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COMPOSITION OF VOLATILE OILS AND ANTIOXIDANT ACTIVITY OF WATER EXTRACTS OF LEAVES OF *Ziziphus jujuba* MILL. FROM CENTRAL IRAN

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ABSTRACT

This study is designed to investigate the volatile oils composition of the leaves of *Ziziphus jujuba* Mill from Qom province, central region of Iran, in two phenology stages: flowering and fruiting and examine antioxidant activity of its water extract. Gas Chromatography (GC) and Gas Chromatography-Mass Spectrometry (GC-MS) analysis indicated that Phytol (16.63%), (E)-2-Hexenal (11.26%) and Eugenol (9.58%) were the major constituents of the obtained volatile oil in the flowering period and (E)-2-Hexenal (37.05%) and n-Octane (7.44%) were of the fruiting period. The number of identified compounds in the volatile oil from the flowering season was 19 while the number in the fruiting season was 24. In addition, the antioxidant activity of water extracts of *Z. jujuba* leaves in two phenology stages were evaluated via 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging test and beta-carotene bleaching method. Also total phenolic compounds were estimated. The results show there is no significant differences in antioxidant properties of leaves' water extract in the two phenology stages, but total phenolic compounds content of leaf extract of this plant is dependent on the phenology stage that the leaf is from.

Key words: Volatile oil, Water extract, Antioxidant activity, *Ziziphus jujuba*

INTRODUCTION

Ziziphus is a genus of the family Rhamnaceae which consists of about 40 species and is a small spiny shrub, distributed in warm, temperate and subtropical regions throughout the world (Kathleen, 1995). In Iran, *Ziziphus* is mostly found in central regions such as Qom and Khorasan and Golestan provinces.

Ziziphus is considered edible, holds an special place in traditional medicine and its fruit as a medicinal herb with high nutritional values is used both fresh and dried (Omid Beigi, 1997).

The drupe and stone of *Ziziphus* contain its most medicinal properties, but the leaves have also shown healing properties. The infusion of its leaves is usually gargled to treat sore throat and bleeding gums and if used topical can heal joint pain (Amin, 1997; Omid Beigi, 1997; Usher, 1971).

Kurihara *et al.* (1998) extracted saponin and ziziphin from the dried leaves *of Z. jujuba.* Leaves of *Z. jujuba* due to the existence of the active substance ziziphin suppress the sweet taste sensation in flies (Pharmaregina), rats and hamsters (Kurihara *et al.,* 1988).

In a study conducted by Zahra Shirdel *et al.,* it was determined that the ethanolic extract of leaves of *Z. jujuba* has hypoglycemic effect in diabetes mellitus patients and its effect is similar to that of Glibenclamide (Shirdel *et al.,* 2011).

The anti-allergic activity of the water extract of leaves of *Z. jujuba* was studied by measuring its inhibitory effect on hyaluronidase (bovine testes) activation *in vitro. Z. jujuba* showed strong anti-allergic activity (Mahajan *et al.,* 2009).

The leaf extract of *Z. jujuba* was found to stimulate chemotactic, phagocytic and intracellular killing potency of human neutrophils (infection fighting white blood cells) at 5-50 micro g/ml (Ganachari *et al.,* 2004).

Our searches in the literature have not led to any report regarding chemical constituents of the volatile oil of the leaves of *Z. jujuba*. About its antioxidative properties, to the best of our knowledge, there is only one report on its methanolic extract (Kar *et al.,* 2013) that shows high antioxidtive activity of its leaves by two methods of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric ion reducing capacities.

In regard to this fact that medicinal application of plants in traditional medicine is usually by

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infusing them or brewing with water, the water extract is very important. There is no report in the literature on the water extract of leaves of *Z. jujuba*, so the present study, is about the antioxidative properties of the mentioned water extract by two methods, beta-carotene bleaching and DPPH radical scavenging tests and also, isolating and identifying the chemical composition of the volatile oil of the leaves in two phenology stages.

MATERIAL AND METHODS

Materials

The fresh leaves of *Ziziphus jujuba* were collected from Qom province, center of Iran, in two phenology stages: flowering (April) and fruiting (June), in 2014 year. The plant materials were authenticated by Department of Botany (Islamic Azad University, Qom Branch).

Samples were dried and subsequently ground in a blender to obtain fine powder. All reagents and chemicals used in this study were from Merck or Sigma Companies.

