Iranian Food Science and Technology Research Journal
Vol. 18, No. 3, Aug. Sep. 2022, p. 53-68

نشریه پژوهشهای علوم و صنایع غذایی ایران
جلد ۱۸، شماره ۳، مرداد - شهریور ۱۴۰۱، ص. ۶۸-۵۳

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Full Research Paper

Antioxidant and antibacterial properties of borage (*Echium amoenum* L.) and hollyhock (*Althaea rosea* var. *Nigra*) extracts obtained through soaking and ultrasonic-assisted extraction methods

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Received: 2022.04.26
Accepted: 2022.06.08

How to cite this article:
Zamankhani, M., Moeini, S., Mahasti Shotorbani, P., Mirsaeedghazi, H., Jafarpour, A. (2022). Antioxidant and antibacterial properties of borage (*Echium amoenum* L.) and hollyhock (*Althaea rosea* var. *Nigra*) extracts obtained through soaking and ultrasonic-assisted extraction methods. *Iranian Food Science and Technology Research Journal*. 18 (3), 53-68.

Abstract

This study aimed to investigate antimicrobial and antioxidant activities of borage (*Echium amoenum* L.) and hollyhock (*Althaea rosea* var. *Nigra*) extracts. The extracts were obtained through soaking and ultrasound-assisted methods using water or methanol as a solvent. The total phenols and flavonoid, anthocyanin content, free radical scavenging activity, ferric reducing antioxidant power, and antibacterial capacity of the extracts were determined. Phenolic acids were identified using the HPLC chromatogram. It was found that the ultrasound-assisted extraction was more efficient compared to the soaking method. The results showed that in the TPC, anthocyanins, and the FRAP tests, the highest amount was related to the samples extracted using the ultrasound- assisted method with water as solvent. The highest amount of TFC was obtained through a soaking method using methanol as the solvent. Anti- radical activity of the samples indicated that using water as a solvent in the optimum method resulted in a higher antioxidant activity. Furthermore, bacterial alpha amylase inhibition test signified that the inhibitory effect was boosted by increasing the extract concentration. The HPLC analysis of the borage and hollyhock extracts revealed that gallic acid and Syringic acid were the most prominent phenolic compounds. Generally, the results showed a good antibacterial property against *Staphylococcus aureus* for borage and hollyhock extracts. The results give us valuable insight into the potential therapeutic and medicinal applications of borage and hollyhock as a natural preservative to improve immunity.

Keywords: Natural extracts, Soaking method, Ultrasound-assisted extraction, Polyphenols.

Introduction

A variety of palnt materials are known to be a rich sources of natural antioxidants. Dietary and medicinal plants, vegetables, and fruits can provide health benefits and inhibit oxidative damage through scavenging reactive free radicals (Shariffar et al., 2009). Furthermore, the application of antioxidant preservatives in the food processing leads to preserve freshness

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DOI: 10.22067/IFSTRJ.2022.76413.1167

while avoiding browning and rancidity of foods. Such antioxidants can be used in particular to inhibit and delay deterioration of lipid products during processing, transportation, and storage (Al-Juhaimi et al., 2018). Historically, medicinal plants have been applied to control the microorganisms responsible for food spoilage in order to enhance the safety of products and also to prolong their shelf life (Nabavi et al, 2015). Borage (*Echium amoenum* L.) is a member of the *Boraginaceae* family and also one of the popular annual herbs used in traditional medicine in many countries. It is a member of t. Borage that is scattered in the northern Iran, Europe, and Mediterranean basin. The leaves and the stems of this herb are bristly and hairy with star- shaped bright blue flowers. Most parts of the plants are used for medical purposes (Abolhassani, 2010). The plant is commonly applied as a decoction whether used alone or combined with other herbs (Pilerood and Prakash, 2014). Traditionally, the plant is used also as an antidepressant, antifebrile, sedative, anti- inflammatory, and to treat influenza, infectious diseases, pulmonary and cardiovascular diseases. It is believed that the plant can be used for different types of cancers (Abolhassani, 2010). Researchers have conducted numerous in vitro, in vivo, and clinical studies to show the therapeutic effects assigned to the borage plant. The studies have shown that the flowers of this herb contain antioxidants (Abolhassani, 2010), antiviral (Ranjbar et al., 2006), and antibacterial activities against Gram-positive and Gram-negative bacteria. It also demonstrates anti-inflammatory effects (Karimi et al., 2018). Hollyhock is another important medicinal plant indigenous to Asia, Europe, and the United States which is traditionally used to treat throat irritation, dry cough, insect bites, mild gastritis, and skin burns. In addition, it can be used to treat inflammation, wound, abscess, constipation and diarrhea (Shah et al., 2011). Different parts of this plant contain various bioactive compounds such as mucilaginous compounds, starch, sucrose, betaine, flavonoids, coumarins, phenolic acid, essential fats, vitamin C, pectin, and carotene. A special variety of hollyhock, known as black hollyhock or *Althaea rosea* var. *Nigra* is widely used in traditional medicine because its dark purple flowers are rich in biologically active molecules especially anthocyanins such as delphinidin, petunidin, and malvidin (Dudek et al., 2006; Hosaka et al., 2012). The extraction yield and the bioactivity of the extracts from medicinal plants depend on the solvent and the method of extraction. Therefore, different methods and various solvents with different polarities such as water, methanol, ethanol, ethyl acetate, and petroleum ether have been used to obtain maximum yield and produce extract with good biological activity (Azwanida, 2015). The decoction of borage and hollyhock flower has been applied to treat a wide range of different illnesses and disorders in Iranian traditional medicine. Additionally, potential and industrial applications of these flowers need more extensive research. Therefore, the current study was conducted to assess anti- radical activity, bioactive compounds, and antibacterial properties of the extracts prepared from borage and hollyhock flower using soaking and ultrasonic-assisted methods in the presence of water or methanol as a solvent.

