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Seasonal Variation in Volatile Oil, Polyphenol Content and Antioxidant Activity in Extract of *Laurus nobilis* Grown in Iran

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Abstract: The leaves of *Laurus nobilis* were collected in the middle of four seasons (spring, summer, autumn, and winter) to determine the best harvesting time for obtaining the highest oil yield, 1,8-cineole and polyphenol content and antioxidant activity. After drying the plant materials in shade, their EOs (essential oils) were obtained by hydro-distillation method. Analysis of variance showed harvesting time had significant effect on the oil yields of *L. nobilis*. Seventy one components were identified in the oils of *L. nobilis* with 1, 8-cineole (5.7%-42.6%), α-terpinyl acetate (3.2%-13.1%), sabinene (2.3%-12.0%), beta-elemene (0.2%-17.7%) and (E)-caryophyllene (0.2%-16.9%) as the main constituents in different seasons. The predominant phenolic constituents in *L. nobilis* were cinnamic acid, carvacrol, quercetin and coumarin, respectively. Gallic acid, catechin, caffeic acid, chloregenic acid, p-Comaric acid and rutin were not detected in any seasons. The best antioxidant activity was in spring (268.6 μg/mL) and the lowest one was in winter (702.1 μg/mL).

Key words: *Laurus nobilis*, EO, seasonal variation, polyphenol, antioxidant.

1. Introduction

Laurus nobilis L. belongs to the family *Lauraceae*, which comprises numerous aromatic and medicinal plants [1]. *Laurus nobilis* L. native to Mediterranean regions is also known as sweet bay, bay laurel, Grecian laurel, true bay, and bay. *Laurus nobilis* L. is an evergreen shrub indigenous to the south parts of Europe and the Mediterranean area. It is cultivated in the north of Iran [2]. In Iranian folk medicine, the leaves of this plant have been used to treat epilepsy [2, 3], neuralgia and parkinsonism [3]. The essential oil obtained from its leaves has been used for relieving hemorrhoid and rheumatic pains [2]. It also has diuretic [2, 3], antifungal [4] and antibacterial [5] activities. Leaves of this plant produce a yellow oil known for many therapeutic indications. Leaves and their essential oil increase gastric fluid secretion and work against digestive disorders such as flatulent colic [6]. Anticonvulsive and antiepileptic activities [7] of the leaf extract have been confirmed. Recently Simic et al. [8] tested the antioxidative activity of methanolic extracts of leaves, bark and fruits. The most significant activity was obtained with the bark extract. The volatile compounds of wood and bark of this species have been studied [9]. Monoterpenes (in particular 1, 8-cineole and eugenol) were identified as the main class constituent in the bark oil, whereas sesquiterpenes (dehydrocostunolide) were the most important constituents of the wood extract. Fang et al. [10] reported their results concerning the isolation of six sesquiterpene lactones from the methanol extract of dried leaves of *L. nobilis*, responsible for inducing apoptosis. The leaf essential oil of the species under investigation showed also a very strong antibacterial

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activity against foodborne pathogens [11]. Moreover, pharmacological activity, which includes antifungal, anti-diabetic, and anti-inflammatory activities [10], has been also demonstrated. The chemical composition of the leaf essential oil obtained from different methods of isolation, were extensively studied by many researchers [12-16]. Generally, the yield and composition of the oil varies, depending upon the origin, the collection period, and the growth stage of the plant [17]. The study of the seasonal and geographical variation in yield and composition of leaf essential oil of *L. nobilis* collected from various areas in Tunisia showed that a seasonal variation both in yield and composition revealed, whereas geographical variability did not pronounce, and no chemotypes observed [18]. Seasonal variation in oil composition of *L. nobilis* grown in Portugal showed that during the period of highest essential oil yield (end of August), lower percentages of hydrocarbons and higher percentages of oxygenated compounds found [19]. Phenolic compounds in plants provide an array of natural sources of antioxidants for use in foods and nutraceuticals. Since natural sources contain a variety of phenolic compounds with varying antioxidant activity and exert their effects via different mechanisms, their efficacy in bulk oils, emulsions and composite foods might be greater than that of individual compounds. Furthermore, in addition to their safety advantages, food phenolics may augment body's source of natural antioxidants [20]. Phenols are able to donate H-atoms of phenol hydroxyl groups in reaction with peroxyl radicals that can produce stabilized phenoxyl radicals, thus terminating lipid peroxidation chain reactions. The antioxidant activity of phenols depends on the electronic and steric effects of the ring, substituents, and the strength of hydrogen-bonding interactions between the phenol and the solvent [21-23]. A few papers have dealt with the antioxidant activity and phenolic constituents of laurel leaves [24-27]. As a sample the antioxidant activity of the oil of *L. nobilis* collected in Tunisia found more effective than the synthetic antioxidant (BHT) at 200 ppm and could be attributed to the active compounds eugenol, elemicin and methyl eugenol present in this essential oil [28]. But there is no previous study on the seasonal changes of polyphenol content and antioxidant activity in this plant. The objective of this study was to investigate the seasonal changes in the chemical composition of oil, polyphenol content and antioxidant activity in extract of *L. nobilis*.

