

Original Research Article

Extraction and Identification of Compounds of *Althaea officinalis* L. and *Anchusa italica* Retz. Native to Ilam Province by Using HS-SPME, GC-MS, and FTIR, and Study of Their Antioxidant Capacity

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ABSTRACT

Medicinal plants, plant extracts, and essential oils are critically important for the pharmaceutical, health, and food industries. Chemical analysis and identification of compounds of medicinal plants has drawn a great deal of attention. In May 2022, the samples of two aromatic species, borage (*Anchusa italica* Retz.) and marshmallow (*Althaea officinalis* L.), native to Dehloran in the south of Ilam province, western Iran, were collected. The medicinal plants were dried, and then pulverized. Their essential oils were extracted by using headspace-solid phase microextraction and their chemical compounds were identified by gas chromatography-mass spectrometry (GC-MS). The functional groups of the plants were also identified by using Fourier-transform infrared spectroscopy. Total antioxidant capacity was measured based on divalent iron reducing ability and by single electron transfer mechanism. The GC-MS results showed that *A. italica* contains 42 chemical compounds including trans-caryophyllene (13.26%), allospathulenol (11.27%), germacrene D (10.47%), bicyclogermacrene (9.77%), safranal (7.62%), δ -cadinene (6.42%), and α -pinene (4.38%) are α -copaene (3.96%) and β -myrcene (3.75%). *A. officinalis* also contains 39 chemical compounds such as tetradecane (22.54%), α -pinene (15.50%), hexadecane (10.46%), 2-hexenal (8.48%), dodecane (7.28%), β -Ionone (3.50%), and trans-geranylacetone (3.26%). After measurement of the wavelengths at 570 nm, the antioxidant property of *A. officinalis* was calculated at 1.2 mmol Fe²⁺/L and that of *A. italica* at 2.9 mmol Fe²⁺/L. Our results showed that the two studied medicinal plants have a wide range of chemical compounds such as phenols, flavonoids, alkaloids, polysaccharides, mucilage, and saponin with the potential antioxidant properties. Therefore, given the antioxidant properties of these plants, this research can be a starting point for the additional experiments regarding the use of essential oils and extracts of these two medicinal plants. Taken together, the potential of these two plants, as two important medicinal plants occurring in Iran, for use in traditional medicine, modern medicine, and industries can be promising with respect to their trade and cultivation, and can stimulate comprehensive research in their pharmaceutical, cosmetic, and medical applications in clinical pharmacy so that they would be introduced into the booming and popular market of medicines of natural origin.

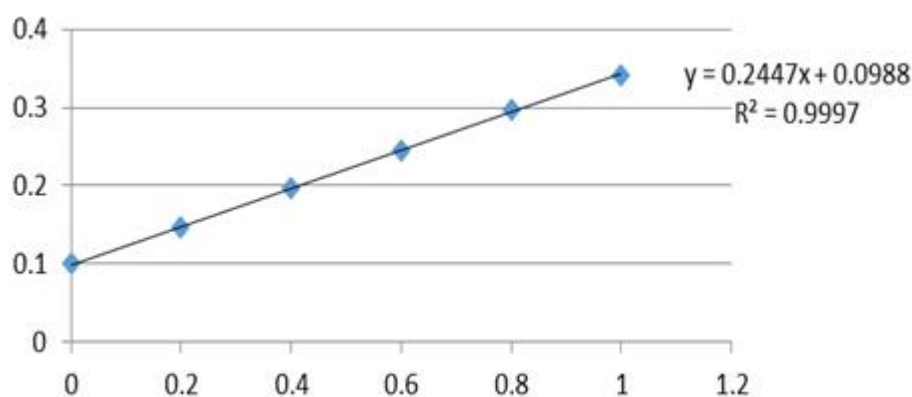
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GRAPHICAL ABSTRACT



Introduction

Medicinal plants are an important source of drugs for various types of diseases [1]. Humans have long used medicinal plants for healthcare and treatment [1,2]. Certain reasons such as population increase, side effects of synthetic drugs, and the development of drug resistance to infectious diseases, the insufficient supply of drugs have led to the use of plant-derived materials as an important source of the inexpensive drugs with fewer side effects than chemical drugs [3,4].