Materials and methods

Methanol, Folin- Ciocalteu, Sodium Carbonate, Gallic Acid, Hydrochloric Acid, Potassium Chloride, Sodium Acetate, TPTZ, Acarbose, α- amylase, Quercetin, Aluminum Trichloride, 2, 2- Diphenyl- 1- Picrylhydrazyl (DPPH), and Muller Hinton Broth were procured from Sigma-Aldrich (St. Louis, MO, USA). Borage was collected from the mountains of Mazandaran Province and Hollyhock was supplied by Zarband Pharmaceutical Company (Tehran, Iran).

Extraction methods

Echium Amoenum L. and *Althaea rosea* var. *Nigra* plants were oven- dried completely at 50°C, they were then ground before being stored in airtight containers. To extract using an

ultrasonic device, 10 g of the powder was homogenized and mixed in a ratio 1: 100 of solvent (water or methanol) in a cold oven and was then subjected to an ultrasonic device (AMMM, M.P.Interconsulting, Switzerland) for 10 minutes at 20°C. The solution tube was protected entirely by an aluminum foil against the light. Then, the extracts were placed on a shaker (Snijder, 34533) and after 24 hours, they were smoothed and kept at 4°C (Rabiei et al, 2012). In order to extract through the soaking method, 10 g of the powdered plant was mixed with 100 ml of the solvent (water or methanol) and stirred for 24h at ambient temperature. After extraction, the extracts were filtered and the solvents were removed through a rotary evaporator. Finally, the concentrated extracts were stored for further experiments at 4°C (Rafiee et al., 2012).

Extraction efficiency

The average extraction efficiency of the extracts from the soaking and ultrasound-assisted methods was determined using water and methanol solvents with four replicates per 100 g powder of the plant. For this purpose, the difference in the weight of the evaporative balloon (empty and after evaporation) was obtained by dividing it by the weight of the sample.

Total phenolic content (TPC)

Following Kiselova et al. with a few modifications, the total phenolic content of extracts was measured through the Folin-Ciocalteu colorimetric method. For this purpose, 1.0 g of each extract was mixed with methanol 80% and then it was centrifuged for 5 min at 3000 rpm. After that, 0.1 ml of the supernatant was mixed with 0.4 ml of methanol and 2.5 ml of Folin- Ciocalteu reagent (10% v/v) and it was incubated for 5 min at ambient temperature in a dark place. After that, 2 ml of sodium carbonate solution of 7.5% (w/v) was added to the mixtures. Following 2hrs of incubation at ambient temperature, the absorption level of the samples was determined at 765 nm using a UV/ Vis spectrophotometer. The results were expressed as milligrams of Gallic acid equivalent per gram of the extract (mg GAE/g) using the standard curve of Gallic acid (Kiselova et al., 2006).

Total flavonoid content (TFC)

To determine FC, 5.0 ml of 2% aluminum trichloride ($AlCl_3$) in methanol was mixed with the same volume of the given extracts. Then, the absorbance of the mixtures was measured at 415 nm using a spectrophotometer for 10 min against a blank sample composed of extract solution and 5.0 ml methanol without $AlCl_3$. Different concentrations of quercetin were used to prepare the standard curve. Therefore, flavonoids volume in the extract was determined as mg of quercetin equivalent per gram of extract (mg QUE/g) (Pilerood and Prakash, 2014).

Total anthocyanin content (TAC)

The anthocyanin content was identified qualitatively using ammonia HCl test according to Egbuna et al. 2 mL of the extract was mixed with ammonia and 2 mL of 2 N HCl. The change in color from pink -red to blue- violet was considered as an indicative of anthocyanin (Egbuna et al., 2018). Then, the total content of anthocyanin was specified using the pH differential method which is based on the structural changes in absorbance measurements at pH 1.0 and 4.5 and also chemical forms of anthocyanin. In total, 0.025 M hydrochloric acid potassium chloride buffer (pH= 1) and 0.4 M sodium acetate buffer (pH= 4.5) were used to dilute separately the crude extracts. To give an absorbance reading between 0.2 and 1.4 each sample was diluted with the buffers. The UV-Vis spectrophotometer showed the mixture absorbance at 700 nm. The total amount of anthocyanin content was expressed in the form of cyanidin- 3 glucoside equivalents according to the following equation (Anuar et al., 2013; Maran and Sivakumar, 2014; Shah et al., 2011; Zuo et al., 2002).

$$\text{Anthocyanin pigment (mg/L)} = \frac{A \times MW \times DF \times V \times 1000}{a \times l \times m} \quad (1)$$

In this equation, DF represents the dilution factor, MW represents the molecular weight of cyanidin- 3 -glucosode (449.2 g/mol), A represents the absorbance, V represents the solvent volume (mL), l is the cell path length (1 cm), and *a* represents the molar absorptivity (26,900 L. mol^{-1}. cm^{-1}).

Anti-radical activity of the extract based on DPPH method

Calculation of the anti-radical activity of the sample was conducted according to Oliveira et al. by using DPPH free radicals. In this method, 0.2 ml of the sample of different concentrations was mixed with 4 ml of DPPH methanolic solution with concentration of 6×10^{-5} mol/l and kept at dark place for 120 min. Then, the solution absorbance was read at 517 nm by a spectrophotometer. In this case, one sample containing 0.2 ml of methanol was used plus 4 ml of DPPH as a control. The radical inhibition activity rate was obtained as follows:

Inhibition (%)= $(A_{control}- A_{sample} /A_{control})\times 100$ (2)

In this formula, A_{blank} and A_{sample} represent the absorbance of the control and various concentrations of the extracts respectively. Then, the concentration of extract with a 50% radical inhibitory concentration was calculated by the graph and reported as IC_{50}. Clearly, the lower IC_{50} indicates a higher radical scavenging activity as a measure of antioxidant property of the extracts (Oliveira et al., 2008).

