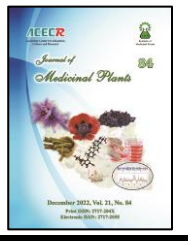
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Research Article

Antimicrobial and antioxidant activities of the leaf extract of some cultivated Iranian licorice populations

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ABSTRACT

Background: Licorice (*Glycyrrhiza glabra* L.) is a well-known commercial medicinal plant with wide usage. **Objective:** During our ongoing aims for the selection and breeding programs of Iranian licorice (*Glycyrrhiza glabra* L.) populations, in the present study, total phenol content (TPC), total flavonoid content (TFC), and biological activities of the leaf extract of eight selected plant populations cultivated in the north of Tehran were investigated. **Methods:** The TPC and TFC were determined by Folin-Ciocalteu and aluminum chloride reagents, respectively. The antioxidant potential of the leaf extracts was measured through the evaluation of their power to reduce the Fe^{3+}-TPTZ complex to Fe^{2+}-TPTZ. The antibacterial activity was also assessed according to the broth micro-dilution method to determine minimum inhibitory concentration. **Results:** The results indicated that TPC varied from 6.18 ± 0.33 (mg GAE/g DW) in Bajgah population to 14.91 ± 1.17 (mg GAE/g DW) in Ilam population. The highest TFC was observed in Ilam (17.04 ± 1.25 mg rutin/g DW) and Marvest (15.06 ± 1.77 mg rutin/g DW) populations, respectively, without statistically significant differences. The maximum antioxidant activity was associated with the Ilam (532.18 ± 12.61 µmol Fe/g DW) population, confirming the antioxidant potential of phenolic and flavonoids compounds. The leaf extracts of Eghlid, Marvest, Ilam, and Bojnourd populations were exhibited outstanding antibacterial activity against S. *aureus*. **Conclusion:** The licorice leaf extracts showed more inhibitory effect against *Staphylococcus aureus* than *Escherichia coli*.

1. Introduction

Infectious diseases cause the death of about 57 million people around the world annually [1]. The number of synthetic antibiotics produced by pharmaceutical companies is increasing annually. However, multiple drug resistance and

Abbreviations: TPC, Total Phenol Content; TFC, Total Flavonoid Content; MIC, Minimum Inhibitory Concentration; MAPs, Medicinal and Aromatic Plants; DMSO, Dimethyl Sulfoxide; TPTZ, 2,4,6-Tripyridyl-S-triazine; $AlCl_3$, Aluminum Chloride; ROS, Reactive Oxygen Species. FRAP, Ferric Reduction Antioxidant Potential

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side effects have overshadowed the effectiveness of synthetic antibiotics, which has driven researchers to develop natural and unimpaired antibiotics [2]. On the other hand, growing concerns about the use of synthetic antioxidants have increased the importance of the plant as a source of natural antioxidants. Natural antioxidants not only barricade the generation of reactive oxygen species (ROS) but also scavenge the generated free radical molecules, protecting DNA and protein damage as well as lipid peroxidation [3].

Medicinal and aromatic plants (MAPs) have long been used to disinfect and reduce adverse effects of microbial agents, attracting researcher's attention of researchers to discover and extract antimicrobial compounds. The discovery of new antimicrobial compounds from the MAPs will help reduce dependence on synthetic antibiotics and seems to reduce the multi-drug resistant of microorganisms [4]. Among plant compounds, phenols and flavonoids have shown a major role in the emergence of antioxidant properties [5]. Reducing disease incidence has been proven by consuming diets rich in antioxidants [6].

Licorice (*Glycyrrhiza glabra* L.), belongs to the Fabaceae family, has been consumed as a drug for a long time. Despite the focus of research on the underground part of the plant, its aerial part bring up as the neglected area of the research. In addition to the well-known pharmaceutical properties of the underground part of the plant, its leaves have traditionally been used to treat wounds [7]. Licorice leaves have also been consumed as livestock fodder, soil fertilizer, and more recently as a source of extraction of antibacterial and antifungal compounds [8, 9]. Phytochemical quantitative measurements indicated the predominance of polyphenols such as licoflavanone, grabranin, pinocembrin and dihydrostilbenes in the leaf extract of the licorice [10].

The first step in cultivating a species is to assess natural populations in order to identify a population with desirable biological and phytochemical characteristics to meet the needs of industries. Various studies has been performed on endemic MAPs to identify compounds with specific biological properties. Actually, relying on the exploitation of indigenous plants in order to use their biological properties mainly antioxidant and antimicrobial characterization can be conspicuously important.

