

Investigation of chemical keys for relationship between plants and their unifloral honeys by hydrodistillation and SPME and biological activities of honeys

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Abstract Volatile compounds of unifloral honeys and their plants essential oils obtained by hydrodistillation (EOH) and solid phase micro extraction (EOS) methods were investigated and compared with each other for the first time. The results exhibited presence of volatile compounds of plant such as *o*-cymene and carvacrol in *Thymus*, *cis*-linalool oxide in *Citrus*, aliphatic hydrocarbons in *Citrus* and *Astragalus* and hexadecanoic acid (palmitic acid) in *Astragalus* and *Medicago* honeys. The amounts of terpenes were decreased by increasing molecular weight in EOS, while this pattern does not occur in EOH. Total phenols and antioxidant activities were increased from *Citrus* to *Thymus* honey (*Citrus* < *Medicago* < *Astragalus* < *Thymus*). In antibacterial assay, *Thymus* honey showed the most potential inhibition to all the experimented strains of bacteria. Increase in phenol content may be an effective factor for antibacterial activity of honey against *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*. It seems that active compounds with antioxidant properties were responsible for growth inhibition effect on *E. coli* and *S. aureus*.

Keywords Floral origin honeys · Volatile oils · Total phenol · FRAP antioxidant activity · Antibacterial assay · SPME

Introduction

Honey is one of the oldest medicinal foods with great variability of taste and flavor. The properties of honey are associated with its botanical origin, phenol compounds, minerals, proteins, amino acids, enzymes and vitamins [1]. The aroma profile of unifloral honeys represent of nectar origin, processing and storage conditions [2].

Applying hydrodistillation for isolation of honey volatiles generates artifacts because of the effects of heat. Solid phase microextraction (SPME) is a method which does not need too high temperature and can isolate selectively head-space volatiles [3].

During the past decade, several therapeutic effects of honey such as antibacterial, anti-inflammatory and antitumor properties have been identified [4, 5]. Recent views proposed honey as a valuable dietary source of antioxidants similar to many fruits and vegetables [6]. Several studies have demonstrated a strong correlation between the content of phenolic compounds in different botanical honeys and their antioxidant and antibacterial capacities [6–9].

There are very few data about composition, total phenol, antioxidant and antibacterial activities of Iranian nectar honeys. The aim of this work is comparison of volatiles of unifloral honeys with essential oils of related plants by two methods of hydrodistillation and SPME. In addition, total phenol, antioxidant and antibacterial activities of various botanical honeys were determined.

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Materials and methods

Chemicals

Vitamin E 97 % was achieved from Sigma-Aldrich Chemie GmbH, Germany. Butylatedhydroxylanizole (BHA), sodium acetate, 2,4,6-tripyridyl-s-triazine (TPTZ), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, Folin-Ciocalteu phenol reagent and HCl were purchased from Merck, Germany.

Plant and honey materials

The flowers of *Citrus orientalis* were collected in May 2012 from Mazandaran Province, and the top flowered aerial parts of *Thymus* spp, *Astragalus* spp and *Medicago* spp were collected in August 2012 from Teharan Province of Iran. The plants were dried in shade and powdered separately. The unifloral honeys were prepared from honey producers in the same origins.

Bacteria and media

The various bacteria including *S. aureus* ATCC6538 and *B. subtilis* ATCC 6633 as Gram-positive and *Pseudomonas aeruginosa* ATCC9027 and *E. coli* ATCC8739 as Gram-negative bacteria were obtained from Department of Drug and Food Control, Faculty of Pharmacy, Tehran University of Medical Sciences. Soybean Casein Digest Agar (Merck, Germany) was used as medium for the growth of bacterial strains.

Isolation of essential oil by hydrodistillation

The flowers of *Citrus* and top flowered of the other plants were separately subjected to hydrodistillation for 4 h using a Clevenger-type apparatus. The oils were collected separately, dried on anhydrous sodium sulfate and kept in refrigerator for further investigations.

SPME procedure

A manual SPME holder and polydimethylsiloxane (PDMS) fiber were purchased from Supelco (Bellefonte, USA). The fiber was exposed at 250 °C for 5 min in GC injector prior to use in order to remove contaminants. One gram of powdered plants and 10 g of honey samples were placed into a 20-ml glass bottle sealed with septum caps separately. Then, 0.5 g of anhydrous sodium sulfate was added to honey samples.

For each extraction, the samples were heated at 70 °C for 30 min, and then, the fiber was extended through the SPME needle. After extraction time (20–30 min), the SPME fiber was withdrawn from the vial and extracted analytes on the

fiber were desorbed at GC injector and then analyzed by GC/MS.