Extraction of the volatile oil

The volatile oils of leaves of *Z. jujuba* were extracted from dried leaves samples via hydro-distillation using Simultaneous Distillation Extraction (SDE) method (Wei *et al.,* 2010) and n-hexane was used as the organic solvent for 2 hours. The n-hexane solution was dried over anhydrous sodium sulfate, the solvent was removed and the extracted oil was stored at 4°C in a sealed vial until analyzed.

Preparation of extracts

According to the reflux method, 50 g of dried leaves of the herb (*Z. jujuba*) were powdered and exhaustively refluxed with water (1000 mL) for 2 h. The extract was filtered out, concentrated, and dried using a rotavapor in a pre-weighed flask.

Gas Chromatography analysis

Analytical gas chromatography of the volatile oil was carried out using a Hewlett-Packard 5975B series gas chromatograph with Agilent HP-5 capillary column (30 m x 0.25 mm, f.t 0.25 μm); carrier gas, He; split ratio, 1:10, and using a flame ionization detector. The column temperature was adjusted at 50°C which was unchanging for 10 min and was programmed to rise up to 240°C at the rate of 4°C/min and then stay constant at that temperature for 15 min. GC/MS was performed on a HP 5975B with a Hewlett–Packard 5973 quadruple detector, on capillary column HP-5 (30 m x 0.25 mm; f.t 0.25 μm);

The MS operated at 70 eV ionization energy. Retention indices were calculated using retention time of n-alkanes that were injected after volatile oil at the same chromatographic conditions. Quantitative data were obtained from the electronic integration of the FID peak areas. Acquisition mass range was 40-400 m/z.

The components of the oils were identified by comparing their mass spectra and kovats indexes with Wiley library and published books, data bases available and credible websites (Adams 2001).

Antioxidant activity

DPPH radical assay

Radical-scavenging activities of the plant extract were determined using a published DPPH radical scavenging activity assaying method with minor modifications (Foti *et al.,* 2004; Huang *et al.,* 2005; Garcia *et al.,* 2012). Briefly, stock solutions (10 mg/ml each) of the extract and the synthetic standard antioxidant BHT were prepared in methanol. Dilutions were made to obtain concentrations ranging from 0.8 to $5x10^{-4}$ mg/ml. Diluted solutions (1 ml each) were mixed with 1 ml of a freshly prepared 1 mg/ml DPPH radical methanol solution and allowed to stand for 30 min in the dark at room temperature for reactions to take place. Absorbance values of these solutions were recorded on an ultraviolet and visible (UV–Vis) spectrometer at 517 nm using a blank containing the same concentration of the extract or BHT without DPPH radicals. Inhibition of DPPH radical in percent (I %) was calculated as follows (Akhbari *et al.,* 2014):

$$I\% = [(A_{blank} - A_{sample}) / A_{blank}] \times 100$$

Where A blank is the absorbance value of the control reaction (containing all reagents except the test compound) and A sample is the absorbance values of the test compounds. The sample's concentration is expressed in terms of IC_{50} which was calculated by drawing the chart of inhibitory percentages against concentrations of the sample. All the tests were carried out in triplicate and IC_{50} values were reported as means ± SD.

β-carotene /linoleic acid bleaching assay

In the β-carotene /linoleic acid test, the antioxidant competes with β-carotene for transferring hydrogen atoms to the proxy radicals (R1R2HCOO.) formed from the oxidation of linoleic acid in the presence of molecular oxygen (O_2) and converts them to hydroperoxides (R1R2HCOOH) leaving the β-carotene molecules intact (Huang *et al.,* 2005). Assaying the remained β-carotene gives an estimation of anti-oxidative potential of the

sample. A mixture of β-carotene and linoleic acid was prepared by adding 0.5 mg of β-carotene to 1 ml of chloroform (HPLC grade), 50 mg of linoleic acid and 200 mg of Tween 40. The chloroform was then completely evaporated under vacuum and 100 ml of oxygenated distilled water were subsequently added to the residue and mixed gently to form a clear yellowish emulsion. The extract and BHT (positive control) were individually dissolved in methanol (2 g/l) and 350 µl of each of them were added to 2.5 ml of the above mentioned emulsion in test tubes and mixed thoroughly. The test tubes were incubated in a water bath at 50°C for 2 h together with a negative control (blank) that contained the same volume of methanol instead of the extracts. The absorbance values were measured at 470 nm on an ultraviolet and visible (UV–Vis) spectrometer. Antioxidant activities (inhibition percentages, I %) of the samples were calculated using the following equation:

$$I\% = (A_{\beta\text{-carotene after 2 h assay}} / A_{\text{initial } \beta\text{-carotene}}) \times 100$$

Where $A_{\beta\text{-carotene after 2 h assay}}$ is the absorbance values of β-carotene after 2 h assay remaining in the samples and $A_{\text{initial } \beta\text{-carotene}}$ is the absorbance value of β-carotene at the beginning of the experiments. All the tests were carried out in triplicate and inhibition percentages were reported as means ± SD of triplicates.