They are found in natural substances in medicinal plants such as the primary and secondary metabolites [5]. The primary metabolites such as carbohydrates, fats, various amino acids, nucleoproteins, enzymes, etc. are essential for living organisms to survive [5,6]. The secondary metabolites include glycosides, alkaloids, essential oils, flavonoids, mucilages, tannins, etc. [5,6] each of which has the specific therapeutic and medicinal properties.

Iran is a privileged and highly ranked country with respect to plant richness and biodiversity, so that it contains 11 climates out of 13 known climates across the globe. The estimated number of plant species occurring in Iran is about 8,000, of which over 2,300 species have medicinal, aromatic, spice, and cosmetic properties. Ilam province, west of Iran, hosts 1000 species of the

plants, 400 of which have the medicinal and aromatic properties [7].

Althaea officinalis L. and *Anchusa italica* Retz .are two of aromatic medicinal plants native to Ilam province [7] that contain aromatic substances and are valuable due to their active ingredients [7,8]. Many plants occur in Ilam province that have medicinal properties, including *A. officinalis* and *A. italica*. *A. officinalis* belongs to the Boraginaceae family. The plant contains substances such as sugars, starches, asparagine, pectin, mucilage, polysaccharides, flavonoids, terpenes, terpenoids, sterols, phenolic compounds, and volatiles [9-11].

The properties of *A. officinalis* include cough-treating, expectorant, and emollient of the skin, and respiratory tract. It is also used in traditional medicine for gastroenterological diseases. Likewise, its antibacterial, antiviral, and antifungal effects have been reported [12-14].

The results of a study in the northern parts of Iran (Khalkhal, Ardabil province) showed that *A. officinalis* extract contains omega-3. Its essential oil is also distilled with compounds such as palmitic acid (13%), heptacosane (9.3%), nonacosane (11.2%), palmitic acid (16.8%), linoleic acid (28%), and naphthalene decahydro 2, 6-dimethyl (16.4%) [15].

A. italica belongs to the Boraginaceae family [15]. Triterpenes, alkaloids, lignans, megasigmans, and flavonoids have been isolated from the plant

[15]. Borage is a two-year plant with a height of 30-60 cm, paniculate branches in the upper part, and standing stem with basal leaves 16-20 cm long and 2-5 cm wide, and finally leafy Calyx usually up to 15 mm long. In addition, the inflorescence first mass was examined [15].

The other compound isolated from *A. italica* include anchusoside-3, viscoside A, 21 β - (β -D-glucopyranosyloxy) -2 α , 3 α -dihydroxyolean-12-28-oic acid-, accigenin, niga-ichigoside F1, glucosyl pinfaensate, daidzein, tormentic acid, and pinfaenoic acid [16-22]. Research shows that this plant has anti-inflammatory, anti-cancer, and antiviral effects.

The results of studies show that chronic diseases such as cancer, Alzheimer's disease, atherosclerosis, Parkinson's disease, and aging are caused by the oxidative stress due to the impacts of free radicals in the body [23]. The antioxidants destroy free radicals. Free radicals cause body molecules to be damaged and lose their normal function. The antioxidants are responsible for the primary defense barrier to oxidative damage [24]. So far, the chemical compounds of *A. officinalis* and *A. italica* native to Ilam have not been studied by using headspace-solid phase microextraction (HS-SPME). Therefore, the present study aimed to identify the chemical compounds in the two plants by using HS-SPME, gas chromatography-mass spectrometry (GC-MS) and Fourier-transform infrared spectroscopy (FTIR), as well as to evaluate their antioxidant capacity.

Materials and Methods

Plant preparation

The plant samples were collected from the southern region of Ilam province. Identification and approval of plant species were conducted based on the Atlas of Plant Flora of Ilam Province (Mozaffarian) at the Biotechnology and Medicinal Plants Research Center of Ilam University of Medical Sciences (By Dr. Mahmoud Bahmani). The botanical and geological information of the area is summarized in Table 1.

Plant drying

In the spring, the flowers of the medicinal plants in question were collected and cleaned.

Plant pulverization

The dried plants were pulverized separately by a special mill (Best 350A, Iran).

Extraction and identification of chemical compounds

First, the essential oils of medicinal plants were extracted by using HS-SPME, and then analyzed by GC-MS. Compounds and functional groups were also evaluated by FTIR.

