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Optimization of Anthocyanin Extraction from Iranian Borage (*Echium amoenum*) Using Microwave-Assisted Methods and Evaluation of Its Stability under Different Storage Conditions

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ABSTRACT

Echium amoenum, commonly known as Iranian borage, is a medicinal plant from the Boraginaceae family. It is traditionally recognized for its anti-inflammatory, sedative, and anxiolytic properties. One of its key bioactive compounds is anthocyanin. Anthocyanins are water-soluble, non-toxic pigments with a wide range of colors, making them promising natural alternatives to synthetic food and pharmaceutical dyes. To extract anthocyanins from *Echium amoenum*, a microwave-assisted extraction method was employed. The experiment was conducted as a factorial design within a completely randomized framework with three replicates. Extraction variables included microwave power levels (90, 180, and 360 watts) and durations (30, 60, and 90 seconds). Total anthocyanin content was quantified. Additionally, the stability of anthocyanins was evaluated in the presence of approved food additives over 14 days, and under varying light and temperature conditions over a 28-day period.The highest anthocyanin concentration (4.89 mg/L) was observed at 90 watts microwave power and 90 seconds extraction time. Among the additives tested, sorbitol significantly enhanced anthocyanin stability. Furthermore, storage in dark conditions and under refrigeration notably improved the pigment's stability. Microwave-assisted extraction offers several advantages, including higher yield, reduced processing time, and better preservation of anthocyanin properties. Stability assessments under light, temperature, and additive exposure provide valuable insights for optimizing anthocyanin applications in scientific and industrial contexts.

1- INTRODUCTION

Iranian borage (*Echium amoenum*) is a medicinal plant belonging to the Boraginaceae family. This herbaceous, perennial plant has soft hairs covering its organs. Its blue-purple flowers are arranged unilaterally on simple, elongated spike-like inflorescences. This plant species grows best in temperate mountainous climates as well as the temperate Caspian climate. Generally, cold and semi-humid mountainous regions with suitable soil and relatively regular spring and summer rainfall provide favorable conditions for its cultivation. Iranian borage exhibits good growth and flowering at altitudes of approximately 1500–2000 meters above sea level [1]. Iranian borage is recognized as a valuable medicinal plant in Iran, and its cultivation is mainly concentrated in the northern provinces, including Gilan and Mazandaran, the Kandovan and Gorgan regions, as well as the Heyran and Qazvin highlands. Estimates indicate that the annual production of this plant in Iran is about 300–350 tons [2]. The petals of borage contain about 13% anthocyanins, nearly 0.15% aglycone flavonoids, and small amounts of alkaloids have also been reported [3]. After chlorophyll, anthocyanins are recognized as one of the main groups of natural pigments. They are non-toxic, water-soluble, and widely present in the cytoplasm of plant cells. These colored compounds play an essential role in producing red, blue, and purple colors in many fruits, vegetables, and flowers [4]. Despite the high diversity of anthocyanin compounds, these pigments generally exhibit specific light absorption patterns. The color characteristics of anthocyanins are strongly influenced by the pH of the environment, producing different hues at various pH levels, and their stability also depends on factors such as temperature, light intensity, and storage conditions. Chemically, anthocyanins are classified as polyphenolic compounds and a subgroup of flavonoids. These compounds are major components of color systems and, due to their safety, can be used as food colorants [5]. An increase in the pH of solutions leads to a decrease in color intensity, concentration, and pigment stability. In contrast, the results of some studies have shown that polymerization reactions can improve the color stability of anthocyanins. At near-neutral pH, stable purple quinonoid anions are formed. Anthocyanins are synthesized in plants and stored mainly in cell vacuoles [6]. Typically, acidic environments and solvents such as methanol, ethanol, acetone, and water are used to extract these compounds [7]. Extraction is defined as a process in which effective medicinal compounds of plants are separated from other plant components using suitable solvents and standard methods [8]. One of the most advanced and efficient extraction methods is the use of microwave technology. Microwave radiation effectively affects various compounds, producing both thermal and non-thermal effects, depending on the heating rate, ion acceleration, and molecular collisions. Furthermore, microwave application can significantly reduce processing time and serves as an energy-efficient method in extraction processes [9]. Microwave-assisted extraction offers multiple advantages, including faster separation of bioactive compounds, higher yield compared to traditional extraction methods, and reduced thermal gradients [10]. Due to shorter processing time, lower solvent consumption, and higher anthocyanin extraction yield, the microwave-assisted method is highly effective for recovering heat-resistant bioactive compounds from by-products. Reduced solvent use also makes the processing of anthocyanin extracts more economical [11].