Ferric reducing antioxidant power (FRAP) assay

The decreasing power of the compound (antioxidant) as described formerly was the main factor in the assay. Some potential antioxidants reduce the ferric ion (Fe^{3+}) to the ferrous ion (Fe^{2+}); a blue complex (Fe^{2+}/TPTZ) is formed by the latter and increases the absorption at 593 nm. Breifly, the preparation of FRAP reagent was done by mixing acetate buffer (300 µM, pH 3.6), 10 µM TPTZ in 20 µM HCl, and 10 µM $FeCl_3$ at 10:1:1 (v/v/v). The sample solutions (10 µL) and reagent (300 µL) were mixed completely. The absorbance was measuredat 593 nm after 10 min. Different concentrations of Trolox were used to prepare the standard curve and then, the results of dilution were corrected and stated in micromolar Trolox per 100 g of dry weight (DW). The calculations were repeated three times.

α- Amylase inhibition assay

Based on the procedure outlined by Nowicka et al. the α-amylase inhibitory effect exerted by edible flowers extracts was assayed. By measuring the decreasing groups released from starch, the inhibition of α-amylase activity was determined. Acarbose was incorporated as the positive control. Reading of the results was done at 540 nm (α-amylase). The assays of enzyme inhibition were stated as IC50 value (mg/mL). The values expressed in mg/mL represents a quantitative measure indicating the concentration of edible flowers (mg/mL) required to inhibit, in vitro, a specific biological component solution by 50% (1 U/mL) (Nowicka et al., 2016).

Quantitative estimation of phenolic acids by HPLC

Dionex Ultimate 3000 liquid chromatography (Germany) including a manual sample injection valve equipped with a 20 µl loop, a diode array detector (DAD 3000) with 5 cm flow cell, and Chromeleon 6.8 system manager was used to conduct HPLC analyses. The fractionation process was conducted by a reversed-phase Acclaim TM 120 C18 column (5 µm particle size, 4.6× 250 mm). Additionally, following Hajlaoui et al. (2019), the HPLC was used to estimate phenolic acids.

Antimicrobial activity

In order to determine the minimum inhibitory concentration (MIC), 40 mg/ml of each extract was prepared. A series of ten tubes were used for each extract. Eight tubes were considered for different concentrations of each extract, one tube as positive control, and one

tube as negative control. To each of the tubes, 9 ml of the Muller Hinton Broth was added with a specific concentration of extract and 1ml of suspension of microorganisms (*Staphylococcus aureus*). The tubes were then placed in an oven at 35°C for 24hrs. After incubation, the turbidity caused by growth of microorganism in the tubes was determined. Among the tubes without bacterial growth, the one containing the lowest concentrations of herbal extracts was considered as MIC. In order to determine the minimum bactericidal concentration (MBC), one ml of tube without bacterial growth was mixed with 15 ml of Muller Hinton Broth and a molten agar with a temperature about 48°C was poured on the surface of plate. After agar being solidified, discs impregnated with different concentrations of extract were placed on the plate and incubated at 35°C for 24 hours. The least extract concentration without observing any bacterial growth was considered as the MBC of the extract (Rezaei et al, 2015).

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) carried out in the SPSS statistical package (SPSS 16.00). Duncan's multiple range test at $p< 0.05$ was also used to determine significant differences between the mean scores.

Results and discussion

Efficiency of extraction

According to the results (Fig. 1), the extraction efficiency for the hollyhock plant was significantly more than that of borage. Clearly, in both of the plants (i.e. borage and hollyhock), the ultrasound- assisted extraction was more efficient compared to the soaking method. Moreover, it was found that the higher extraction efficiency achieved by using methanol as a solvent compared to the water. Therefore, the highest extraction efficiency was found with the hollyhock extract which was prepared by ultrasonic- assisted method using methanol as a solvent.

The higher efficiency for ultrasound-assisted method compared to the soaking can be due to the fact that the ultrasound-assisted extraction method uses the shear force resulting from the cavitation bubbles implosion of ultrasonic waves in order to change material properties. Thus, it disrupts plant tissues and facilitates the extraction procedure to a greater extent (Dzah et al., 2020).

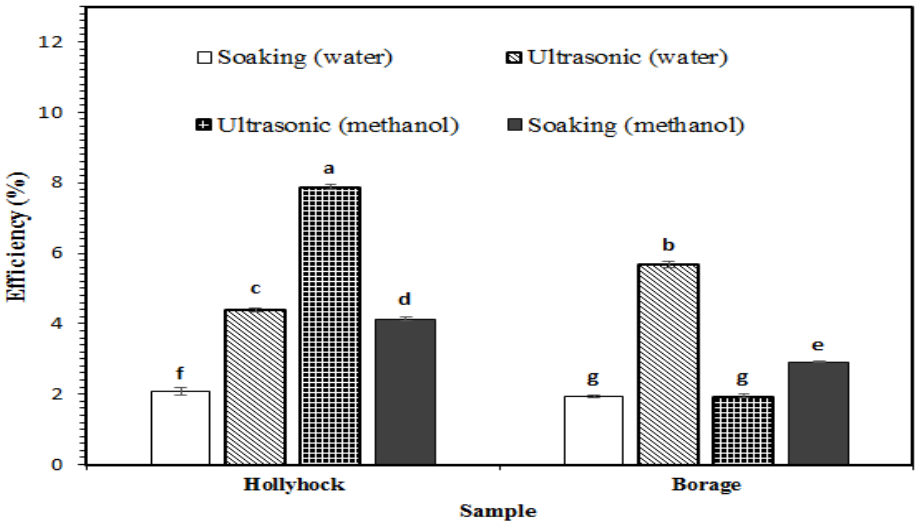

Fig. 1. The effects of the extraction method on the extraction efficiency
Means followed by different letters are significantly different ($p < 0.05$)

Therefore, the higher extraction efficiency of ultrasound-assisted method is due to solvent penetration, enhancement of cell disruption, and mass transfer (Chukwumah et al., 2009).

Consistent with our findings, Safdar et al. used different methods (ultrasound and maceration) of extracting polyphenols from mango peel (Safdar et al., 2017). According to their report, the highest extraction yield was associated with ultrasound-assisted extraction using methanol as a solvent. Generally, it can be concluded that the extraction efficiency of extracts from borage and hollyhock was a function of many factors including the type of method and the polarity of the solvent.