Our research group already studied identification, collection and phytochemical and genetic analysis of natural licorice populations [11, 12]. Literature survey revealed that biological activity of licorice has already been reported [13-16], but to the best of our knowledge, antioxidant and antimicrobial activities of Iranian licorice populations have not yet been studied. So, the present study was aimed to explore antibacterial, antifungal, and antioxidant activities of some cultivated licorice populations in the same area. The results of this work can be taken into consideration to identify superior populations for the continuation of breeding works and commercial exploitations.

2. Materials and methods

2.1. Chemicals and reagents

The standard of gallic acid and rutin were purchased from Sigma-Aldrich Company, Germany. Folin–Ciocalteu reagent ethanol 96%, dimethyl sulfoxide (DMSO), 2, 4, 6-Tripyridyl-S-triazine (TPTZ) and aluminum chloride (AlCl3) were prepared from Merck (Darmstadt, Germany).

2.2. Plant materials and cultivation site

The rhizomes of licorice populations were cultivated in the randomized complete block design (RCBD) experiment with three replications at the research farm of Medicinal

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Plants and Drugs Research Institute (MPDRI), Shahid Beheshti University, Evin, Tehran, Iran. The plant density was 30 × 70 cm. The plants were watered every ten days. The aerial part of the plants were harvested at the initial stage of flowering and were transferred to the laboratory for shade-drying. The climatic conditions and soil characteristics of the plant cultivation site are also presented in Table 1.

2.3. Preparation of leaf extracts

The licorice leaf extraction was performed using methanol according to method previously described [11], and then filtered and used for TPC, TFC and antioxidant measurement. The methanolic extracts were then dried and dissolved in DMSO with concentration of 300 mg/ml for antimicrobial assay.

2.4. Determination of total phenol and total flavonoid content

Total phenol content was determined using a colorimetric method in which 25 µl of extract was added with 125 µl Folin-Ciocalteu reagent 10% and 100 µl sodium carbonate solution 7.5% (V/V), and then placed on a dark place for 2 hours. The absorbance of the samples was then measured at 760 nm [17]. The total flavonoid content (TFC) was calculated according to the previously described method by Zhishen et al. [18] using aluminum chloride (AlCl3) reagent. Briefly, 25 µL of the sample solution, along with 100 µL of distilled water and 7.5 µL of NaNO2 solution (n = 3) were poured into 96 plate well. After six minutes, 7.5 µL of AlCl3, 100 µL of NaOH, and 10 µL of distilled water were added to each well. The absorption was read after 15 min at a wavelength of 510 by the spectrophotometer. Different concentrations of gallic acid and rutin were used to draw the calibration curves for TPC and TFC, respectively.

Table 1. Climatic conditions and soil characteristics of the plant cultivation site

Meteorological and climatic conditions	Latitude (N)	35°48'20.6"
	Longitude (E)	51°23'35.3"
	Altitude (m)	1190
	Mean annual temperature (°C)	17.3
	Average minimum annual temperature (°C)	13.3
	Average maximum annual temperature (°C)	22.9
	Mean Annual Precipitation (mm/year)	395.5
	Climate	Cold semi-arid
Edaphological characteristics	N_{total} (%)	0.26
	P_{ava} (ppm)	66.5
	K_{ava} (ppm)	350
	pH	7.8
	EC (ds/m)	0.71
	OC (%)	2.04
	% Sand	49
	% Silt	32
	% Clay	19
	Texture	Loamy

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2.5. Ferric Reduction Antioxidant Potential (FRAP) assay

The antioxidant potential of the leaf extracts was measured through the evaluation of their power to reduce the Fe^{3+} - TPTZ complex to Fe^{2+} - TPTZ according to the Benzie and Strain [19] method. In summary, 300 mM of acetate buffer (pH 3.6), 20 mM of ferric chloride in water and 10 mM of TPTZ in 40 mM of hydrogen chloride adequately mixed with a ratio of 10:1:1 to prepare the FRAP solution. Then, 175 µl of FRAP solution was added to 25 µl of extracts and incubated in darkness for 7 min. The absorbance of the samples was read at a 593 nm by Powerwave XS Microplate spectrophotometer (Bio-Tek Instruments, Inc., USA) device. Different concentrations (250, 500, 1000, 1500, 2000 µM) of ferrous sulfate ($FeSO_4.7H_2O$) were used for plotting a calibration curve. The antioxidant activity was reported as micromole ferrous sulfate per gram of plant dry weight (µmol Fe/g DW).

