evaluated. The result of the free amino acid content in the plant pre and at blooming period time, showed that the harvesting time of the plant is important. *Artemisia abrotanum* L. should be harvested in blossom time. Both protein determination methods could be used for the evaluation of the protein content of the plant. On the other hand, the Dumas method was easier to apply and gave detailed information about the structure of the plant. Another advantage of this method is its contribution to green chemistry with its low time consumption and chemical usage.

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Ethics

Ethics Committee Approval: Ethics committee approval is not required due to the type of study.

Peer-review: Externally peer reviewed.

Authorship Contributions

Concept: Ö.S., Design: Ö.S., Data Collection or Processing: İ.C., Analysis or Interpretation: İ.C., Ö.S., Literature Search: İ.C., Ö.S., Writing: İ.C.

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