GC/MS analysis

Essential oil analyses were performed using a Hewlett Packard 6890 gas chromatograph system coupled with a HP 5973 mass spectrometer. The compounds were separated on a HP-5MS capillary column (30 m × 0.25 mm, film thickness 0.25 μm). For both distillation and SPME samples, split ratio was 10 ml/min and the thermal program was 40 °C (5 min) to 250 °C at 3 °C/min, and then was stable at 250 °C (10 min). Injector and detector temperatures were 250 °C. The flow rate of helium as carrier gas was 1.2 ml/min. The ionizing energy was 70 eV. The percentage compositions of the identified compounds were computed from the GC peak areas.

The compounds were identified by comparison of their mass spectra with the Wiley library or with published data and comparison of their Kovats indices (KI) [10, 11].

Total phenol assay (Folin-Ciocalteu)

The determination of phenol content of honeys was performed according to the Folin-Ciocalteu method with slight modifications [12]. Honey samples were diluted with distilled water and filtered separately. 0.2 ml of each sample (100 μg/ml) was mixed with 2.5 ml of Folin-Ciocalteu reagent (1:10 diluted with distilled water), and after 5 min, 2 ml of Na_2CO_3 solution (75 g/l) was added. The absorbance of mixtures was measured at 760 nm after incubation at room temperature for 2 h. Gallic acid (0–100 μg/ml) is used as standard to produce the calibration curve. Total phenol content was expressed as mg of gallic acid equivalents GAE/100 g of honey. All determinations were performed in triplicate.

Ferric reducing antioxidant power assay (FRAP)

Total antioxidant capacity was determined using FRAP procedure with adjustment for analysis of honey [13]. Fifty microliters of each aqueous honey solution (100 μg/ml) was added to 1.5 ml of freshly prepared FRAP reagent. The FRAP reagent was prepared by mixing 2.5 ml of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl with 2.5 ml of 20 mM FeCl_3 and 25 ml of 0.3 M acetate buffer, then pH adjusted to 3.6. After 10 min incubation at 37 °C, the absorbance was measured at 593 nm against the blank of FRAP reagent. A standard aqueous solution of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (in a range of 125–1,000 μmol/l) was prepared to obtain calibration curve. The results from triple replicate were expressed as mmol Fe^{2+} /100 g of honeys.

Antibacterial assay

The antibacterial activity of the different floral origin honeys was studied by cup-plate diffusion method as described by Warnock DW [14]. Each organism was separately suspended in normal saline solution, which was equal to 10^8 CFU/ml. For preparing base plates, 25 ml of cooled media was poured into the sterile Petri dishes and inoculated with one of the microorganisms by spreading microbial suspension over the plate with a sterile cotton swab. Then in each plate, holes of 7 mm in diameter were made at equal distances using sterile cork borer. Different concentrations of honey aqueous solutions were prepared. Hundred microliters of each honey was added to each hole on the medium, and all plates were incubated at 35 °C for 24 h. The diameter of zone of inhibition (mm) and minimum inhibitory concentration (MIC) was measured after incubation time and compared with gentamycin (5 mg/ml) as positive control.

Results and discussion

Honeys and plants volatiles

Four different botanical honey samples were studied in terms of investigation of volatile components and biological activities. The chemical composition of plants essential oils was determined by hydrodistillation (EOH) and SPME (EOS) methods and compared to related honeys. The amount of compounds is expressed as percentage of the obtained peak area, compared with total area of all peaks in the chromatograms.

Table 1 demonstrated volatile compounds of plants, which were origin of botanical honeys.

The *Thymus* EOH contained monoterpenes (87.94 %), sesquiterpenes (10.46 %), nonterpenes (0.71 %) and phenyl propanoids (0.18 %), which were similar to *Thymus* EOS sample included monoterpenes (88.69 %), sesquiterpenes (11.11 %) and nonterpenes (0.38 %). According to the results, two methods of extraction of *Thymus* essential oils gave approximately same compounds. The similar major volatile components were α -thujene, α -pinene, camphene, β -pinene, β -myrcene, phellandrene, *o*-cymene, thymol and carvacrol. The abundant compounds of EOH were carvacrol (18.7 %) and thymol (12.66 %), and the predominant volatiles of EOS were *o*-cymene (26.21 %) and carvacrol (15.37 %). Aromatic profiles of *Thymus hyemalis* and Spanish *T. vulgaris* essential oils showed that linalool, borneol, thymol, carvacrol and β -damascenone were the compounds with most aroma impact for *T. hyemalis*, while thymol, carvacrol, 1,8-cineol, eucalyptol, borneol, terpinyl acetate and β -damascenone were abundant for *T. vulgaris* [15].