Phenolic content of extracts

Table 1 lists the TPC in different samples. In fact, results showed that as for the hollyhock, the greatest TPC was related to the extract which was prepared by ultrasound- assisted method with water as solvent suggesting high water solubility for the phenolic compounds of hollyhock extract. For the borage, the TPC content of extract prepared by ultrasound-assisted method was higher compared to those prepared by soaking method; however, the effect of solvent type (water and methanol) on the TPC of extracts produced by ultrasound-assisted extraction was insignificant ($p > 0.05$). Moreover, according to the results, the lowest TPC was related to the extracts which were prepared by soaking method with methanol as a solvent. Generally, it seems that the ultrasound-assisted extraction using water or methanol as a solvent is more efficient compared to a soaking method to produce extracts from borage and hollyhock with high content of phenolic compounds. The point that is consistent with our findings is that a higher TPC was reported for aqueous extract of *Echium amoenum* L. compared with the extract produced by acetone as a solvent (Pilerood and Prakash, 2014). This was attributed to the high degree of solubility of borage polyphenols in water. In addition, Goli et al. used different methods and solvents to prepare extracts from the pistachio green hull. Consistent with our findings, they also reported that the highest TPC was related to the extracts which were produced by ultrasound-assisted extraction using methanol or water as a solvent (Goli et al., 2005). In conclusion, with respect to the high TPC of extracts from borage and hollyhock as well as the many beneficial attributes of phenolic compounds such antioxidant and antimicrobial activity, the extracts produced in this study can be used as natural antioxidant and antimicrobial agents for developing food products with a wide range of health- promoting properties.

Flavonoid content of the extracts

The TFC of different extract samples prepared using soaking and ultrasound- assisted extraction methods using water and methanol as solvents is shown in Table 1. In both of the examined plants, the results showed that the extracts obtained by the soaking method using methanol as the solvent had the highest level of flavonoids. This observation suggested that the soaking method is a more efficient method in comparison with ultrasound-assisted extraction to prepare extracts with high content of flavonoids. The lower TFC of extracts through ultrasound-assisted method might be due to the formation of free radicals which may have an effect on the active unstable phytochemicals such as flavonoids (Azwanida, 2015). The results also indicated that methanol was a better solvent to extract flavonoids from borage and hollyhock compared to water. Therefore, it seems that the flavonoids found in these plants had a higher affinity to methanol compared to water. Consistent with our findings, Karimi et al. found a significant difference existed between methanol, ethanol, and water extracts of *Borago officinalis* L. flower for TFC (Karimi et al., 2018). The greatest content of flavonoids was found in the methanolic extract. This was ascribed to the greater polarity index of methanol relative to water and ethanol resulting in the extraction of more flavonoid compounds.

Anti-radical activity of the extracts

Antioxidants are chemicals that prevent and control the effects of free radicals thus helping to protect the human body against infections and diseases. Determining the antioxidant activity using DPPH assay as a common method of assessing the free radical scavenging

activity of plant extracts provides helpful information about the antioxidant potential that occur in plant materials (Fraczek et al., 2019; Safdar et al., 2017). The results showed the adequate ability of all extract samples to scavenge the free radicals of DPPH. The findings also showed that the extraction method and the solvents also had a significant effect on the antioxidant activity of the resulting extracts. As for hollyhock, the extracts prepared through the soaking method had a higher antioxidant activity compared to the extracts obtained through the ultrasound- assisted method. For borage, the anti- radical activity of extracts prepared using ultrasound- assisted extraction was higher than those extracted through soaking approaches. However, in both examined plants, using water as a solvent in the optimum method resulted in greater antioxidant activity than methanol. The antioxidant properties of these herbs may be connected to their biological active components such as phenolic and flavonoid compounds which are well-known as potent antioxidant and anti-radical agents (Hosaka et al., 2015; Karimi et al., 2018). Safdar et al. also indicated a higher DPPH radical scavenging activity in aqueous extracts of mango peel compared to those extracted by other solvents such as methanol and ethanol (Safdar et al., 2017). Pilerood and Prakash (2014) found that decreased power of extract and the free radical scavenging activity were measures of the antioxidant activity and associated with the type of the solvent which was employed to obtain the extracts. Generally, these findings suggested that the extracts from borage and hollyhock could be effectively applied as the natural antioxidant agents found in food products instead of the synthetic antioxidants which have many disadvantages.

Table 1- Total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity of different extract samples

Extract sample	TPC (mg GAE/g)	TFC (mg QUE /g)	IC_{50} (mg/ml)
Hollyhock + ultrasound (water)	535.07 ± 16.98^{a}	115.48 ± 1.89^{d}	0.50 ± 0.01^{bc}
Hollyhock + soaking (water)	421.80 ± 12.80^{b}	107.76 ± 5.84^{e}	0.32 ± 0.03^{cd}
Hollyhock + ultrasound (methanol)	332.50 ± 4.07^{e}	23.40 ± 0.70^{f}	0.67 ± 0.06^{b}
Hollyhock + soaking (methanol)	221.76 ± 2.97^{f}	337.03 ± 3.61^{c}	0.40 ± 0.00^{bcd}
Borage + ultrasound (water)	354.57 ± 5.66^{cd}	3.35 ± 0.21^{g}	0.13 ± 0.06^{d}
Borage + soaking (water)	344.93 ± 12.93^{de}	383.54 ± 3.27^{b}	1.70 ± 0.25^{a}
Borage + ultrasound (methanol)	363.03 ± 6.14^{c}	5.10 ± 0.11^{g}	0.18 ± 0.03^{d}
Borage + soaking (methanol)	117.52 ± 1.73^{g}	547.49 ± 2.05^{a}	1.83 ± 0.29^{a}

*Different superscripts in each column represent a significant difference ($p < 0.05$)

Anthocyanin content of the extracts

Total anthocyanidins are conventionally extracted from polar organic solvents. As shown in Figure 2 in the case of Hollyhock and Borage which were examined, the results showed that the extracts obtained by ultrasound- assisted method using water as a solvent had the highest level of Anthocyanin. In addition, these variations can be associated with the conditions of the extraction, raw material, and analysis conditions.