2.6. Determination of antimicrobial potential

The antibacterial and antifungal potential of leaf licorice extracts against models of Gram-positive (*Staphylococcus aureus* ATCC25923) and Gram-negative (*Escherichia coli* ATCC1399) bacteria and *Candida albicans* ATCC11006 as human pathogenic yeast model was measured by determination of MIC values. In brief, Broth micro-dilution method was performed for determination of minimum inhibitory concentrations; MICs values, according to the standard protocols recommended by CLSI (Clinical Laboratory Standard Institute) ad as the lowest concentration of each assessed compound required for inhibition of visible growth of the tested microorganism. In brief, two-fold serial dilutions of each compound were made in a concentration range of 0.015-32 mg/ml in sterile plastic micro-dilution trays containing Mueller Hinton broth medium. Then microbial suspensions of each bacterial and fungal strain were prepared from freshly cultured cells in sterile normal saline that were adjusted to 0.5 McFarland standard turbidity. The suspension was diluted (1:100 and 1:1000 for bacteria and yeast, respectively) by sterile Mueller-Hinton broth (MHB) just before adding to the trays containing a serial dilution of each compound. So each concentration of compounds was evaluated against about 0.5-1×106 bacterial cells. 96 well plates were incubated at 37 °C for 24 hrs. Resazurin was used as a growth indicator. In brief, 4 microliters of 4 mg/ml stock solution of reagent in sterile D.W. was added to each well. Pinkish wells indicate the growth of bacteria in wells. Cefixime and Nystatin were used as standard antimicrobial agents against bacteria and yeast, respectively. Cefixime was assessed as standard antibiotic and Nystatin was evaluated as standard antifungal agent [20].

2.7. Statistical analysis

The raw data were analyzed by SPSS software (version 16.0) and their mean values were compared using Duncan's test.

3. Results

3.1. Total phenol (TPC) and total flavonoid content (TFC)

The results of this study indicated that TPC was varied from 6.18 ± 0.33 (mg GAE/g DW) in Bajgah population to 14.91 ± 1.17 in Ilam population. After the Ilam population, Kashmar (12.67 ± 0.67 mg GAE/g DW) and Marvest (11.70 ± 0.30 mg GAE/g DW) populations were dedicated the highest TPC (Fig. 1).

The highest TFC was observed in Ilam (17.04 ± 1.25 mg rutin/g DW) and Marvest (15.06 ±

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1.77 mg rutin/g DW) populations, respectively, without statistically significant differences. The lowest TFC was also observed in Bajgah population (5.95 ± 1.52 mg rutin/g DW). In total, Ilam population was recognized as the most representative population in terms of TPC and TFC (Fig. 1).

3.2. Antioxidant activity

The antioxidant potential of the leaf extracts of different licorice populations was measured by the FRAP method and was expressed based on µmol Fe/g DW of the plant. The findings revealed that the maximum and minimum antioxidant power was associated to Ilam (532.18 ± 12.61 µmol Fe/g DW) and Bajgah (197.44 ± 15.08 µmol Fe/g DW) populations, respectively (Fig. 2). As mentioned above, the Ilam population had the highest TPC and TFC, while the Bajgah population showed the lowest value in this respect, which confirms the antioxidant potential of phenolic and flavonoid compounds.

3.3. Antimicrobial activity

The minimum inhibitory concentration (MIC) values for all studied populations are shown in Table 2, wherein the leaf extracts of Eghlid, Marvest, Ilam, and Bojnourd populations were exhibited outstanding antibacterial activity against *S. aureus*. The leaf extracts of Takestan, Taft, Marvest, and Ilam populations were also more effective that the rest of populations against *E. coli*. The results of antifungal activity also indicated that the leaf extract of Marvest population (MIC = 0.125) was more effective than other licorice populations (Table 2).