Previous Investigation proposed 1,3-diphenyl-2-propanone, (3-methylbutyl) benzene, 3,4,5-trimethoxy benzaldehyde, 3,4-dimethoxy benzaldehyde, vanillin and thymol as markers for *Thymus capitatus* honey [16]. In our experiment, it was exhibited that *o*-cymene and carvacrol were existed in both *Thymus* essential oils and related honey.

Comparison of essential oils of *Citrus* flowers showed that the amount of nonterpenes in EOH was more than EOS. On the other hand, the monoterpenes content of EOS was considerable. In detail, *Citrus* EOH contained monoterpenes (30.09 %), sesquiterpenes (3.26 %) and nonterpenes (52.67 %) while EOS included monoterpenes (85.34 %), sesquiterpenes (12.09 %) and nonterpenes (1.39 %). The identical compounds in both *Citrus* EOH and EOS were α -pinene, β -myrcene, limonene, benzene acetaldehyde, trans- β -ocimene and linalool. The GC analysis proved that the predominant compounds of *Citrus* EOH were hexadecanoic acid (palmitic acid) and linalool (25.53 and 19.04 %, respectively), while limonene was major component of *Citrus* EOS (67.18 %). Previous analysis of flowers volatile extracts of four *Citrus* species was showed linalool as predominant compound [17]. *Cis*-linalool oxide and aliphatic hydrocarbons such as tetradecane, hexadecane, heptadecane, nonadecane and heneicosane were similar in *Citrus* honey and flowers volatiles. Recent studies reported linalool, (*Z*, *E*)-linalool oxide, α -terpineol, terpinene and isomers of lilac aldehyde and lilac alcohol as considerable markers for *Citrus* honey [18, 19]; among them, existence of *cis*-linalool oxide (2.11 %) in our tested honey confirmed previous results.

The deliberation on *Astragalus* volatile oils demonstrated the existence of phytol as abundant part of EOH (17.6 %). It was obvious from results, EOH included monoterpenes (22.41 %), sesquiterpenes (3.89 %), diterpenes (17.6 %), miscellaneous compounds of terpenoid origin (1.08 %) and nonterpenes (44.65 %). The EOS contained monoterpenes (12.67 %), sesquiterpenes (12.22 %) and nonterpenes (47.53 %). Hexanal, 1-hexanol, octanal, nonanal and hexadecane as aliphatic hydrocarbons; *cis*-3-hexenyl benzoate as ester; hexadecanoic acid as saturated fatty acid, and trans- β -caryophyllene and β -bisabolene as sesquiterpenes were the same compounds in *Astragalus* EOH and EOS. Alcohols and aldehydes, especially saturated and unsaturated C6 volatile constituents, provide a defense mechanism against mechanical damage [20]. It was interesting that carvacrol (16.55 %), decanal (14.16 %) and hexadecanoic acid (12.64 %) were principal portion of *Astragalus* EOH, but nonanal (10.31 %) was the chief compound of EOS. Comparison of *Astragalus* volatiles exhibited existence of nonanal, decanal, tetradecane, pentadecane and hexadecanoic acid in both honey and essential oils. It was the first report for composition of *Astragalus* essential oil and its honey.

Table 1 Volatile composition of plants (origin of honeys) by hydrodistillation and SPME