2-Literature Review

In one study, in addition to traditional solvents such as water, methanol, and ethanol, a glycerol-based solvent was used to extract anthocyanin pigments from borage petals. The results showed that the most common anthocyanins in borage include cyanidin-3-glucoside, cyanin chloride, cyanidin-3-rutinoside, and pelargonidin-3-glucoside. It was also found that

choline chloride and the glycerol-based solvent are effective and environmentally friendly options for extracting anthocyanins [12]. In another study, microwave-assisted extraction was used to extract anthocyanins and evaluate their stability from Chinese bayberry leaves. The optimal extraction conditions for achieving total anthocyanin content were a solid-to-solvent ratio of 1:50, a temperature of 80 °C, and a time of 15 minutes. Under these conditions, the total anthocyanin content was 2.95 ± 0.08 mg/mL, and the antioxidant activity was 279.96 ± 0.1 µmol/mL based on dry weight. These findings indicate that microwave-assisted extraction is a highly efficient method that can reduce process time, although it may lead to anthocyanin degradation under some conditions [13]. In another study, microwave-assisted extraction was used to extract polyphenols and caffeine from green tea leaves. The results showed that this method significantly increased extraction yield in only 4 minutes compared to room-temperature extraction for 20 hours. Furthermore, microwave technology was also used to extract ginsenosides from ginseng roots, improving extraction yield in 15 minutes compared to the conventional solvent method, which took 10 hours [14]. In a study comparing anthocyanin extraction from blueberry powder using microwave-assisted extraction and hot water extraction, ethanol was used as a solvent. Microwave radiation increased the release and solubility of anthocyanins in blueberry powder, while particle kinetic energy was higher in the hot water extraction method [15]. The results indicated that anthocyanin content increased with temperature up to 41.2 °C, and temperatures above 41.2 °C caused anthocyanin degradation. High temperatures lead to aggregation and decomposition of anthocyanins, whereas the microwave method, by generating pressure and indirect heating through an electromagnetic field, reduces the negative effects of temperature on anthocyanin decomposition. The present study investigates the extraction of anthocyanins from Iranian borage using microwave technology. This method significantly reduces extraction time and solvent consumption compared to conventional techniques while increasing anthocyanin yield. In addition, the use of microwaves helps preserve the physiological and chemical properties of these valuable compounds, whereas conventional thermal methods may cause anthocyanin degradation. Moreover, assessing anthocyanin stability against light, temperature, and permitted additives enables optimization of industrial and research processes in the field of medicinal plants and the food industry.

3-Materials and Methods

3-1-Sample Preparation

Iranian borage petals were freshly picked and collected from the Asiabar region (E 36.8670011, N 49.8356188) in Gilan province and then dried in the shade [16]. The dried flowers were ground and passed through a No. 80 sieve. The resulting powders were stored in tightly sealed containers until the experiments were conducted [17].

3-2- Anthocyanin Extraction

Anthocyanin extraction was performed using a microwave-assisted method with a solvent and a microwave oven (LG-MA3884VC). The extraction variables included microwave power (90, 180, and 360 W) and extraction time (30, 60, and 90 s). The solvent (water) was used at a ratio of 1:30 (solvent (mL): plant material (g)). The sample was mixed with the solvent, placed in the microwave, and the extraction was performed. After the extraction process, equal volumes of the solution containing the sample and solvent were transferred into 15-mL Falcon tubes and then centrifuged at 3500 rpm for 20 minutes [18].

3-3-Measurement of Total Anthocyanin Content

To determine the total anthocyanin content, the differential absorption method at different pH levels was used. A 0.5-mL aliquot of the

extracted extract was added to 2.5 mL of each buffer solution of pH 1.0 (containing 0.2 M potassium chloride and 0.2 M hydrochloric acid) and pH 4.5 (containing 0.2 M sodium acetate and 0.2 M acetic acid). The absorbance was measured using a spectrophotometer (T80+ UV/VIS Spectrometer, PG Instrument) at wavelengths of 510 and 700 nm, and the total anthocyanin content was calculated using the following formula:

$$A = (A_{510}\ pH1.0 - A_{700}\ pH1.0) - (A_{510}\ pH4.5 - A_{700}\ pH4.5)$$

$$\text{Total anthocyanin (mg/L)} = (A \times MW \times DF \times 1000) / \varepsilon$$

In the above formula, A is the absorbance, MW is the molecular weight of the predominant anthocyanin (cyanidin-3-glucoside = 449.2), 1000 is the conversion factor to liters, ε is the molar extinction coefficient of cyanidin-3-glucoside (26,900), and DF is the dilution factor [19].