Identification of chemical compounds by HS-SPME

Essential oils were extracted by using HS-SPME. Briefly, about 2 g of dried plant and plant powder were placed in a vial and the vial temperature was set at 60-70 °C.

Table 1. Characterization of *Althaea officinalis* L. and *Anchusa italica* Retz.

Plant's name	Scientific name	Herbal family	Collection area	Geographical coordinates
Khatmi	<i>Althaea officinalis</i> L	Boraginaceae	Dehloran	32° 41' 28" North, 47° 15' 58" East
Gavzaban	<i>Anchusa Italica</i> Retz.	Malvaceae	Dehloran	32° 41' 28" North, 47° 15' 58" East

These temperature conditions were optimal so that the vapors of the material in the plant essential oil would be saturated in the space above the solid surface. The SPME syringe was placed in the upper space of the container with the lid closed and the materials in the plant vapors were absorbed by the silica phase in the device's needle.

After sufficient time and saturation of silica fiber from the volatile compounds, the fiber was placed directly in the input part of the GC-MS device and due to the temperature of the input part, the materials in the fiber were desorbed, entered the GC-MS device, and were identified [25].

The conditions of device were as follows

The gas chromatograph (Agilent 6890N) was coupled to the Agilent 5973 mass detector. Column: HP-5. (30 m length 5 0.25 mm (ID) 5 0.25 μm) fixed phase thickness), injection type: split/gap, and column temperature program: 50 $^{\circ}\text{C}$, holding time 0.00 min and - $^{\circ}\text{C}/\text{min}$ rate, temperature: 200 $^{\circ}\text{C}$, denaturation time, 0.00 min, rate 5 $^{\circ}\text{C}/\text{min}$, temperature 240 $^{\circ}\text{C}$, denaturation time 0.00 min, and rate 10 $^{\circ}\text{C}/\text{min}$. Carrier gas: He (99.999%); injection type: no gap, Library: Wiley 7n, injector temperature: 250 $^{\circ}\text{C}$, and flow rate: 0.9 ml/min. Extraction mode: (HSSPME), SMPE fibers: PDMS thickness: 100 μm (SUPELCO), sample's weight: 0.5 g, Extraction temperature: 60 $^{\circ}\text{C}$, extraction time: 20 minutes, ultrasound time: 10 minutes (Euronda ultrasound instrument, Italy), and disposal time in port GC-MS injector: 3 minutes [26].

Identification of functional groups by FTIR method

Infrared Fourier transform spectroscopy (FTIR) was used to identify the functional groups. Since the sample used in this study was in the powder form, the KBR (potassium bromide) tablet preparation method was used.

To prepare the sample by KBR (potassium bromide) tablet preparation method, the solid

sample was completely pulverized and mixed with powdered potassium bromide, and then the mixture was turned into a small tablet under the pressure of 10 tons of the device.

The reason for using potassium bromide is that this compound creates no peaks in the range of 650-3900 cm^{-1} . Finally, the sample was exposed to radiation and the spectrum was obtained from the Fourier-transform infrared spectroscopy (FTIR) [27]. Therefore, only the peaks of plant extracts would be revealed in the spectroscopy [7].

Determination of plant antioxidant activity

Preparation of plant samples

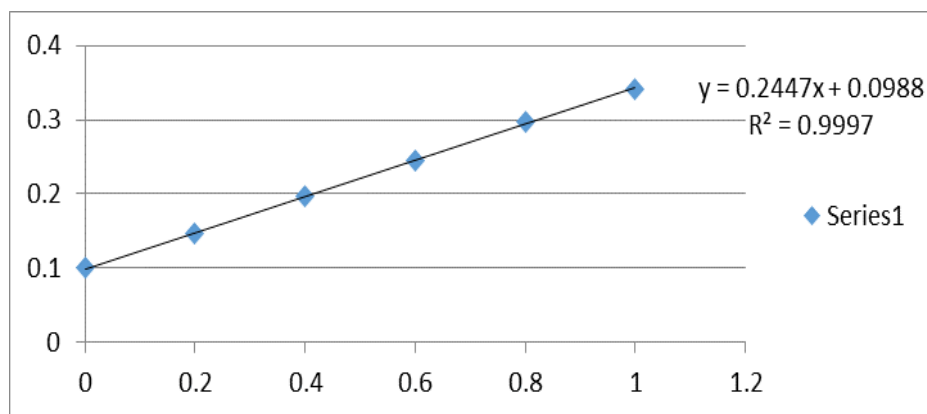
After drying flowers of two plants, 1 g of the dry powder of each plants was homogenized by addition of 100 mL of methanol solution and shaking of the resulting solution for 6 hours. The resulting solution was then poured into a plastic falcon and centrifuged at 6000 rpm for 10 minutes. The resulting solution was used as a sample.