Assay for total phenolic

Total phenolic constituents of water extract of leaves of *Z. jujuba* were determined by literature methods involving Folin–Ciocalteu's phenol reagent and Gallic acid standard (Slinkard *et al.,* 1977, Trouillas *et al.,* 2003). A solution of the extract (0.1 ml) containing 1000 µg of the extract was pipetted into a 50 ml volumetric flask, then 46 ml of distilled water and 1 ml of Folin–Ciocalteu's phenol reagent were added to it, and the flask was shaken thoroughly. After 3 min, 3 ml of 2% Na_2CO_3 solution were added and the mixture was allowed to stand for 2 h with intermittent shaking. Absorbance values were measured at 760 nm. The same procedure was repeated for all the standard Gallic acid solutions (0–1000 µg / 0.1 ml) and a standard curve was obtained from the following equation:

$$\text{Absorbance} = 0.0012 \times \text{Gallic acid } (\mu g) + 0.0033$$

Total phenols of the extract, as gallic acid equivalent, were determined using the absorbance value of the extract measured at 760 nm as input to the standard curve and the equation. The test was carried out in triplicate and gallic acid equivalent value was reported as mean ± SD of triplicate.

RESULTS AND DISCUSSION

Chemical composition of the volatile oil

By applying Simultaneous Distillation Extraction (SDE) the volatile oil of the Leaves of *Z. jujuba* was extracted in the two mentioned seasons and this procedure resulted in 0.15% and 0.12% for the flowering and fruiting periods respectively. The number of compounds found in the volatile oil from the flowering season was 19 and in the fruiting season was 24 which were separated and identified by GC/FID and GC/Mass analysis. Total identified constituents of the volatile oils were 81.31% and 83.80% for the flowering and fruiting seasons respectively (Table 1). After studying the extracted compounds from the leaves acquired in the two phenology seasons, a big difference was observed in the obtained compounds. For instance the primary compounds of this volatile oil in the flowering season were phytol (16.63%), (E)-2-Hexenal (11.26%) and Eugenol (9.58%), while the primary compounds in the fruiting season were (E)-2-Hexenal (37.05%) and n-Octane (7.44%).

Comparing the volatile oils composition of the leaves of *Z. jujub*a in the two seasons, we found that volatile oil in the fruiting season is richer in oxygenated hydrocarbons than the flowering season (Table 2).

Considering that Eugenol (9.58%), Cadinene (6.31%) and Phytol (16.63%) can be seen only in the volatile oil of the leaves of *Z. jujuba* in flowering season and because of the phytochemical properties of these materials, it is expected that the leaves of this plant in flowering season have a significant physiological properties. Therefore, it is suggested for further research.

As it is indicated in Table 2 the volatile oil of the leaves in the flowering season has more oxygenated terpene compounds compared to volatile oil compounds of the leaves in the fruiting season; and also the volatile oil in the fruiting season is rich in non-terpene hydrocarbon compounds.

Antioxidant activity

By using reflux method and taking water as solvent, the process of extraction was done which was then used to perform antioxidant tests and the output of extraction from the flowering season was 5.22% and from the fruiting season was 5.46%.

DPPH radical scavenging activity potential and β-carotene bleaching tests for assessment of antioxidant capacities were done on the water extract of leaves of *Z. jujuba* in the two phenology seasons and the results are presented in Table 3. In DPPH radical scavenging test the water extract of the leaves of the plant from the flowering season

Table 1. Chemical composition of the leaves volatile oils of *Z. jujuba* in two phenology stages: flowering and fruiting