No.	Compounds	KI	T EOH (%)	T EOS (%)	C EOH (%)	C EOS (%)	A EOH (%)	A EOS (%)	M EOH (%)	M EOS (%)	Methods of Identification
1	Hexanal	801	–	–	–	–	1.44	0.88	0.74	8.3	MS-KI
2	2-Hexenal	846	–	–	–	–	–	–	2.64	6.16	MS-KI
3	3-Hexen-1-ol, (Z)	850	–	–	–	–	–	–	1.18	–	MS-KI
4	2-Hexen-1-ol, (E)	854	–	–	–	–	–	–	0.59	–	MS-KI
5	1-Hexanol	863	–	–	–	–	0.79	0.57	3.32	–	MS-KI
6	Nonane	900	–	–	–	–	1.01	–	–	–	MS-KI
7	Heptanal	901	–	–	–	–	0.18	–	–	–	MS-KI
8	α -Thujene	924	4.94	6.1	–	–	–	–	–	–	MS-KI
9	α -Pinene	932	4.54	7.25	0.12	0.14	–	–	–	–	MS-KI
10	Camphene	946	2.23	6.02	–	–	–	–	–	–	MS-KI
11	Sabinene	969	0.66	–	–	–	–	–	–	–	MS-KI
12	β -Pinene	974	0.73	1.69	0.77	–	–	–	–	–	MS-KI
13	1-Octen-3-ol	974	–	–	–	–	–	–	8.95	–	MS-KI
14	3-Octanone	979	–	–	–	–	–	–	1.3	15.4	MS-KI
15	β -Myrcene	988	6.03	1.84	0.2	0.73	–	–	–	–	MS-KI
16	3-Octanol	988	0.47	–	–	–	–	–	0.95	–	MS-KI
17	Octanal	998	–	–	–	–	0.98	0.29	–	–	MS-KI
18	Phellandrene	1,002	0.46	0.21	–	–	–	–	–	–	MS-KI
19	3-Hexen-1-ol, acetate, (Z)	1,004	–	–	–	–	–	–	0.74	–	MS-KI
20	δ -3-Carene	1,008	0.2	–	–	–	–	–	–	–	MS-KI
21	α -Terpinene	1,014	2.71	–	–	–	–	–	–	–	MS-KI
22	<i>o</i> -Cymene	1,022	7.55	26.21	–	–	0.52	–	–	–	MS-KI
23	Limonene	1,024	–	2.85	0.96	67.18	–	0.16	–	–	MS-KI
24	β -Phellandrene	1,025	1.46	–	–	–	–	–	–	–	MS-KI
25	1,8-Cineol	1,032	3.13	0.49	–	–	–	–	–	–	MS-KI
26	<i>cis</i> - β -Ocimene	1,032	–	–	–	2.85	0.71	–	–	–	MS-KI
27	Benzeneacetaldehyde	1,036	–	–	0.2	0.42	–	–	–	–	MS-KI
28	<i>trans</i> - β -Ocimene	1,044	–	–	0.43	0.12	–	0.23	–	–	MS-KI
29	γ -Terpinene	1,054	8.13	3.25	–	0.04	0.83	–	–	–	MS-KI
30	Acetophenone	1,059	–	–	–	–	1.1	–	–	–	MS-KI
31	1-Octanol	1,063	–	–	–	–	0.4	–	–	–	MS-KI
32	<i>cis</i> -Sabinene hydrate	1,065	2.53	2.32	–	–	–	–	–	–	MS-KI
33	<i>cis</i> -Linaloloxide	1,067	–	–	0.2	–	–	–	–	–	MS-KI
34	α -Terpinolene	1,086	0.38	0.78	–	–	–	–	–	–	MS-KI
35	Linalool	1,095	0.9	1.08	19.04	13.76	–	10.31	2.61	–	MS-KI
36	Nonanal	1,100	0.24	0.12	–	–	4.22	1.83	0.6	–	MS-KI
37	Phenethyl alcohol	1,106	–	–	–	0.06	–	–	–	–	MS-KI
38	Fenchyl alcohol	1,118	–	0.22	–	–	–	–	–	–	MS-KI
39	Benzene acetonitrile	1,134	–	–	–	0.46	–	–	–	–	MS-KI
40	Citronellal	1,153	–	–	–	0.44	–	–	–	–	MS-KI
41	Borneoll	1,165	1.76	1.64	–	–	1.71	–	–	–	MS-KI
42	Lavandulol	1,165	–	–	–	–	–	–	0.35	–	MS-KI
43	Octanoic acid (Caprylic acid)	1,167	–	–	–	–	–	–	–	–	MS-KI
44	4-Terpineol	1,174	1.05	1.26	–	–	0.15	–	–	–	MS-KI
45	Benzene acetic acid methyl ester	1,175	–	–	–	0.04	–	–	–	–	MS-KI
46	Naphthalene	1,178	–	–	–	0.02	–	–	–	–	MS-KI
47	α -Terpineol	1,186	–	–	1.33	0.02	–	–	0.34	–	MS-KI