Ravanfar et al. (2015) reported that ultrasonic energy was effective in intensification and yields improvement of the anthocyanins' extraction. The type of plant pigments having relevant roles in plant defense mechanisms, plant propagation, and the color of fruits and vegetables is anthocyanins. As shown by studies, they have positive effects on human health. Pilerood and Prakash (2014) evaluated anthocyanins and the antioxidant activity of Borage (*Echium amoenum*). They indicated that borage is rich source of anthocyanins (104.4 mg/ 100 g). As for anthocyanin content, studies have reported that fruits contain anthocyanin in various amounts. For example, Liu et al. (2020) reported that the anthocyanin content of raspberries ranged from

0.17 to 57.0 mg/ 100 g, this range for grapes was reported by Bridle and Timberlake to be 30– 750 mg/ 100 g.

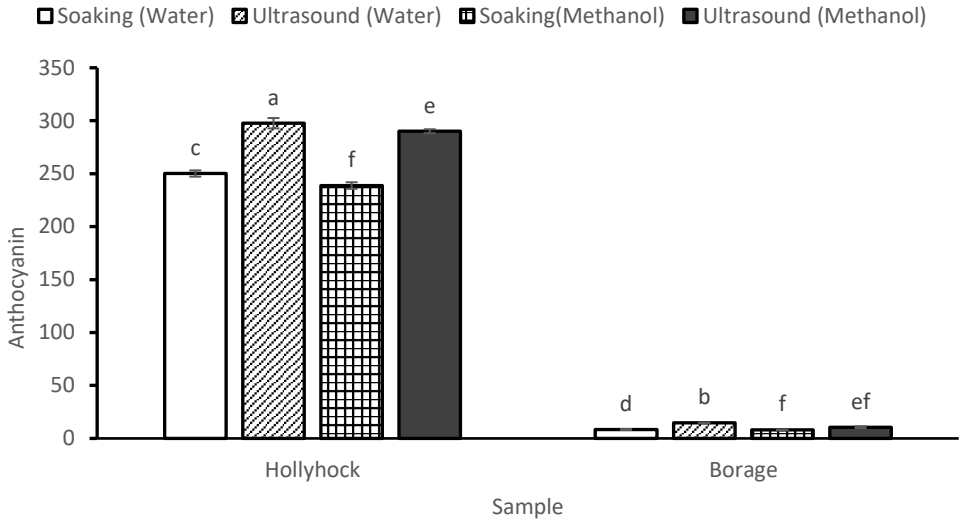

Fig. 2. The effects of the extraction method on the anthocyanin content of the extracts.
Means followed by different letters are significantly different ($p < 0.05$)

Ferric reducing antioxidant power (FRAP)

Total antioxidant activity results, as calculated using the FRAP method, are listed in Table 2 and compared to Vitamin C and TBHQ. Ultrasound- assisted method and water as a solvent had the main role and showed higher power in total antioxidant activity compared to natural and artificial antioxidants.

Table 2- Ferric reducing antioxidant power (FRAP) of different extract samples

Extract sample	FRAP
Hollyhock+ soaking (water)	1.88 ± 0.07^{b}
Hollyhock+ ultrasound (water)	2.19 ± 0.08^{a}
Borage+ soaking (water)	1.5 ± 0.07^{d}
Borage+ ultrasound (water)	1.61 ± 0.06^{c}
Hollyhock+ soaking (methanol)	1.68 ± 0.03^{c}
Hollyhock+ ultrasound (methanol)	1.92 ± 0.06^{b}
Borage+ soaking (methanol)	1.3 ± 0.08^{e}
Borage+ ultrasound (methanol)	1.49 ± 0.03^{d}
Vitamin C	1.31 ± 0.03^{e}
TBHQ	1.37 ± 0.03^{e}

*Different superscripts in each column represent a significant difference ($p < 0.05$)

Chaouche et al. (2011) indicated antioxidant activity in hydromethanolic root extracts of *E. pycnanthum* collected in southern Algeria. *E. vulgare* and *E. rubrum* have been tested in terms of antioxidant activity through metal-chelating (Fe^{2+}), FRAP, TAC, OH radical, DPPH and ABTS radical scavenging assays. In addition, the results indicated the high potency of *E. vulgare*, due to its high TPC and TFC values.

Bacterial alpha amylase inhibition

The α-amylase inhibition was used to investigate the edible flowers extracts in terms

of their inhibitory effect at various concentrations. Their biological activity was established and the IC50 values were calculated (Table 3). As for the human body, pancreatic α-amylase hydrolyzes dietary carbohydrates into monosaccharides which are considered suitable for absorption. One strategy applied to counteract metabolic abnormalities connected to type 2 diabetes and hyperglycemia is the inhibition of these enzymes, and thus using phytoextracts such as α-amylase inhibitors may provide an alternative to prevent diabetes mellitus. As shown in Fig 3., the inhibitory effect increases with increasing extract concentration.