Stock solution preparation

2.2 mL of R2b solution was added to the parent bottle R2a and vortexed until complete dissolution and R2 solution was obtained. Then, the R2 solution was mixed in a ratio of 1:1 and after vortexing, was added to R1 solution in a ratio of 5:1. The resulting solution served as the stock solution of the antioxidant assay kit.

Standard solution preparation

The standard solution at 0, 0.2, 0.4, 0.6, 0.8, and 1 was also prepared. The linear equation obtained from the standard solution at different concentrations is displayed in Figure 1.



Conc.

Figure 1. Linear equation obtained from standard solution at different concentrations

$$Y = 0.2447x + 0.0988, R^2 = 0.9997$$

Procedure

First, 5 μL of the prepared plant solution was added to each well, and then 250 μL of the working solution was added to each well containing the plant solution. The microplate was then incubated at 35-50 $^{\circ}\text{C}$ for 30 minutes, and finally it was read at 570 nm with the ELISA reader.

Results

In this study, HS-SPME was performed to extract the essential oils of *A. officinalis* and *A. italica*. Likewise, the essential oil analysis of the two medicinal plants was conducted by using the GC-MS. The results of GC-MS showed that *A. officinalis* contains 39 chemical compounds including tetradecane (22.54%), alpha-pinene (15.50%), hexadecane (10.46%), 2-hexenal (8.48%),

dodecane (7.28 %), β -ionone (3.50%), and trans-geranylacetone (3.26%) (Table 2).

Figure 2 and Table 3 demonstrate the chromatograms of *A. officinalis* functional groups. The plant has 15 peaks.

The GC-MS results showed that *A. italica* contains 42 chemical compounds including trans-caryophyllene (13.26%), allospathulenol (11.27%), germacrene D (10.47%), bicyclogermacrene (9.77%), safranal (7.62%), δ -cadinene (6.42%), α -pinene (4.38%), α -copaene (3.96%), and β -myrcene (3.75%) (Table 4).

Figure 3 and Table 5 present the composition chromatogram of *A. officinalis* essential oil. The plant has 12 peaks.

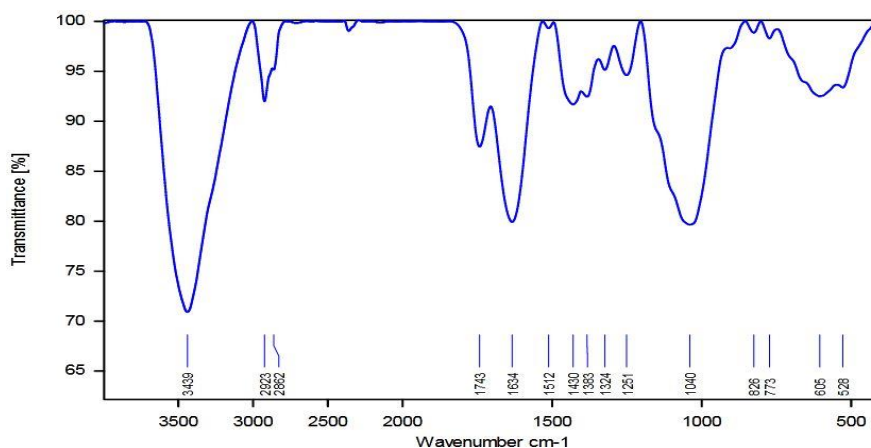


Figure 2. Chromatogram of *Althaea officinalis* L.