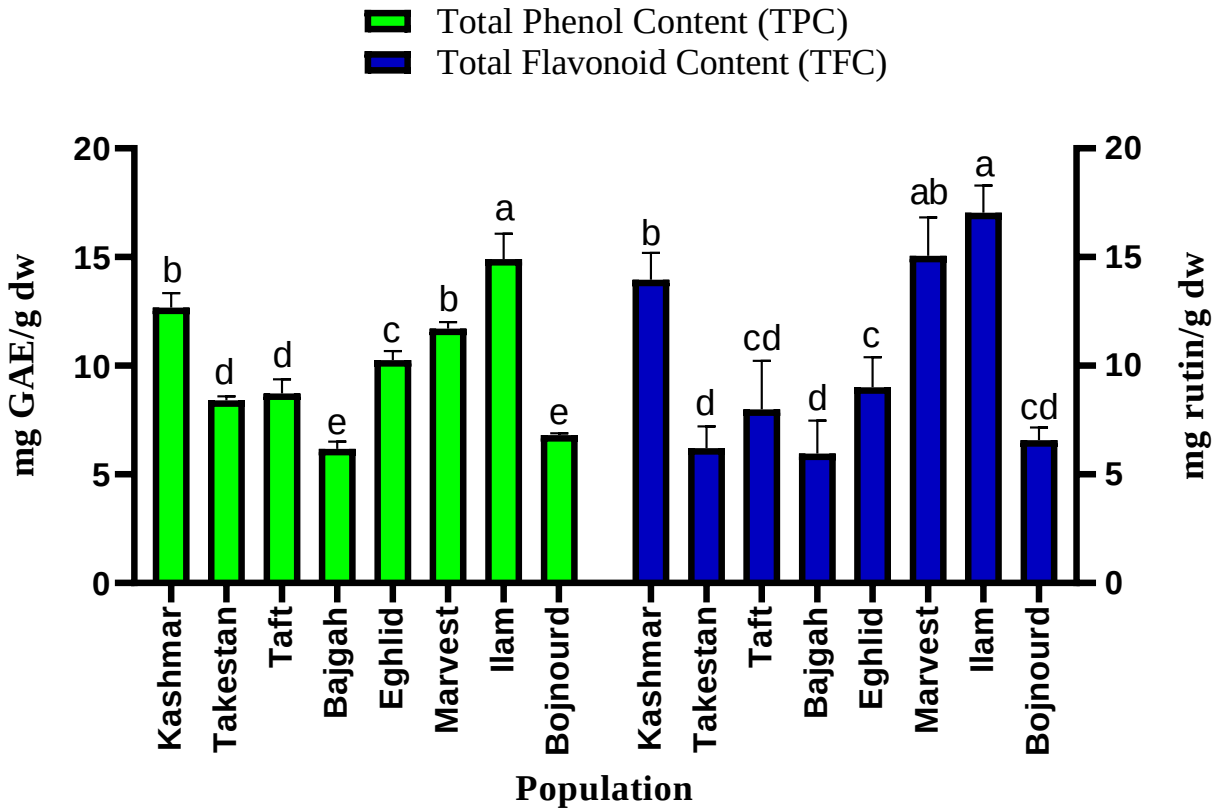


Fig. 1. Total phenolic and total flavonoid content of cultivated licorice populations

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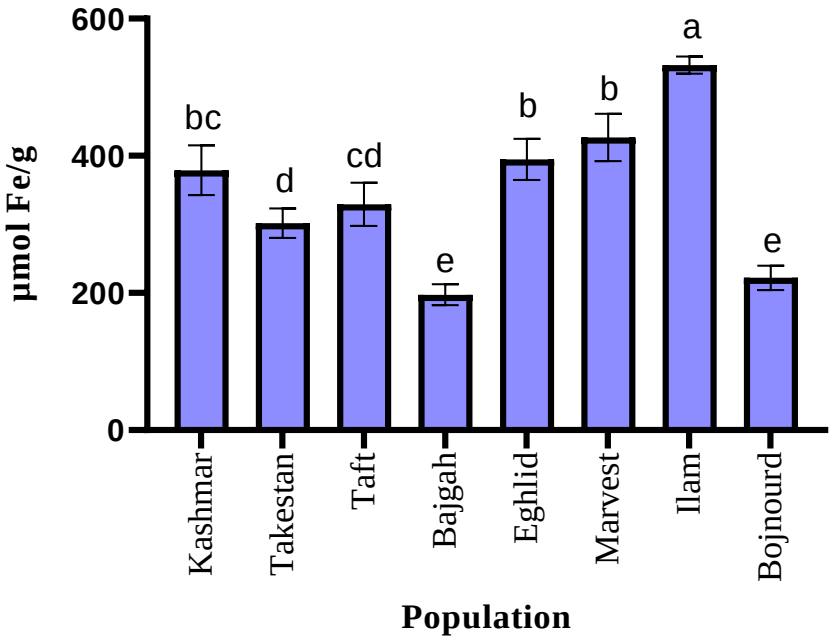


Fig. 2. Antioxidant activity of cultivated licorice populations

Table 2. Antibacterial activity of Iranian licorice leaf extract based on MIC.