Table 1 continued

No.	Compounds	KI	T EOH (%)	T EOS (%)	C EOH (%)	C EOS (%)	A EOH (%)	A EOS (%)	M EOH (%)	M EOS (%)	Methods of Identification
48	Methyl salicylate	1,190	–	0.26	–	–	0.37	–	0.43	1.6	MS-KI
49	Dodecane	1,200	–	–	–	–	–	0.48	–	–	MS-KI
50	n-Decanal	1,201	–	–	–	–	14.16	4.7	0.42	0.65	MS-KI
51	Nerol	1,230	–	–	0.4	–	–	–	0.2	–	MS-KI
52	Thymol methyl ether	1,232	1.79	0.41	–	–	–	–	–	–	MS-KI
53	Carvacrol methyl ether	1,241	4.04	0.65	–	–	0.22	–	–	–	MS-KI
54	trans-Geraniol	1,249	–	–	–	–	–	–	0.26	–	MS-KI
55	Linalyl acetate	1,257	–	–	5.18	–	–	–	–	–	MS-KI
56	2-Decenal, (E)	1,260	–	–	–	–	0.46	–	–	–	MS-KI
57	Ethyl salicylate	1,266	–	–	–	–	0.58	–	–	–	MS-KI
58	Nonanoic acid (Pelargonic acid)	1,267	–	–	1.02	–	–	–	–	–	MS-KI
59	Thymol	1,289	12.66	9.00	–	–	0.8	–	–	–	MS-KI
60	Carvacrol	1,298	18.7	15.37	–	–	16.55	–	–	–	MS-KI
61	Tridecane	1,300	–	–	0.51	–	–	1.39	–	8.29	MS-KI
62	β -Citronellal	1,312	–	–	–	0.06	–	–	–	–	MS-KI
63	<i>cis</i> -3-Hexenyl tiglate	1,319	–	–	–	–	–	–	0.24	–	MS-KI
64	Eugenol	1,356	0.18	–	–	–	–	–	–	–	MS-KI
65	Neryl acetate	1,359	–	–	0.63	–	–	–	–	–	MS-KI
66	Decanoic acid (Capric acid)	1,364	–	–	1.19	–	–	–	–	–	MS-KI
67	Carvacryl acetate	1,370	1.36	–	–	–	–	–	–	–	MS-KI
68	Geranyl acetate	1,379	–	–	0.83	–	–	–	–	–	MS-KI
69	β -Bourbonene	1,387	–	0.46	–	–	–	–	9.79	–	MS-KI
70	Tetradecane	1,400	–	–	0.49	–	–	8.87	–	–	MS-KI
71	<i>cis</i> - α -Bergamotene	1,411	–	–	–	0.1	–	–	–	–	MS-KI
72	β -Funebrene	1,413	–	–	–	–	0.35	–	–	–	MS-KI
73	trans-Caryophyllene	1,417	3.88	4.69	–	2.87	0.45	–	18.09	–	MS-KI
74	β -Gurjunene	1,431	–	0.45	–	–	–	–	–	–	MS-KI
75	trans- α -Bergamotene	1,432	–	–	–	2.05	–	–	–	–	MS-KI
76	α -Humulene	1,452	–	–	–	0.31	–	–	3.31	–	MS-KI
77	trans-Geranylacetone	1,453	–	–	–	–	0.92	–	–	–	MS-KI
78	trans- β -Farnesene	1,454	1.01	–	–	0.06	0.47	–	–	–	MS-KI
79	Alloaromadendrene	1,458	–	0.95	–	–	–	–	–	–	MS-KI
80	ethyl E-2-Z-4-decadienoate	1,467	–	–	–	–	–	4.59	–	–	MS-KI
81	Germacrene D	1,484	–	–	–	0.47	–	–	7.6	–	MS-KI
82	trans- β -Ionone	1,487	–	–	–	–	1.08	–	–	6.09	MS-KI
83	β -Selinene	1,489	–	0.5	–	–	–	–	–	–	MS-KI
84	Pentadecane	1,500	–	–	–	–	–	7.26	–	–	MS-KI
85	Bicyclogermacrene	1,500	–	–	–	–	–	–	6.36	–	MS-KI
86	β -Bisabolene	1,505	1.26	1.89	–	4.97	0.67	2.7	–	–	MS-KI
87	(E,E)- α -Farnesene	1,505	–	–	–	–	–	–	–	3.58	MS-KI
88	<i>cis</i> - α -Bisabolene	1,506	0.7	–	–	0.24	–	–	–	–	MS-KI
89	γ -Cadinene	1,513	0.71	0.81	–	–	–	–	1.72	–	MS-KI
90	δ -Cadinene	1,522	–	0.08	–	0.32	–	–	–	–	MS-KI
91	<i>cis</i> -Calamenene	1,528	–	0.56	–	–	–	–	–	–	MS-KI
92	phenol, 2-4-bis[1,1 dimethyl-ethyl]	1,536	–	–	2.5	–	–	–	–	–	MS-KI
93	α -Cadinene	1,537	–	0.07	–	–	–	–	–	–	MS-KI