Table 2. Identified compounds of *Althaea officinalis* L. essential oil by headspace-solid phase microextraction/gas chromatography-mass spectrometry

Retention time	Compound	Area	KI	%
4.925	2-Hexenal	9387236	930	8.48
6.669	α -Pinene	1544025	938	1.40
8.298	β -Myrcene	1701546	990	1.54
9.561	<i>dl</i> -Limonene	2898658	1046	2.62
12.018	Nonanal	1604956	1104	1.45
13.578	Decamethyl- Cyclopentasiloxane	1926425	1123	1.74
13.761	Menthone	1252023	1138	1.13
14.533	Menthol	731367	1150	0.66
15.19	Dodecane	8057024	1178	7.28
15.487	Decanal	899104	1206	0.81
16.345	<i>Z</i> -3-hexenyl isopentanoate	1405280	1232	1.27
18.459	Menthyl acetate	1268828	1285	1.15
18.557	Tridecane	1146098	1300	1.04
19.482	<i>Z</i> -3-hexenyl tiglate	1876078	1315	1.70
20.311	5-Methyltridecane	2007364	1342	1.81
20.843	2-Methyltetradecane	1419047	1369	1.28
21.666	Cyclotetradecane	2356423	1386	2.13
21.774	Tetradecane	24943030	1400	22.54
22.46	Trans-Caryophyllene	3090965	1419	2.79
22.877	α -Zingiberene	384241	1436	0.35
23.415	Trans-Geranylacetone	3605037	1358	3.26
23.557	Pentatriacontane	1353364	1371	1.22
23.803	7-n-Hexyldocosane	792208	1385	0.72
24.398	β -Ionone	3876745	1392	3.50
24.615	Pentadecane	1532376	1500	1.38
25.369	δ -Cadinene	1009004	1532	0.91
25.752	Dihydroactinidiolide	1414986	1546	1.28
25.832	Methylundecane	1926047	1568	1.74
25.986	5-Methylpentadecane	2229475	1579	2.01
26.455	3-Methylpentadecane	1780320	1585	1.62
27.192	Hexadecane	11574562	1600	10.46
28.358	Phytane	1650986	1710	1.49
28.558	Lanol	575342	1769	0.52
29.656	3-Cyclohexen-1-ol, 3-methyl-	2711086	1910	2.45
31.37	Octadecane	1132035	2020	1.02
31.485	Tritetracontane	943789	2100	0.85
31.965	Tetrahydrogeranylacetone	1255038	2122	1.13
33.153	Dibutyl phthalate	1380405	2154	1.25

Table 3. *Althaea officinalis* L. functional groups in Fourier-transform infrared spectroscopy

Wavelength	Factor group	Range	Type
3439	O-H	3550-3200	Stretching - Alcohol
2923	N-H	3330-3250	Stretching - Alcohol
2862	N-H	3000-2800	Tensile - Amine salt
1743	C=O	1770-1780	Tensile - vinyl / phenyl ester
1634	C=C	1662-1626	Tensile - Alken
1512	N-O	1550-1500	Tensile - Nitro composition
1430	O-H	1440-1395	Bending - Carboxylic acid
1383	S=O	1415-1380	Tensile - Sulfate
1324	O-H	1390-1310	Bending - Phenol
1251	C-O	1275-1200	Tensile - Aromatic ester
1040	C-O	1075-1020	Stretch - Vanilla Ether
826	C-Cl	850-550	Tensile - Halide composition
773	C-Cl	850-550	Tensile - Halide composition
605	C-Br	690-515	Tensile - Halide composition
528	C-I	600-500	Tensile - Halide composition

Table 4. Identified compounds of *Anchusa italica* Retz. essential oil by headspace-solid phase microextraction/gas chromatography–mass spectrometry

Retention time	Compound	Area	KI	%
6.388	α -Thujene	40228932	922	0.78
6.629	α -Pinene	226645613	928	4.38
7.72	Sabinene	57068792	969	1.10
7.823	β -Pinene	123756369	973	2.39
8.269	β -Myrcene	194236836	987	3.75
8.663	1-Phellandrene	17411736	992	0.34
8.823	Δ -3-Carene	19444062	1013	0.38
9.349	p-Cymene	10108986	1022	0.20
9.509	Safranal	394230206	1032	7.62
9.743	<i>Cis</i> -Ocimene	64270983	1045	1.24
10.424	γ -Terpinene	8678774	1082	0.17
10.852	Trans-Sabinene hydrate	23529116	1092	0.45
11.967	Linalool	46106333	1120	0.89
14.63	4-Terpineol	30769965	1182	0.59
15.179	α -Terpineol	67769632	1196	1.31
16.367	Nerol	5798145	1225	0.11
19.374	Carvacrol	41041352	1302	0.79
19.837	Bicycloelemene	19580785	1328	0.38
20.248	α -Cubebene	9660568	1345	0.19
21.145	α -Copaene	204733623	1366	3.96
21.414	β - Bourbonene	20822562	1382	0.40
21.654	β - Elemene	24204526	1387	0.47
22.591	<i>Trans</i> -Caryophyllene	685772236	1419	13.26
23.534	<i>Trans</i> -.beta.-Farnesene	196566397	1448	3.80
24.357	Germacrene- <i>D</i>	541863238	1481	10.47
24.466	Aromadendrene	85278608	1489	1.65
24.769	Bicyclogermacrene	505238789	1494	9.77