Population	Minimum Inhibitory Concentration (MIC mg/ml)		
	Staphylococcus aureus	***Escherichia coli***	***Candida albicans***
Kashmar	0.25	16	4
Takestan	0.125	8	1
Taft	0.25	8	1
Bajgah	0.125	16	0.25
Eghlid	0.062	16	0.25
Marvest	0.062	8	0.125
Ilam	0.062	8	1
Bojnourd	0.062	16	4
Cefixime (µg/ml)	0.5	2	-
Nystatin (µg/ml)	-	-	16

4. Discussion

In this study, variation in TPC (6.18 ± 0.33 - 14.91 ± 1.17 mg GAE/g DW) and TFC (5.95 ± 1.52 - 17.04 ± 1.25 mg rutin/g DW) was observed among cultivated licorice populations. Siracusa et al. [10] identified 30 metabolites from the *g. glabra* leaves by different extraction methods, which mainly belonged to dihydrostilbenes and flavonoids classes. They also measured the TPC in licorice leaf extract and reported that ethyl acetate extract (297.25 ± 15.65 µg/mg) had richer TPC than the methanol (104.09 ± 5.25 µg/mg) and n-hexane (111.53 ± 6.53 µg/mg) extracts. Licorice leaf flavonoids have been classified into two major groups, including isoquercitrin-type and rutin-type by Hayashi et al. [21]. Our previous study on this plant revealed that Iranian licorice has the flavonoid rutin in its aerial parts

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and thus it's placed in the rutin-type category [11]. Total phenol content (TPC) and TFC of licorice extract from locality of Fruska Gora (Serbia) has been reported in the range of 13.23-37.27 (mg GAE/g DW) and 2.69-5.90 (mg QE/g DW), respectively [22]. In the previous study, the measurement of TFC in licorice root and leaf extracts showed that the TFC in licorice leaf (384.75 ± 4.11 mg Catechin equiv/g) was much higher than its root (91.75 ± 6.61 mg Catechin equiv/g), which caused the leaf extract to have more effective antioxidant activity than the root [23]. Our finding showed that the Ilam population with maximum TPC and TFC had the highest antioxidant activity. Clearly, the antioxidant potency of phenolic and flavonoid compounds and direct relationship between these compounds and antioxidant activity have been reported in various studies [24-27]. On the other hand, Iranian licorices are rich in bioflavonoid rutin, which has been proven to have powerful antioxidant activity [28]. Strong antioxidant properties of licorice leaf extract have been previously reported, and this ability to scavenge free radicals has been attributed to the presence of either hydroxyl groups or prenyl groups in flavanone backbone [29]. In a study, three different methods, including DPPH, ABTS, and FRAP were performed to determine the antioxidant potential of G. glabra leaf extract, with values of EC50=116.17 ± 0.55 (µg/ml), 672.19 ± 5.06 (mM Trolox/Mm), and 477.42 ± 13.00 (mmol Fe^{2+}/g), respectively.

In general, leaf licorice extracts showed a stronger inhibitory effect against gram-positive (*S. aureus)* than gram-negative (*E. coli*) bacteria, as previously reported by researchers [30, 31]. Also, the antibacterial activity of licorice leaf extract against *S. aureus* has been reported to be more effective than its root extract [31]. In this study, Iranian leaf licorice extracts showed significant activity against *S. aureus* bacterium with a MIC range of 0.062-0.250, whereas the antibacterial potential of ethanolic leaf extract of *G. glabra* was previously reported with MIC of 1.25 mg/ml against *S. aureus* [30]. Aggarwal et al. [32] showed the antibacterial effect of licorice leaf extract against *E. coli* with Mic of 1 µg/ml. They related this antibacterial activity to the presence of tannins in the leaves of the plant. Other phytochemicals, besides tannin, have been isolated and identified as antimicrobial agents in licorice. Hermann et al. [9] proved the effectiveness of licorice leaf extract on plant pathogenic bacteria and fungi. They also showed the *in vitro* and *in vivo* fungicide and bactericide properties of licorice leaf extract. As previously reported, stilbenes are one of the main metabolites in licorice leaves [10], which as natural factors protect the plant against the attack of viruses, microbes and diseases [33]. Due to the complexity and heterogeneity of plant extracts, it is difficult to determine the level of involvement of a pure substance in antimicrobial activity.