Table 1 continued

No.	Compounds	KI	T EOH (%)	T EOS (%)	C EOH (%)	C EOS (%)	A EOH (%)	A EOS (%)	M EOH (%)	M EOS (%)	Methods of Identification
94	trans-Nerolidol	1,561	–	–	1.82	0.62	4.64	6.15	0.5	–	MS-KI
95	Dodecanoic acid (Lauric acid)	1,565	–	–	1.34	–	–	–	–	–	MS-KI
96	3-Hexen-1-ol, benzoate, (Z)	1,565	–	–	–	–	–	–	–	1.07	MS-KI
97	Caryophyllene oxide	1,570	–	0.47	–	–	–	–	–	–	MS-KI
98	Spathulenol	1,577	0.31	–	–	0.25	–	–	2.08	–	MS-KI
99	Caryophyllene oxide	1,582	0.4	–	–	0.11	–	–	2.28	–	MS-KI
100	Hexadecane	1,600	–	–	0.68	–	0.28	7.15	–	–	MS-KI
101	Benzophenone	1,626	–	–	0.36	–	–	–	–	–	MS-KI
102	Bicyclosquiphellandrene	1,645	1.91	–	–	–	–	–	–	–	MS-KI
103	β -Eudesmol	1,649	–	–	–	0.53	–	–	–	–	MS-KI
104	α -Cadinol	1,652	0.15	–	–	–	–	–	–	–	MS-KI
105	Tetradecanol	1,671	–	–	0.76	–	–	–	–	–	MS-KI
106	α -Bisabolol	1,685	0.13	2.76	–	–	–	–	–	–	MS-KI
107	Heptadecane	1,700	–	–	0.57	0.11	–	–	–	1.33	MS-KI
108	(2Z,6E)-Farnesol	1,722	–	–	–	–	0.57	–	–	–	MS-KI
109	(2E,6E)-Farnesol	1,742	–	–	1.44	–	–	–	–	–	MS-KI
110	Benzyl benzoate	1,759	–	–	0.54	–	1.4	–	0.23	–	MS-KI
111	Tetradecanoic acid	1,768	–	–	3.72	–	–	–	–	–	MS
112	Hexadecanal (Palmitaldehyde)	1,794	–	–	–	–	–	–	0.95	2.43	MS
113	Octadecane	1,800	–	–	0.7	–	–	–	–	–	MS-KI
114	Pentadecanoic acid	1,866	–	–	1.71	–	–	–	–	–	MS
115	Nonadecane	1,900	–	–	–	0.07	–	–	–	–	MS-KI
116	Methyl hexadecanoate	1,921	–	–	–	0.21	–	–	–	9.22	MS-KI
117	Phytol	1,942	–	–	–	–	17.6	–	11.91	–	MS-KI
118	Hexadecanoic acid (Palmitic acid)	1,959	–	–	25.53	–	12.64	3.37	5.29	–	MS-KI
119	Heneicosane	2,100	–	–	0.77	–	–	–	–	–	MS-KI
120	Octadecenoic acid (Oleic acid)	2,141	–	–	2.61	–	–	–	–	–	MS-KI
121	Octadecanoic acid (Stearic acid)	2,165	–	–	9.85	–	–	–	–	–	MS
122	Pentacosane	2,500	–	–	0.12	–	–	–	–	–	MS-KI
123	Heptacosane	2,700	–	–	–	–	–	–	–	0.73	MS-KI

EOH essential oil by hydrodistillation, EOS essential oil by SPME, T: *Thymus*, C: *Citrus*, A: *Astragalus*, M: *Medicago*

Investigation of *Medicago* essential oils demonstrated that EOH contained oxygenated monoterpenes (3.76 %), sesquiterpenes (51.73 %), diterpenes (11.91 %) and nonterpenes (28.57 %). In EOS, 64.85 % of all peaks were identified including hydrocarbon sesquiterpenes (3.58 %), miscellaneous compounds of terpenoid origin (6.09 %) and nonterpenes (55.18 %). β -caryophyllene (18.09 %) and phytol (11.91 %) of EOH and 3-octanone (15.4 %) and methyl hexadecanoate (9.22 %) of EOS were the most intense aromas which observed. Recent studies reported trans-2-hexenal as the most abundant volatile in all genotypes of *Medicago sativa* [21]. *n*-Hexanal, 2-hexenal, 3-octanone, methyl salicylate, *n*-decanal and hexadecanal

(palmitaldehyde) were the same compounds in *Medicago* EOH and EOS. Previous studies revealed the existence of (Z)-3-hexenol, 1-octen-3-ol, linalool, α -terpineol, nerol, nerolidol, tetradecanoic acid, pentadecanoic acid, palmitoleic acid and phytol in *Medicago rugosa* essential oil [22]. Hexadecanoic acid was the only volatile compound of plant, which existed in the *Medicago* honey.

Table 2 showed identified volatiles of different floral origin honeys and two honeys in which one of them was collected from north of Iran forests and the other was purchased from market. Our investigation demonstrated all types of honey were rich in nonterpenes. Aliphatic hydrocarbons, alcohols and acids were the most part of identified