24.992	β -Bisabolene	65803532	1516	1.27
25.289	β -selinene	43394526	1548	0.84
25.443	δ -Cadinene	356456954	1562	6.89
26.226	Caryophyllene oxide	20518009	1581	0.40
27.049	Allospathulenol	582964229	1618	11.27
27.324	γ -Gurjunene	68172362	1628	1.32
27.964	Alloaromadendrene oxide	24879436	1636	0.48
28.198	Valencene	37462774	1644	0.72
28.444	Isospathulenol	23142357	1654	0.45
28.541	Phytol	75411996	1682	1.46
28.855	<i>t</i> -Muurolol	45118432	1720	0.87
29.627	Vulgarol B	100793745	1811	1.95
31.124	Tetradecanoic acid	36694625	1846	0.71
31.719	Isopropyl myristate	28004475	1888	0.54

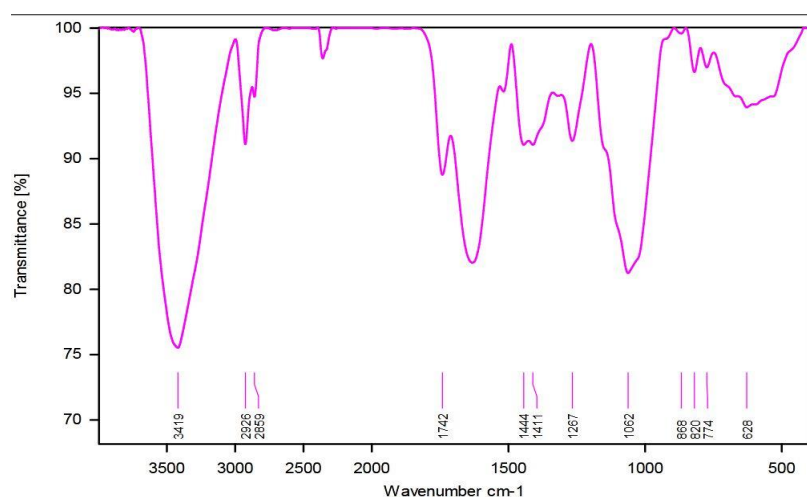


Figure 3. Chromatogram of *Anchusa italica* Retz. essential oil

Table 5. The Fourier-transform infrared spectroscopy results for *Anchusa italica* Retz.

Wavelength	Factor group	Range	Type
3419	O-H	3550-3200	Stretching - Alcohol
2926	C-H	3000-2840	Stretching - Alkan
2859	C-H	3000-2840	Stretching - Alkan
1724	C=O	1740-1720	Stretching - Alloid
1444	C=C	1648-1638	Tensile - Alken
1411	O-H	1440-1395	Bending - Carboxylic acid
1267	C-O	1275-1200	Tensile - Aromatic ester
1062	C-O	1075-1020	Stretching - Alkyl Aryl Ether
868	C-H	880±20	Bending
820	C-Cl	850-550	Tensile - Halide composition
774	C-Cl	850-550	Tensile - Halide composition
628	C-Br	690-515	Tensile - Halide composition

Recently, to eliminate or reduce chemical and synthetic compounds in food, much research has been done to replace them with natural compounds. In this regard, many efforts have been made to detect natural antioxidants in the plant sources. In this study, total antioxidant

capacity was measured by using the FRAP and with a single-electron transfer via Naxifer™ kit. The results regarding the total antioxidant capacity of the two studied plants are presented in Table 6.

Table 6. Total antioxidant capacity of *Althaea officinalis* L. and *Anchusa Italica* Retz.