3-4- Stability of Extracted Anthocyanin

3-4-1- Stability against Light and Temperature

Using the optimal microwave power and extraction time determined based on total anthocyanin content, an anthocyanin extract was prepared from Iranian borage and centrifuged. Equal volumes of the extract were then poured into Falcon tubes and stored for 28 days under different conditions: direct light (23 ± 2 °C), indirect light (23 ± 2 °C), darkness (23 ± 2 °C), ambient temperature (23 ± 2 °C), and refrigeration (4 °C). To compare the stability of the extract under these conditions, anthocyanin absorbance was measured on days 0, 7, 14, and 28 using a spectrophotometer, and the total anthocyanin content was calculated and analyzed [20].

3-4-2- Stability in the Presence of Permitted Additives

Equal volumes (10 mL) of the extracted extract were placed into Falcon tubes, and to each sample, a permitted food additive was added at a ratio of 1:10. These additives included 1 g glucose, 1 g sorbitol, and 1 mL of 0.01 M citric acid. Additionally, a pure sample was considered as a control. For each additive and the control sample, half of the samples were stored under indirect light (23 ± 2 °C) and half in darkness (23 ± 2 °C). Anthocyanin absorbance was measured on days 0 and 14 using a spectrophotometer [21].

3-5- Statistical Analysis

Data analysis included descriptive and inferential stages. In the descriptive part, means and standard deviations were calculated, and in the inferential part, analysis of variance (ANOVA) and Tukey's multiple range tests were performed at a 95% confidence level. All calculations were conducted using SAS software version 9.4.

4-Results

4-1- Total Anthocyanin Content

The ANOVA results, presented in Table 1, showed that both microwave power and

extraction time had a significant effect on the total anthocyanin content of Iranian borage.

Table 1. Analysis of variance (ANOVA) of the effects of microwave power and extraction time on total anthocyanin content

Anthocyanin	df	Source of variation
26.81**	2	Power
1.17**	2	Time
0.65**	4	Power × time
0.05	18	Error
7.70	–	CV

** Values indicate significance at the 1% probability level; ns denotes no significant difference.

The results of the interaction between microwave power and extraction time on total anthocyanin content showed that the highest anthocyanin content was obtained at 90 W power and 90 s extraction time. Under these conditions, the extracted anthocyanin content was 4.89 mg/L (Table 2).

Table 2. Comparison of means for the effects of microwave power and extraction time on total anthocyanin content

Total anthocyanin	Treatments	
mg/L	Time (s)	Power (W)
4.56 a	30	90
4.61 a	60	90
4.89 a	90	90
3.35 b	30	180
3.93 b	60	180
2.26 c	90	180
1.98 c	30	360
1.15 d	60	360
0.58 d	90	360

Means sharing at least one common letter are not significantly different at the 5% level

4-2- Stability of Anthocyanin Extract

4-2-1- Permitted Additives

The ANOVA results showed that the three-way interaction of additives (glucose, sorbitol, and citric acid) with light conditions (half of the samples in darkness and half under indirect light) and time (anthocyanin absorbance values on days 0 and 14) had no significant effect on the total anthocyanin content of Iranian borage extract (Table 3). However, the two-way effects of additives under light conditions and their two-way effects over time showed significant differences in total anthocyanin content at the 0.05 probability level.

Table 3. Analysis of variance (ANOVA) of the effects of different treatments on anthocyanin stability in Iranian Borage (*Echium amoenum*) extract

Anthocyanin	df	Source of variation
**2.90	3	Permitted additives
0.01 ns	1	Light conditions
**0.11	3	Light conditions × Permitted additives
0.02	14	Main plot error
**31.5	1	Time
**3.30	3	Time × Permitted additives
ns 0.05	1	Time × Light conditions
ns 0.06	3	Time × Permitted additives × Light conditions
0.02	16	*Residual error*
8.54	–	CV

** Values indicate significance at the 1% probability level; ns denotes no significant difference.

Table 4. Comparison of means for the dual effects of light and different additives on total anthocyanin in Iranian Borage (*Echium amoenum*)

Total anthocyanin	Treatments	
mg/L	Permitted additives	Light conditions
1.51 [c]	Control	Indirect light
1.37 [d]	Control	Darkness
1.68 [c]	Glucose	Indirect light
1.65 [c]	Glucose	Darkness
2.34 [b]	Sorbitol	Indirect light
2.66 [a]	Sorbitol	Darkness
1.48 [c]	Citric acid	Indirect light
1.51 [c]	Citric acid	Darkness

Means sharing at least one common letter are not significantly different at the 5% level

Samples treated with permitted additives (glucose, sorbitol, and citric acid) exhibited better color stability than the control samples.