Table 2 Volatile composition of different types of honey

No.	Compounds	KI	<i>Thymus</i> (%)	<i>Citrus</i> (%)	<i>Astragalus</i> (%)	<i>Medicago</i> (%)	Forest (%)	Market (%)
1	<i>o</i> -Cymene	1,022	2.95	–	–	–	–	–
2	Benzeneacetaldehyde	1,036	–	–	–	–	1.15	–
3	Nonanal	1,100	–	–	0.72	–	2.46	–
4	Octanoic acid (Caprylic acid)	1,167	2.79	–	–	–	–	–
5	<i>cis</i> -Linalool oxide	1,170	–	2.11	–	1.56	–	–
6	dodecane	1,200	–	–	–	–	2.92	0.16
7	Decanal	1,201	–	0.83	1.56	–	1.1	0.65
8	Nonanoic acid (Pelargonic acid)	1,267	11.51	–	2.17	12.09	–	2.58
9	Carvacrol	1,298	2.09	–	–	–	–	–
10	Decanoic acid (Capric acid)	1,364	1.15	–	0.86	–	–	–
11	dodecane,3- methyl	1,367	–	–	–	–	–	1.84
12	tetradecane	1,400	0.46	0.97	0.24	1.58	–	0.71
13	Dodecanal	1,408	–	0.41	–	–	0.27	–
14	1-Dodecanol	1,469	–	–	–	0.88	–	–
15	Pentadecane	1,500	1.4	1.15	0.37	1.22	–	2.01
16	Dodecanoic acid (Lauric acid)	1,565	4.7	–	5.82	4.05	–	–
17	Hexadecane	1,600	0.33	1.28	–	0.4	5.04	1.74
18	Heptadecane	1,700	–	4.79	–	–	2.99	6.03
19	Tetradecanoic acid	1,768	6.37	–	5.62	6.39	–	–
20	Pentadecanoic acid	1,866	3.87	–	3.25	2.32	–	–
21	Nonadecane	1,900	–	12.29	–	–	7.8	5.06
22	9-Hexadecenoic acid	1,901	–	–	8.31	–	–	–
23	Hexadecanoic acid (Palmitic acid)	1,959	26.63	–	27.1	12.94	–	–
24	Eicosane	2,000	–	1.91	0.77	4.02	–	–
25	1-Octadecanol	2,077	–	–	–	–	0.32	–
26	Heneicosane	2,100	0.35	14.01	0.81	–	8.84	–
27	Octadecenoic acid (Oleic acid)	2,141	5.77	–	9.72	–	–	–
28	Octadecanoic acid (Stearic acid)	2,176	2.09	–	4.98	–	–	–
29	Tricosane	2,300	–	–	1.1	–	–	–
30	Pentacosane	2,500	–	–	–	–	–	9.85
31	Octacosane	2,800	–	2.92	–	–	–	–

Table 3 Total phenol and antioxidant activity of various floral origin honeys

Sample	FRAP (mmol Fe ²⁺ /100 g)	Total phenol (GAE/100 g honey)
<i>Citrus</i> honey	59.2 ± 3.58	320.0 ± 0.04
<i>Medicago</i> honey	207.0 ± 2.33	661.0 ± 0.02
<i>Astragalus</i> honey	267.5 ± 2.58	656.5 ± 0.01
<i>Thymus</i> honey	274.0 ± 1.53	658.0 ± 0.08
Market honey	50.0 ± 1.20	263.0 ± 0.03
Vitamin E	313.7 ± 2.17	–
BHA	880.3 ± 6.43	–

– Not examined

components in all honeys. It was so amazing that fatty acids exist as major part of volatiles in floral origin honeys, but there were in a little amount in forest and market honeys.

Hexadecanoic acid was an abundant component of *Thymus*, *Astragalus* and *Medicago* honeys (26.63, 27.1 and 12.94 %, respectively). The existence of octadecenoic acid (oleic acid, 9.72 %) in *Astragalus* honey was considerable. There were reports for existence of noticeable amounts of Hexadecanoic acid and oleic acid in honey as the principle factor for anti proliferative effect in keloid fibroblasts [23, 24]. Nonanoic acid (pelargonic acid) was the second valuable volatile of *Thymus* (11.51 %) and *Medicago* (12.09 %) honeys. Previous studies confirmed the existence of nonanoic acid in floral origin honeys [25]. There was no evidence for existence of fatty acids in *Citrus* honey. The most aromas of *Citrus* honey belong to aliphatic alkan group as heneicosane (14.01 %), although previous studies suggested linalool derivatives as significant proportion of *Citrus* honey [17, 19].

Total phenol content

The results of total phenol content of analyzed honeys were represented in Table 3 and calculated based on gallic acid standard curve ($y = 0.007X$, $R^2 = 0.999$). High content of total phenols was found in the samples (263–658 mg GAE/100 g honey) in comparison with previous researches [26, 27]. The data revealed that phenol content of honeys was dependent on the floral sources of them and increased in following order: *Citrus* < *Medicago* < *Astragalus* < *Thymus*.