No	Components	Flowering period	Fruiting period	RI[a]
1	n-Octane	9.10	7.44	800
2	(E)-2-Hexenal	11.26	37.05	851
3	o-xylene	–	2.48	874
4	α-pinene	–	0.41	936
5	Benzaldehyde	–	0.93	964
6	2-Methyl-3-octanone	–	1.19	986
7	6-Methyl-5-heptene-2-one	1.80	2.87	991
8	2-Pentylfuran	–	0.78	995
9	cis-3-Hexenyl Acetate	–	1.23	1010
10	Linalool oxide	1.04	0.80	1075
11	β-Linalool	–	3.88	1103
12	Terpinolene	2.26	–	1104
13	(3E)-6-Methyl-3,5-heptadien-2-one	0.61	–	1110
14	3-hexenyl ester Butyric acid	–	3.57	1189
15	Fenchyl alcoho	0.95	–	1197
16	(-)-alpha-Terpineol	–	1.02	1198
17	β-Cyclocitral	–	0.72	1226
18	(Z)-3-hexenyl-2-methylbutanoate	–	2.47	1235
19	Eugenol	9.58	–	1367
20	cis-β-Hexenyl Caproate	–	1.32	1384
21	cis-3-Hexenyllactate	–	1.50	1389
22	Cyclohexane	5.00	–	1396
23	α-Muurolene	–	0.68	1447
24	trans-Geranylacetone	1.67	2.73	1456
25	Alloaromadendrene	–	2.00	1466
26	α-Farnesene	6.13	2.63	1509
27	δ-Cadinene	6.31	–	1528
28	4,5,7,7a-Tetrahydro-4,4,7a-trimethyl-2(6H)benzofuranone	1.27	–	1540
29	Elemol	–	0.82	1549
30	E-Nerolidol	2.85	1.69	1569
31	cis-3-Hexenyl benzoate	–	3.09	1577
32	Pinane	0.82	–	1845
33	Diisobutyl phthalate	0.92	–	1872
34	Butyl carbobutoxymethyl phthalate	0.56	–	1967
35	n-Hexadecanoic acid	2.55	–	1983
36	Phytol	16.63	–	2119
Total		81.31	83.80	

RI[a] : Retention index on a HP-5 MS column

Table 2. The category of chemical composition of the leaves volatile oils of *Z. jujuba* in flowering and fruiting seasons

Category	Flowering season	Fruiting season
Non terpenoid oxygenated	24.87	48.69
Non terpenoid hydrocarbons	14.1	9.92
hemiterpenoid oxygenated	2.41	7.31
Mono terpenoid hydrocarbons	3.08	0.41
Mono terpenoid oxygenated	4.93	9.15
sesquiterpenoid hydrocarbons	12.44	5.31
Oxygenated diterpenoid	16.63	–
Sesqui terpenoid oxygenated	2.85	2.51

Table 3. The antioxidant activities of positive control (BHT) and water extracts of *Z. jujuba* in flowering and fruiting seasons

Sample	DPPH IC_{50} (µg/ml)	β-carotene/linoleic acid Inhibition (%)
Water extract in the fruiting season	177.9 ± 0.89	54.7 ± 0.75
Water extract in the flowering season	166 ± 0.48	50.01 ± 0.64
BHT	19.82 ± 0.52	88.34 ± 0.71
Negative control	NA	5.5 ± 0.52

NA (Not applicable)

showed more antioxidant activity in comparison with the fruiting season; on the other hand β-Carotene/linoleic acid test showed that the extract in the fruiting season had more antioxidant activity when compared to the flowering season. So the results obtained from these methods are different which could be the consequence of different mechanisms of antioxidant tests. Most researchers suggest that the mechanism of DPPH assay is based on electron transfer (ET) while beta-carotene/ linoleic acid assay is one of the antioxidative tests that is categorized under hydrogen atom transfer (HAT) (Ebrahimabadi *et al.,* 2010, Larson, 1977).

Measuring total phenolic constituents

Based on the absorbance value of water extract solution of the leaves reacting with Folin–Ciocalteu phenol reagent and in comparison with the absorbance values of standard solutions of Gallic acid, total phenolic content of water extract of the leaves in the flowering season in terms of equivalence to gallic acid was estimated 17.95 ± 0.87 µg and in the fruiting season 48.91 ± 0.87 µg, and these results are not compatible with the results obtained from DPPH radical scavenging activity but are in fact compatible with the results of beta-carotene assay.

CONCLUSIONS

The results obtained from our analyses, showed significant differences in the chemical compounds extracted from the volatile oil that was obtained from leaves of the plant. The results indicated that oxygenated compounds that exist in the volatile compounds of the leaves in the flowering season equal to 51.69% while they were 67.66% in the fruiting season. Also volatile compounds of the leaves in the flowering season equaled to 42.34% of terpenic compounds but they were only 24.69% in the fruiting season. This reveals that the types of volatile compounds exist in leaves of *Z. jujuba* are highly dependent to the growth stage of the plant and the phenology period it's in.

The results show there is no significant differences in antioxidant properties of leaves' water extract in the two phenology stages, but total phenolic compounds content of leaf extract of this plant is dependent on the phenology stage that the leaf is from.

The entirety of these contents makes the leaves of *Z. jujuba* a suitable candidate for further studies on the structure of its active biological molecules.

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