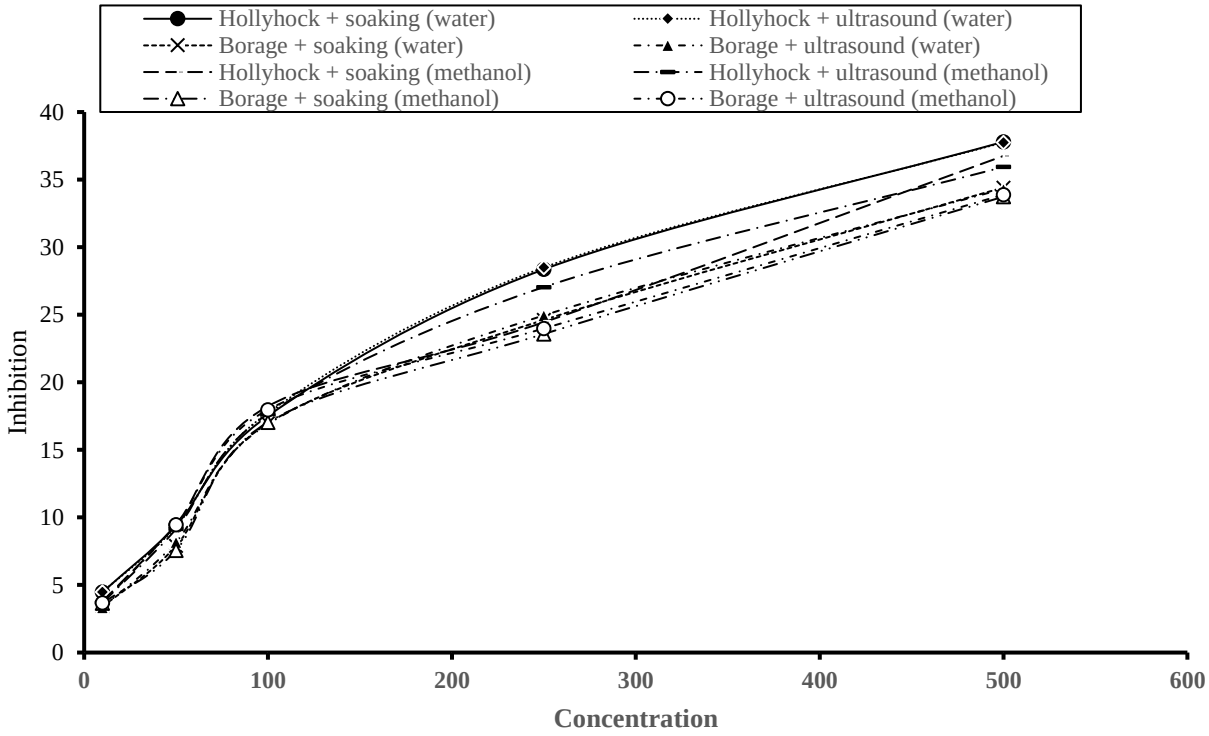

Fig. 3. The effects of Bacterial alpha amylase inhibition of the extracts.

Table 3- Bacterial alpha amylase inhibition of the extract samples

Extract sample	IC50
Hollyhock+ soaking (water)	3166.09 ±86.35^{f}
Hollyhock+ ultrasound (water)	3130.02 ±89.2^{f}
Borage+ soaking (water)	5520.72 ±101.66^{c}
Borage+ ultrasound (water)	5452.87 ±75.66^{c}
Hollyhock+ soaking (methanol)	4305.09 ±78.52^{d}
Hollyhock+ ultrasound (methanol)	3887.96 ±87.66^{e}
Borage+ soaking (methanol)	6715.49 ±70.12^{a}
Borage+ ultrasound (methanol)	6331.26 ±100.29^{b}

The α-amylase inhibition, presented by IC50 values, ranged from 3130.02 to 5520.72 mg/ml. The lowest value was for Hollyhock extract obtained by ultrasound- assisted and water as solvent and highest amount of IC50 was related to Borage extract obtained by ultrasound-assisted method and methanol as solvent.

An in-silico study investigating the inhibitory activities of certain flavonoids and phenolic acids on α-amylase and α-glucosidase, rosmarinic acid exhibited an IC50 value

equivalent to acarbose (Tolmie et al., 2021). McCue and Shetty (2004) also reported in support of these results. According to these researchers, rosmarinic acid has an in vitro inhibitory effect on porcine pancreatic amylase. Some reports in the literature have shown that some extracts contained chlorogenic acid as a major compound and exhibited significant inhibitory activity on digestive enzymes (Chokki et al., 2020; Liu et al., 2020a; Liu et al., 2020b). These findings support those from the present study.

Quantitative HPLC estimation of phenolic contents

Hollyhock+ ultrasound (water)

The HPLC analysis makes it possible to perform simultaneous fractionation and identify an extensive range of phenolics acids in a plant sample. In this study, it was found that an ultrasound- assisted method and water as solvent can extract higher phenolic compounds compared to soaking and methanol solvent. For this reason, the aqueous extract of plants extracted with ultrasound-assisted method was performed to identify phenolic compounds. Figure 4 illustrates the results of HPLC analysis of borage and hollyhock prepared through ultrasonic-assisted method and using water as solvent.

As can be seen in the Figure 4, syringic acid is considered as the main phenolic compound found in Hollyhock. The results showed that after syringic acids, two phenolic compounds, P-hydroxybenzoic acid, and P-coumaric acid had the highest concentrations in final Hollyhock extract. Similar to our research work, Dudek et al. examined the phenolic content of this species through HPLC method and determined the total volume of phenolic acids measured as caffeic acid in the ethereal fractions of the isopropanol extracts from the whole flower (Dudek et al., 2006).

