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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 Çiçeklenme 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 .
Methods: The Dumas and Kjeldahl methods were applied comparatively to determine the total protein amount. In addition, free amino acids were determined in LC-MS/MS in the water:methanol (80:20) containing 0.1% formic acid extract of the samples.
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 were found to 453.41 and 606.18 mg/100g 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. Although both methods gave similar results in total protein determination, it was concluded that the Dumas method is easier to apply than the Kjeldahl method and it contributes more to green chemistry because it consumes less samples, time and chemicals.
Keywords: *Artemisia abrotanum*; Kjeldahl method; Dumas method; protein; free amino acid; LC-MS/MS

ÖZ
Amaç: Bu çalışmanın amacı çiçeklenme öncesi ve çiçeklenme dönemi olmak üzere iki farklı dönemde toplanan *Artemisia abrotanum* L. bitkisinin toplam protein ve serbest amino asit içeriğinin belirlenmesidir.
Yöntemler: Toplam protein miktarının belirlenmesinde Dumas ve Kjeldahl metodu karşılaştırmalı olarak uygulanmıştır. Ayrıca bitki numuneleri % 0,1 formik asit içeren su:metanol (80:20) ile ekstrakte edildikten sonra serbest amino asitler LC-MS/MS 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'dir. 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/100g 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. (2 farklı metod kullanıldığına göre, metotla ilgili bir öneriniz var mıdır?) Kjeldahl ve Dumas metodları ile benzer sonuçlar elde edimesine rağmen bitki protein analizlerinde Dumas yönteminin Kjeldahl yöntemine göre daha uygun olduğu sonucuna varılmıştır. Dumas yöntemi, uygulama bakımından daha kolaydır, ayrıca daha az numune, zaman ve kimyasal madde tüketimi olduğundan dolayı yeşil kimyaya katkısı daha fazladır.
Anahtar Sözcükler: *Artemisia abrotanum*; Kjeldahl metot; Dumas metot; protein; serbest amino asit; LC-MS/MS

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 such as cardiovascular diseases and others (4).

Protein content determination in food is based on the nitrogen content analysis done by the Kjeldahl and Dumas methods. In the Kjeldahl method, the total nitrogen content is found multiplying by a factor to arrive at the protein content (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 (Ala), arginine (Arg), aspartic acid (Asp), cysteine (Cys), glutamic acid (Glu), glycine (Gly), 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 period 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.

Materials and methods

Samples

Artemisia abrotanum L. samples were harvested from XXX near the village of Karakent in Burdur (Turkey). The material collection process was carried out at two different times: pre-blooming and the blooming period. 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 XXX from XXX. (Bitki materyali direk mi kullanıldı? Ekstrak mı edildi?) Methods başlığı altında bitki materyalinin nasıl kullanıldığı açıklanmıştır (Dumas ve Kjeldahl metotlarında numune direkt olarak, serbest amino asit metodunda 0.1% (v/v) formik asit su : metanol (80:20) (v/v) ile ekstrakte edilerek kullanılmıştır). Metod kısmında istenilen değişiklikler yapılmıştır.

Chemicals

The free amino acid (Lysine, Cystine, Histidine, Arginine, Aspartic acid, Serine, Threonine, Glutamic acid, Alanine, Glutamine, Proline, 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, HCl) 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).

Methods

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 1g 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 4000 rpm at 4 °C for 15 min. The upper phase obtained after centrifugation was passed through a 0.2 μm PTFE membrane filter and injected to the UPLC-MS/MS (Dionex Ultimate 3000 - 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 takes 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 period 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 are 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 is 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. determined the N content of *Artemisia abrotanum* L. level, which were grown in Ukraine as 26.9 % by using the Dumas method (13). In our study, the protein content of the plant 13.1% and 13.54% in pre-blooming and blooming period of the plant, respectively by Dumas method..

Pereira (14) and Pe´rez (15) in their studies compared the two methods for the protein analysis. In both studies, a small difference were found between the values of the two methods. . The results of this study have 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 is approximately 13%. Total protein content of *Artemisia abrotanum* L. grown in Turkey shows similar values with other plants.

Elde ettiğiniz sonuçları verdiğiniz bilgilerle tartışınız.. (Veriler karşılaştırılmıştır)

The amino-acids content of *Artemisia abrotanum* L. are 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 pre and blooming period 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 by its content of essential amino acids content. '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. in their study determined the amino acid content of *Artemisia abrotanum* collected from Ukranie (13). The lysine concentration in *Artemisia abrotanum* L. collected from Turkey were found to be lower than those collected from Ukranie (7.39 mg/100g< 19.9 mg/100g). There was information on the N concentration of the plant; therefore, it was impossible to evaluate the lysine and protein content of the plant. The valine concentration was relatively high (120.44 mg/100g >36.9 mg/100g). In this study, the amount of Met + Cys (sulfur amino acids) and Ile + Leu were found to be 9.54 and 93.63 mg/100g and also in Ochkur's et al. study were found to be 33.4 and 22.1 mg/100g respectively. The other essential amino acid Phe concentration was higher in the Turkish *Artemisia abrotanum* L. (75.24 mg/100g > 32.6 mg/100g) (13).

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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Table 1: Instrument parameters (Dumas Method) for nitrogen detection.	
Parameters	**Value**
Combustion Temperature	925 ºC
Reduction Temperature	500 ºC
Thermal Conductivity Detector (TCD) Temperature	82.2 ºC
Separation Column	GC Column SS - 2m 6x5mm (CHNS)
Carrier Gas (Helium) purity	99.999 %
Combustion Gas (Oxygen) purity	99.999 %
Pneumatic Gas (Air) purity	99.995 %

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	+3500 V
Sheath gas	50 Arb
Aux gas	20 Arb
Capillary temp.	270 ºC
Vaporizer temp.	50 ºC
Detection mode	MRM (multiple reaction monitoring)

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

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
Σ 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
Σ essentials			250.70 ± 48.35			326.99 ± 57.87		
SD: Standard deviation								