Table 5. Comparison of means for the dual effects of time and permitted additives on total anthocyanin in Iranian Borage (*Echium amoenum*) extract

Total anthocyanin	Treatments	
mg/L	Permitted additives	day
2.82 [a]	Control	0
0.07 [e]	Control	14
2.80 [a]	Glucose	0
0.53 [d]	Glucose	14
2.80 [a]	Sorbitol	0
2.20 [b]	Sorbitol	14
1.93 [b]	Citric acid	0
1.07 [c]	Citric acid	14

Means sharing at least one common letter are not significantly different at the 5% level

4.2.2. Anthocyanin Stability against Light and Temperature

The ANOVA results for anthocyanin stability under different light and temperature conditions over 28 days showed that the interaction of storage time (day) with treatments (different light and temperature conditions) had a significant effect on anthocyanin stability, and the independent effects of time and treatment were also significant at the 0.01 probability level (Table 6).

Table 6. Analysis of variance (ANOVA) of the effects of storage time and treatment (light and temperature) on anthocyanin stability in Iranian Borage (*Echium amoenum*)

Anthocyanin	df	Source of variation
1.77**	4	Treatment
0.00	10	Main plot error
1.08**	3	Time
0.07**	12	Time × Treatment
0.00	30	error
2.99	-	CV

** Values indicate significance at the 1% probability level; ns denotes no significant difference.

5- Discussions

5-1- Total Anthocyanin Content

The study of total anthocyanin content showed that the highest extraction yield was obtained at 90 W microwave power and 90 s extraction time, and this value was not significantly different from the same power at 30 and 60 s. However, 90 W power showed a significant difference compared to higher powers. The lowest anthocyanin content was observed at 360 W power and 90 s (0.58 mg/L), which was not significantly different from 360 W at 60 s. In general, increasing temperature and extraction time reduced anthocyanin content. The use of microwaves, by directly interacting with polar molecules, increases the extraction efficiency of bioactive compounds from cells and improves process yield, but higher powers can lead to anthocyanin degradation. Specifically, temperatures above 80 °C cause the breakdown of these compounds [22]. Anthocyanin pigments dissolve in polar solvents; therefore, polar solvents are typically used for their extraction from plants. The use of acidic solvents destroys plant cell walls, thereby facilitating pigment dissolution and increasing extraction yield [23]. However, the use of organic solvents such as methanol and acetone is limited in the food industry because they may remain in the product. Therefore, selecting an appropriate solvent for extraction is very important to preserve anthocyanins and allow their safe use in food products. Ideally, the extraction method should obtain the highest amount of anthocyanin pigment with minimal degradation [24]. Although ethanol is a common solvent in many separation processes, water-based anthocyanin extraction is considered a more effective and safer method [25]. Based on this approach, distilled water was used as the solvent in this study. In another study on the extraction of anthocyanins from Persian Golnar, water and ethanol solvents were used, and the results showed that the highest anthocyanin content was achieved when water was chosen as the solvent [26].

5-2- Anthocyanin Stability in the Presence of Permitted Additives

The results of the interaction of different treatments on anthocyanin stability in Iranian borage extract showed that the interaction of permitted additives under light conditions created significant differences. The highest stability of the extracted anthocyanin was observed in the presence of sorbitol and storage in darkness, which was significantly different from other additives such as glucose, citric acid, and the control sample. Furthermore, samples treated with glucose and citric acid had nearly similar stability under both light conditions (indirect light (23 ± 2 °C) and darkness (23 ± 2 °C)), and no significant difference was observed between these two conditions (Table 4). Sorbitol and glucose contribute to anthocyanin stability by reducing water activity. Reduced water activity balances the color chromophores. In a study on elderberry, various additives were also used to stabilize the anthocyanin extract, and the results showed that the presence of sorbitol increased anthocyanin stability. This stabilization and increased stability are mainly due to reduced water activity in the system [27]. The results showed that the stability of the anthocyanin extract increased in the presence of glucose and sorbitol additives, and the highest stability was related to sorbitol combined with storage in darkness (23 ± 2 °C). Sorbitol, a sugar alcohol used in canned foods and products suitable for diabetics, has half the sweetness of sucrose and was able to largely maintain anthocyanin stability over 14 days. Citric acid was also significantly effective in stabilizing anthocyanins. These results indicate that permitted additives play an important and positive role in preserving and stabilizing anthocyanin extracts [28]. Citric acid is a natural acid that acts as a pH regulator and stabilizer. By lowering the acidity of the environment, it prevents anthocyanin degradation under alkaline conditions. Additionally, citric acid can inhibit anthocyanin oxidation by forming metal complexes [29].