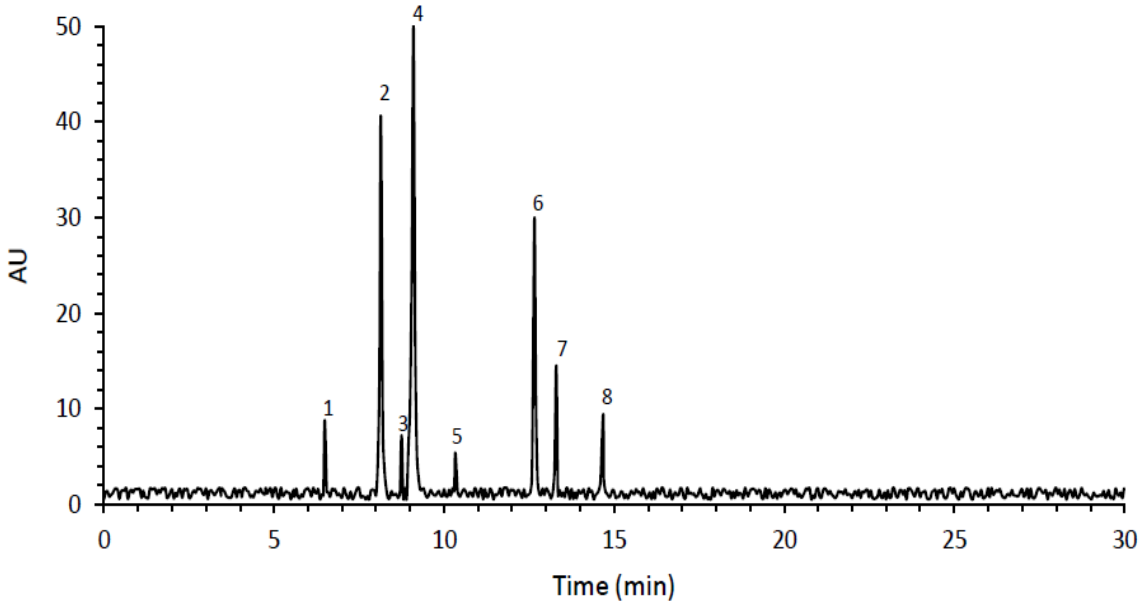

Fig. 4. The HPLC chromatogram of phenolic acids in the Hollyhock + ultrasound (water) Peaks (1) Chlorogenic acids, (2) P-hydroxybenzoic acid, (3) Caffeic acid, (4) Syringic acid, (5) m-hydroxybenzoic acid, (6) p-coumaric acid, (7) Ferulic acid, and (8) Isoferulic acid.

Borage+ ultrasound (water)

The results about identifying the phenolic compound in the aqueous extract of borage extracted by ultrasound-assisted method are presented in Figure 5.

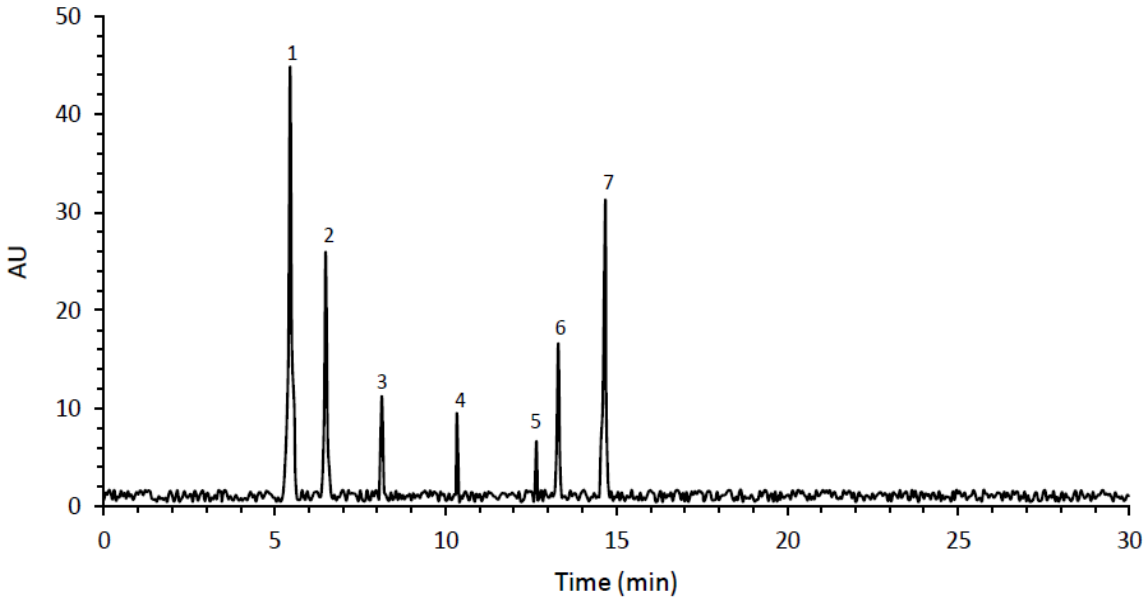

Fig. 5. The HPLC chromatogram of phenolic acids in the Borage + ultrasound (water) Peaks (1) Gallic acid, (2) Chlorogenic acid, (3) P-hydroxybenzoic acid, (4) m-hydroxybenzoic acid, (5) p-coumaric acid, (6) Ferulic acid, and (7) Isoferulic acid.

According to the results, gallic acid was the higher active ingredient in borage extract. The most abundant phenolic acid in medicinal plants is gallic acid. These findings are interesting given the samples pharmacological function. Chlorogenic acid and Isofric acid had the next highest concentrations in the extract. Bandoniene et al. (2005) used HPLC analysis of the aqueous extract of the borage and extracted rosmarinic acid as the major compound along with several minor components. El-Hallous (2019) used HPLC to examine Viper's Bugloss (*Echium Vulgare* L) extract as a natural antioxidant and found that the phenolic acids from *Echium vulgare* extract were gallic acid, benzoic acid, isoferulic acid, chlorogenic acid, vanillic acid, catechol, salicylic acid, ferulic acid, catechin, P-hydroxy-benzoic acid, protocatechuic acid, alpha coumaric acid, and p-coumaric acid.

Antimicrobial activity

The MIC and the MBC of the extracts of borage and hollyhock against *Staphylococcus aureus* are presented in Table 4. The extracts showed an inhibitory effect at concentrations of 1- 5 mg/ml and showed a bactericidal effect at the concentrations between 5- 10 mg/ml on *Staphylococcus aureus*. The results revealed lower MIC and MBC for the hollyhock extract compared to the borage extract. Moreover, it was found that the method and solvent did not have a significant effect on the MIC and MBC. Therefore, it can be concluded that the extract of hollyhock had an antimicrobial effect at lower concentrations and the antibacterial activity of this extract was significantly higher than borage extract. Antimicrobial activity of natural extracts appears to be due to their bioactive compounds especially phenolic and flavonoid compounds (Rezaei et al, 2015). Consistent with our results, Karimi et al. also reported a good antimicrobial activity for *Borago officinalis* L. flower against different foodborne pathogens, which was attributed to the existence of flavonoids and polyphenols in the extract (Karimi et al., 2018). These biologically active compounds possess antibacterial activity through the chemical barrier's induction against the invading microorganisms, interrupting energy supply of the metabolism, cytoplasmic membrane function, disrupting nucleic acid synthesis, non-specific reactions with carbohydrates in the cell

wall, and inactivation of adhesions and protein transport (Oliveira et al, 2008).

Table 4- Minimum inhibitory (MIC) and minimum bactericidal concentration (MBC) of borage and hollyhock extracts prepared using different methods and solvents.