Medicinal plant	Total antioxidant capacity
<i>Althaea officinalis</i> L	mmol Fe ²⁺ /L 1.2
<i>Anchusa Italica</i> Retz.	mmol Fe ²⁺ /L 2.9

Discussion

According to the available evidence, no study has been done on the essential compounds of *A. officinalis* and *A. italica* native to Ilam. A study on the volatile compounds of *Borago officinalis* L. identified 16 chemical compounds including NND-methylethanolamine, 3-6-gluconuro-methyl beta-glycoside, 4-methyl benzaldehyde, 3-hydroxy-tetrafurazane, hexadecanoic acid, heptanoic acid, gamma-butyrolactone, and ethyl octadec-9-enoate [28]. The study of Ghenaet *et al.* (2005) showed that borage contains 28 chemical compounds such as spathulenol, α -pinene, and germacrene D [29].

Phytochemical analysis of the wild borage seeds has revealed that they are rich in saturated fatty acids, unsaturated fatty acids, and alkaloids [30]. Nonacosan (11.2%), heptacosan (9.3%), henicosan (9%), and pentacosan (3.7%) are the most important fatty acids in the hexane extract of marshmallow [31]. Another possible reason for differences in the type and percentage of chemical compounds of marshmallow in the two studies is the use of different parts of these plants. In the above-cited study, flower and seed parts of marshmallow were used, while in our study, the whole plant was examined. Our results differ from the results of other studies in composition, the number of compounds, and the amount of chemical compounds.

A. italica contains 42 compounds including trans-caryophyllene, allospathulenol, germacrene D, bicyclogermacrene, safranal, δ -cadinene, α -

pinene, α -copaene, and β -myrcene. One reason could be the climate and soil of Ilam province [32]. The essential compounds are found in various organs of aromatic medicinal plants and are not chemically homogeneous [33,34].

Studies have indicated that natural compounds and antioxidants in fruits and vegetables are effective in the prevention and treatment of many diseases [35]. Antioxidants are commonly found in medicinal plants that contain phenolic, flavonoids, and flavones [36]. The use of different methods to isolate chemical compounds is one of the important factors for the differences in the identified chemical compounds [37]. However, the techniques used in our study are among the newest and most optimal methods to isolate chemical compounds.

There are so many antioxidant compounds that their identification is difficult. Therefore, the antioxidant capacity of plants should be determined by various measures [34]. Compounds such as tocopherols, phenols, glutathione, carotenoids, and ascorbic acid are the most important natural antioxidants [38]. The secondary metabolites of plant tissues such as flavonols, flavonoids, glycosides, polyacetylenes, and alkaloids can produce therapeutic effects [39]. Synthetic antioxidants are widely used, but they may have detrimental health effects [40,41]. Since herbs are an important and abundant source of natural antioxidants, they do not have the side effects of synthetic antioxidants [42,43]. The total

antioxidant capacity of plants is related to the amount and type of their antioxidant compounds such as phenols, flavonoids, ascorbic acid, and carotenoids [44]. In fact, antioxidants inhibit the activity of free radicals and/or eliminate them [45]. *A. italica* leaf extract can remove the reactive oxygen species and DPPH radicals [46]. The medicinal plants comprise one of the most important bases of complex systems of traditional medicine that have existed for thousands of years [47,48] and continue to provide new treatments to mankind [49,50] mainly due to containing natural antioxidants [51,52].

Conclusion

Our results showed that the two studied medicinal plants have a wide range of chemical compounds such as phenols, flavonoids, alkaloids, polysaccharides, mucilage, and saponin with potential antioxidant properties. Therefore, given the antioxidant properties of these plants, this research can be a starting point for the additional experiments regarding the use of essential oils and extracts of these two medicinal plants. Taken together, the potential of these two plants, as two important medicinal plants occurring in Iran, for use in traditional medicine, modern medicine, and industries can be promising with respect to their trade and cultivation, and can stimulate comprehensive research in their pharmaceutical, cosmetic, and medical applications in clinical pharmacy so that they would be introduced into the booming and popular market of medicines of natural origin.

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Conflict of interest

None of the authors have any conflict of interest.

Consent for publications

All authors approved the final manuscript for publication.

Availability of data and material

Data are available on request from the authors.

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