5-3- Anthocyanin Stability against Light and Temperature

Comparison of the mean interaction of environmental conditions (different temperatures and light) and time (28 days) on this trait showed that these factors significantly influenced anthocyanin stability. The highest anthocyanin content was observed under dark conditions (23 ± 2 °C) on day 0, which was not significantly different from some other conditions, such as storage under indirect light (23 ± 2 °C) and darkness (23 ± 2 °C) on day 7, and storage under indirect light (23 ± 2 °C) on day 14. Conversely, the lowest anthocyanin stability was recorded on day 28 under direct light (23 ± 2 °C), which was significantly different from all other treatments (Figure 1). In general, the results showed that as storage time increased, anthocyanin content decreased and their degradation increased. However, storage under refrigeration (4 °C) and in darkness (23 ± 2 °C) significantly prevented this degradation. This experiment demonstrates that light is one of the main factors affecting the stability and shelf life of anthocyanins and can significantly degrade them, while samples stored in a dark environment (23 ± 2 °C) showed no noticeable color change. The reason for the color change in anthocyanins is the photochemical decomposition of the free hydroxyl group at the fifth carbon of their structure. Additionally, the addition of tannic acid or gallic acid can reduce the effect of light on anthocyanin color [30]. A comparison of samples stored at ambient temperature (23 ± 2 °C) and in a refrigerator (4 °C), both kept in darkness (23 ± 2 °C), showed that lower temperature had a positive effect on anthocyanin stability. In contrast, high temperature causes anthocyanin degradation and color change. Previous studies have also shown a direct relationship between anthocyanin degradation and temperature increase [31]. Anthocyanins degrade rapidly at ambient temperature (23 ± 2 °C). Increasing temperature accelerates chemical reactions, including oxidation and hydrolysis, leading to anthocyanin degradation [32]. Refrigeration at low temperature (4 °C) slows down chemical reactions and thus slows anthocyanin degradation. Refrigeration can significantly increase anthocyanin stability. At refrigerator temperature (4 °C), anthocyanins retain their color and properties for a longer period. This is particularly important in food products and beverages, where maintaining color and product quality is crucial [33].

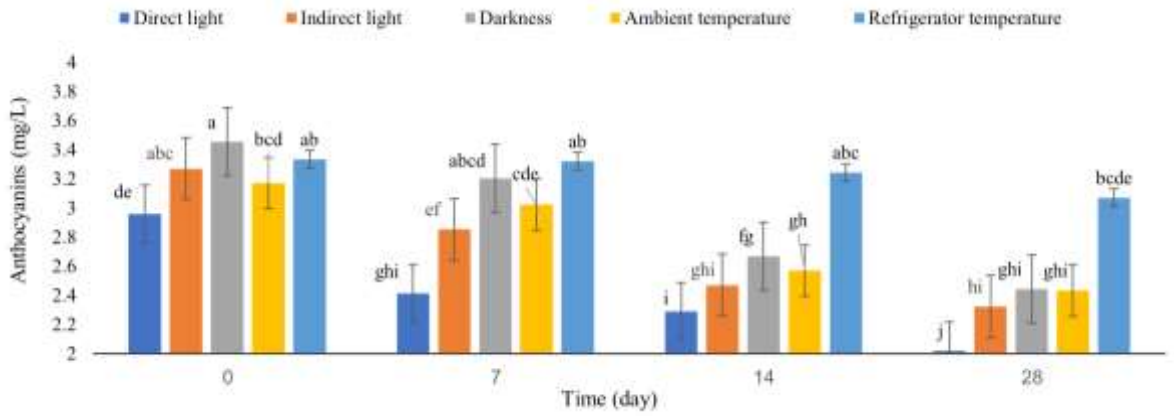

Figure 1. Anthocyanin stability under light and temperature conditions (Different letters indicate significant differences at p < 0.05)