Extract sample	MIC (mg/ml)	MBC (mg/ml)
Hollyhock+ ultrasound (water)	1.33± 0.58^{bc}	4.67± 0.58^{b}
Hollyhock+ soaking (water)	1.33± 0.58^{bc}	4.67± 0.58^{b}
Hollyhock+ ultrasound (methanol)	1.00± 0.00^{c}	4.67± 0.58^{b}
Hollyhock+ soaking (methanol)	2.00± 0.00^{b}	5.00± 0.00^{b}
Borage+ ultrasound (water)	4.67± 0.58^{a}	9.67± 0.58^{a}
Borage+ soaking (water)	4.67± 0.58^{a}	10.00± 0.00^{a}
Borage+ ultrasound (methanol)	5.00± 0.00^{a}	9.67± 0.58^{a}
Borage+ soaking (methanol)	4.67± 0.58^{a}	10.00± 0.00^{a}

*Different superscripts in each column represent a significant difference ($p < 0.05$)

Conclusions

The proper extraction of medicinal plants is essential to meet the increasing pharmaceutical industry demands for biologically active natural extracts. The extracts of medicinal plants with strong antioxidant activity increase pharmacological functions. Borage and hollyhock are rich sources of bioactive compounds with strong antioxidant activity. Different methods (ultrasound- assisted extraction and soaking) and solvents (methanol and water) were used to prepare extracts from these medicinal plants. The results showed that the ultrasound-assisted extraction and methanol were more efficient method and solvent compared to soaking method and water to prepare the extract. Moreover, the content of phenolic and flavonoid compounds as well as the antioxidant activity of the resulting extracts were different depending on the type of the method and the polarity of the solvents. The results of TPC, anthocyanins, and the FRAP tests showed that the highest extract yield was obtained through ultrasound- assisted method with water as solvent. The highest amount of TFC was reached by a soaking method using methanol as solvent. The anti- radical activity tests indicated that using water as a solvent in the optimum method resulted in a higher antioxidant activity. Furthermore, the bacterial alpha amylase inhibition test suggested that the inhibitory effect increased with increasing extract concentration. The HPLC analysis of the borage (*Echium amoenum* L.) and hollyhock (*Althaea rosea* var. *Nigra*) extracts revealed that gallic acid and syringic acid were the most prominent phenolic compounds. Indigenous herbs found in Iran and their effects on infectious agents such as *S. aureus* were addressed in this review. Moreover, the results showed that the borage and hollyhock aqueous extract had remarkable antimicrobial activity against *Staphylococcus aureus* which can be a subject of future studies to find their effective compounds contributing to the antibacterial activity. Therefore, borage and hollyhock can be considered as good sources of antioxidants and antimicrobial compounds aside from their medicinal properties. Thus, they can be applied as natural preservatives supplements in the food formulations in order to enhance the shelf-life through improving their stability against pathogens and by retarding the lipid peroxidation process.

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نشریه پژوهشهای علوم و صنایع غذایی ایران
جلد ۱۸، شماره ۳، مرداد - شهریور ۱۴۰۱، ص. ۶۸-۵۳

Iranian Food Science and Technology Research Journal
Vol. 18, No. 3, Aug. Sep. 2022, p. 53-68

بررسی فعالیت آنتی‌اکسیدانی و ضدمیکروبی عصاره گیاهان گل گاو زبان (*Echium amoenum L.*) و ختمی سیاه (*Althaea rosea var. nigra*) استخراج شده به روش خیساندن و فراصوت

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تاریخ دریافت: ۱۴۰۱/۰۲/۰۶
تاریخ پذیرش: ۱۴۰۱/۰۳/۱۸

چکیده

هدف از این مطالعه بررسی فعالیت آنتی‌اکسیدانی و ضدمیکروبی عصاره گل گاوزبان (*Echium amoenum L*) و ختمی سیاه (*Althaea rosea* var. *Nigra*) بود. عصاره‌ها از طریق روش خیساندن و فراصوت با استفاده از آب یا متانول به‌دست آمد. سپس محتوای فنل و فلاونوئید، فعالیت مهار رادیکال‌های آزاد و ضدباکتریایی عصاره‌ها مورد بررسی قرار گرفت و آنالیز ترکیبات فنولی توسط دستگاه کروماتوگرافی مایع با کارایی بالا انجام گردید. نتایج به‌دست آمده نشان داد که بیشترین میزان ترکیبات فنلی و آنتوسانین و فعالیت آنتی‌اکسیدانی در روش FRAP در نمونه‌ای که به روش فراصوت و حلال آب استخراج شده است، مشاهده گردید و عصاره‌های استخراج شده با روش خیساندن و حلال متانول بیشترین میزان ترکیبات فلانوئیدی را دارا بودند. در بررسی فعالیت به دام‌اندازی رادیکال با دی فنیل پیکریل هیدرازیل، در هر دو عصاره به‌دست آمده به روش فراصوت غلظت کمتری IC50 تعیین گردید. نتایج حاصل از اثر مهارکنندگی عصاره‌ها بر فعالیت آنزیم آلفاآمیلاز نشان داد که با افزایش غلظت عصاره اثر مهارکنندگی آن نیز افزایش یافت. مواد موثره شناسایی شده با دستگاه HPLC نشان داد که اسید سیرینجیک و اسید گالیک ماده موثره اصلی در عصاره ختمی سیاه و گل گاوزبان به‌ترتیب بودند. نتایج به‌دست آمده از این تحقیق حاکی از آن بود که فعالیت ضدباکتریایی عصاره ختمی به‌طور معنی‌داری بیشتر از عصاره گل گاو زبان بوده است. این مطالعه پیشنهاد می‌کند که عصاره‌های گل گاو زبان و ختمی سیاه را می‌توان به‌عنوان یک نگهدارنده طبیعی در فرمول‌های غذایی گنجانید تا ویژگی‌های ارتقادهنده سلامت آنها را بهبود بخشد.

واژه‌های کلیدی: عصاره‌های طبیعی، روش خیساندن، استخراج به کمک فراصوت، پلی‌فنل.

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