5. Conclusion

Our finding showed that Iranian licorice leaf extract have a proper antioxidant activity. The leaf extract of some populations especially Eghlid, Marvest, Ilam, and Bojnourd was also showed a remarkable antibacterial activity against *S. aureus.* In fact, licorice leaf extract can be used as a candidate for the development of natural antioxidant and antibiotic compounds.

Author contribution

H. E: Conceptualization, Investigation, Plant material collection, Statistical analysis, Extraction, and Writing. MH. M: Supervision, Methodology, Validation, Formal analysis, Review and Editing. F. Z: Methodology,

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Validation. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare that they have no competing interests.

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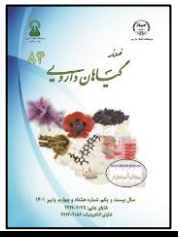
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مقاله تحقیقاتی

ارزیابی فعالیت ضدمیکروبی و آنتی‌اکسیدانی عصاره برگی برخی از جمعیت‌های کشت شده شیرین‌بیان ایران

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اطلاعات مقاله

گل‌واژگان:
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چکیده

مقدمه: شیرین‌بیان یک گیاه دارویی چند ساله تجاری شناخته شده با کاربردهای گسترده است. **هدف:** در ادامه مطالعاتمان جهت انتخاب و برنامه‌های اصلاحی جمعیت‌های شیرین‌بیان ایرانی، در مطالعه حاضر، محتوای تام فنلی، محتوای تام فلاونوئیدی و فعالیت‌های بیولوژیکی عصاره برگی هشت جمعیت منتخب شیرین‌بیان کشت شده در شمال تهران مورد بررسی قرار گرفتند. **روش بررسی:** محتوای تام فنلی، محتوای تام فلاونوئیدی به ترتیب با استفاده از معرف‌های فولین سیوکالتیو و کلرید آلومینیوم به روش رنگ سنجی تعیین شدند. ظرفیت آنتی‌اکسیدانی عصاره‌ها از طریق ارزیابی قدرت آنها در احیای کمپلکس Fe^{3+}-TPTZ به Fe^{2+}-TPTZ اندازه‌گیری شد. فعالیت ضدباکتریایی نیز بر اساس روش میکرورقت براث جهت بدست آوردن حداقل غلظت بازدارنده اندازه‌گیری شد. **نتایج:** نتایج نشان داد که محتوای تام فنلی از ۰/۳۳ ± ۶/۱۸ (میلی‌گرم گالیک اسید بر گرم وزن خشک) در جمعیت باجگاه تا ۱/۱۷ ± ۱۴/۹۱ (میلی‌گرم گالیک اسید بر گرم وزن خشک) در جمعیت ایلام متغیر بود. بیشترین محتوای تام فلاونوئیدی به ترتیب در جمعیت ایلام (۱/۲۵ ± ۱۷/۰۴ میلی‌گرم روتین برگرم وزن خشک) و مروست (۱/۷۷ ± ۱۵/۰۶ میلی‌گرم روتین برگرم وزن خشک) بدون تفاوت معنی‌دار آماری مشاهده شد. بیشترین فعالیت آنتی اکسیدانی مربوط به جمعیت ایلام (۱۲/۶۱ ± ۵۳۲/۱۸ میکرومول آهن بر گرم وزن خشک) بود که پتانسیل آنتی اکسیدانی ترکیبات فنلی و فلاونوئیدی را تأیید می‌کند. عصاره‌های برگ جمعیت‌های اقلید، مروست، ایلام و بجنورد فعالیت ضدباکتریایی فوق‌العاده‌ای بر علیه استافیلوکوکوس اورئوس نشان دادند. **نتیجه‌گیری:** عصاره برگ شیرین‌بیان اثر بازدارندگی بیشتری علیه باکتری گرم مثبت استافیلوکوکوس اورئوس در مقایسه با باکتری گرم منفی اشریشیا کلی نشان داد.

مخففها: TPC، محتوای تام فنلی؛ TFC، محتوای تام فلاونوئیدی؛ MIC، حداقل غلظت بازدارندگی؛ MAPs، گیاهان دارویی و معطر؛ DMSO، دی‌متیل سولفوکساید؛ TPTZ، ۶،۴،۲- تری پیریدیل- اس- تریازین؛ $AlCl_3$، آلومینیوم کلراید؛ ROS، گونه‌های اکسیژن فعال؛ FRAP، ظرفیت آنتی اکسیدانی- احیاکنندگی آهن

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