6- Conclusions

Since anthocyanins are suitable alternatives to synthetic dyes, offering bright and attractive colors as well as anti-cancer, anti-viral, and antioxidant properties beneficial to human

health, this research focused on extracting anthocyanins from Iranian borage using a microwave-assisted method. Iranian borage contains a significant amount of anthocyanins responsible for the red and purple color of its petals. The results showed that the microwave method is effective for extracting anthocyanin pigments from this medicinal plant, and using water as a solvent facilitates the easy extraction of these polar compounds. The results indicated that anthocyanin extraction at 90 W microwave power for 90 s yielded the highest efficiency, whereas longer times and higher powers led to anthocyanin degradation. Permitted additives played an important role in anthocyanin stability; the interaction of additives under different light conditions showed significant differences. The highest stability of the extracted anthocyanin was observed in the presence of sorbitol and storage in darkness (23 ± 2 °C), which was significantly different from other additives, including glucose, citric acid, and the control sample. In samples where glucose and citric acid were used as additives, anthocyanin stability was nearly identical under both storage conditions (indirect light (23 ± 2 °C) and darkness (23 ± 2 °C)), and no significant difference was observed between these conditions. These additives prevent anthocyanin degradation under various environmental conditions by reducing water activity, maintaining moisture, and regulating pH. Therefore, these compounds can be used to maintain the quality and color of food and pharmaceutical products. The study of anthocyanin pigment stability revealed that light and temperature are the two main factors affecting the stability of anthocyanins from Iranian borage. By eliminating direct (23 ± 2 °C) and indirect light (23 ± 2 °C) and avoiding high temperatures, their stability can be significantly increased. Storage in darkness (23 ± 2 °C) effectively prevents light-induced degradation, and refrigeration (4 °C) also increases anthocyanin stability and protects against degradation caused by ambient temperature (23 ± 2 °C). In contrast, direct light (23 ± 2 °C) and ambient temperature accelerate anthocyanin degradation.

Conflict of Interest

The authors have no conflicts of interest

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مقاله علمی_پژوهشی

بهینه سازی استخراج آنتوسیانین از گل گاوزبان ایرانی با استفاده از مایکروویو و ارزیابی پایداری آن تحت شرایط مختلف نگهداری

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کلمات کلیدی:

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چکیده

گیاه گل گاوزبان ایرانی *(Echium amoenum)* یکی از گیاهان دارویی خانواده گاوزبان است که به دلیل خواص ضدالتهابی، آرامش‌بخش و ضداضطرابی شناخته شده و در ساختار شیمیایی خود حاوی آنتوسیانین می‌باشد. آنتوسیانین‌ها ترکیباتی غیرسمی و محلول در آب هستند و به دلیل داشتن رنگ‌های متنوع، جایگزین مناسبی برای رنگ‌های شیمیایی محسوب می‌شوند. برای استخراج عصاره آنتوسیانینی از گل گاوزبان ایرانی به روش مایکروویو، آزمایشی به صورت فاکتوریل و در قالب طرح کاملاً تصادفی با سه تکرار انجام شد.شرایط استخراج شامل توان مایکروویو (۳۶۰، ۱۸۰، ۹۰ وات) و زمان استخراج (۹۰، ۶۰، ۳۰ ثانیه) اعمال شد و محتوی آنتوسیانین کل اندازه‌گیری گردید. پایداری آنتوسیانین در حضور افزودنی‌های مجاز در طی ۱۴ روز و در برابر شرایط نوری و دمایی مختلف در طی ۲۸ روز اندازه‌گیری شد. نتایج نشان داد که بیش‌ترین میزان آنتوسیانین ۴/۸۹ میلی‌گرم در لیتر در تیمار ۹۰ وات (توان مایکروویو) و زمان ۹۰ ثانیه (زمان استخراج) است، همچنین نتایج نشان داد افزودنی‌های مجاز به خصوص سوربیتول نقش مهمی در پایداری آنتوسیانین دارد. همچنین نگه‌داری عصاره آنتوسیانینی در تاریکی و دمای یخچال به پایداری آن کمک می‌کند. مزایای استخراج آنتوسیانین از گل گاوزبان ایرانی به روش مایکروویو شامل افزایش بازده استخراج، کاهش زمان فرایند و حفظ خواص ترکیبات می‌باشد. علاوه بر این، با اندازه‌گیری پایداری آنتوسیانین در برابر نور، دما و افزودنی‌های مجاز، می‌توان به بهبود عملکرد این ترکیب در کاربردهای علمی و صنعتی دست یافت.