Total antioxidant assay

Table 3 showed total antioxidant capacity of honeys by their ability to reduce the Fe^{3+}/Fe^{2+} couple. The antioxidant activity was calculated by plotting the standard curve of $FeSO_4$ ($y = 0.001x + 0.049$, $R^2 = 0.925$). The greatest reducing capacity was belonged to *Thymus* honey (274 mol Fe^{2+}), which was comparable with Vitamin E (313 mol Fe^{2+}). In contrast, *Citrus* honey (59.2 mol Fe^{2+}) showed the lowest antioxidant activity as same as market honey (50 mol Fe^{2+}).

Antibacterial assay

The agar well-diffusion method was used to ascertain antibacterial effects of different botanical honeys, and the results are represented in Table 4. Differences between inhibition zones were observed in four types of honey. In concentration of 60 % w/v, the *Thymus* honey exhibited the largest inhibition to all strains with an overall

mean diameter of 11.75 mm, followed by the *Medicago* (9.12 mm), *Citrus* (8.75 mm) and finally *Astragalus* honey (8.5 mm). The previous evaluation of inhibition activity against pathogenic bacteria demonstrated that the mean of zone diameter of *Thymus* and *Citrus* honeys was 7.7 and 6.4 mm, respectively [28]. The zone diameter of *Thymus*, *Citrus* and *Astragalus* honeys (60 % w/v) against *E. coli* was more than gentamycin as positive control, which means they have potent antibacterial activities. The *Thymus* honey showed the most antibacterial activity against *E. coli* with lowest MIC (195 $\mu\text{g/ml}$). All of the floral origin honeys inhibited the growth of *Ps. aeruginosa* with narrow inhibition zone diameter of 0–10 mm.

Correlations

Correlations between total phenol, antioxidant and antibacterial activities of honeys have been shown in Table 5. A very significant correlation was observed between the amount of total phenols and antioxidant activity in honeys ($y = 0.558x - 118.2$, $R^2 = 0.902$).

Antibacterial activity of honeys against *E. coli*, *S. aureus* and *B. subtilis* demonstrated negative correlation between MIC and the amount of the total phenols. It means increasing phenol content of honey may be a responsible factor for growth inhibition of bacteria. On the other hand, antioxidant activity of honey is correlated with antibacterial efficacy on *E. coli* and *S. aureus* but there was not good dependency against *B. subtilis* and *Ps. aeruginosa*. So antioxidant compounds were effective in growth inhibition of *E. coli* and *S. aureus*.

Table 4 Antibacterial activity of floral origin honeys by cup-plate method

	<i>Thymus</i> Honey				<i>Citrus</i> Honey				<i>Astragalus</i> Honey				<i>Medicago</i> Honey				G
	Zone/Conc. ^a			MIC $\mu\text{g/ml}$	Zone/Conc.			MIC $\mu\text{g/ml}$	Zone/Conc.			MIC $\mu\text{g/ml}$	Zone/Conc.			MIC $\mu\text{g/ml}$	
	20	40	60		20	40	60		20	40	60		20	40	60		
<i>S. aureus</i>	4	10	10	250	2	4.5	8	390	3	6.5	10	250	1.5	3.5	7	250	18
<i>B. subtilis</i>	1.5	4.5	7	310	1.5	3.5	6	420	1.5	3	7	300	1	4	8.5	270	18
<i>E. coli</i>	6	14	20	195	3.5	8	14	320	4.5	9.5	17	210	3.5	8.5	14	210	18
<i>Ps.aeruginosa</i>	0	4	10	270	0	2	7	450	0	0	0	>500	0	0	7	250	19

G Gentamycin (5mg/ml)

aZone (mm) measured in different concentrations (% w/v)

Table 5 Correlation between total phenol, antioxidant and antibacterial activities of honey

Correlation	MIC of <i>E. coli</i>	MIC of <i>S. aureus</i>	MIC of <i>Ps. aeruginosa</i>	MIC of <i>B. subtilis</i>
Total Phenol	$y = -0.339x + 428.6$ $R^2 = 0.984$	$y = -0.413x + 522.3$ $R^2 = 0.999$	$y = -0.330x + 557.3$ $R^2 = 0.197$	$y = -0.375x + 540.2$ $R^2 = 0.937$
FRAP Antioxidant	$y = -0.563x + 347.5$ $R^2 = 0.935$	$y = -0.670x + 420.5$ $R^2 = 0.908$	$y = -0.358x + 440$ $R^2 = 0.080$	$y = -0.555x + 437.2$ $R^2 = 0.710$

Conclusion

The comparison of hydrodistillation and SPME methods of plants essential oils showed difference between type and amount of volatile compounds. There were not high molecular weight terpenes in the essential oils gathered by SPME, while compounds with diterpene skeleton were existed in volatiles by hydrodistillation. The results showed importance of selecting appropriate method for preparing essential oils. The usage of unifloral honeys as therapeutic agents could be determined based on interest properties of their abundant volatile compounds.

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Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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