2. Materials and Methods

2.1 Collection of Plants and Identification

Fresh leaves of *L. nobilis* were collected from its wild habitat Eram garden, Fars province, in the middle of spring, summer, autumn, and winter (2013-2014; Table 1) for determination of oil concentration, chemical constituents and anti oxidant activity. Voucher specimen was deposited at the Herbarium of Fars Research center for agriculture, Shiraz, Iran.

2.2 Preparation of EO and Crude Extract

The plants were shaded at room temperature (20-25 ℃). The EOs of all dried samples (100 g) were isolated by hydrodistillation for 3 h, using a Clevenger-type apparatus according to the method recommended in British Pharmacopoeia [29]. This work was repeated three times. An average of the oils yield was calculated. The distillated oils were dried over anhydrous sodium sulfate and stored in tightly

Table 1 Oil yields, statistical analysis of oil and antioxidant activity in extract of *L. nobilis*.

Species	Collection dates	Oil yield (%)	Duncan's mean separation*	Antioxidante activity (μg/mL)
Laurus nobilis	Spring. May, 2012	0.87	C	268. 6
	Summer. August, 2012	1.3	B	672.5
	Autumn. November, 2012	1.0	BC	418.1
	Winter. January, 2013	2.1	A	702.1

*Oil yields within season followed by the same letter are not significantly different at $P < 0.01$.

closed dark vials until analysis. For extract preparation, the dried plants materials were ground into powder by using a hand mill. The powdered plants (30 g) extracted using 100 mL of methanol by maceration method [29]. The crude extracts were concentrated in vacuo at 40 ℃ using a rota vapor.

2.3 Oil Analysis Procedure

GC analysis was performed using an Agilent gas chromatograph series 7890-A with an FID (flame ionization detector). The analysis was carried out on fused silica capillary HP-5 column (30 m × 0.32 mm i.d.; film thickness 0.25 µm). The injector and detector temperatures were kept at 250 ℃ and 280 ℃, respectively. Nitrogen was used as carrier gas at a flow rate of 1mL/min; oven temperature program was 60-210 ℃ at the rate of 4 ℃/min and then programmed to 240 ℃ at the rate of 20 ℃/min and finally held isothermally for 8.5 min; split ratio was 1:50. GC-MS analysis was carried out by use of Agilent gas chromatograph equipped with fused silica capillary HP-5MS column (30 m × 0.25 mm i.d.; film thickness 0.25 µm) coupled with an Agilent mass spectrometer series 5975-C. Helium was used as carrier gas with ionization voltage of 70 eV. Ion source and interface temperatures were 230 ℃ and 280 ℃, respectively. Mass range was from 45 to 550 amu. Oven temperature program was the same given above for the GC.

2.4 Identification of Compounds

The constituents of the EOs were identified by calculation of their retention indices under temperature-programmed conditions for n-alkanes (C8-C25) and the oil on a HP-5 column under the same chromatographic conditions. Identification of individual compounds was made by comparison of their mass spectra with those of the internal reference mass spectra library or with authentic compounds and confirmed by comparison of their retention indices with authentic compounds or with those of reported in the literature [30]. For quantification purpose, relative area percentages obtained by FID were used without the use of correction factors.

2.5 Extraction of Polyphenol and HPLC Analysis

The procedure for extraction of polyphenols was carried out according to the modified method established by Justesen [31]. Reference standard of 10 polyphenols (gallic acid, catechin, chlorogenic acid, caffeic acid, quercetin, cinnamic acid, coumarin, p-comaric acid, rutin and carvacrol) were purchased from Merck (Darmstadt, Germany). HPLC analysis was carried out on an Agilent 1200 series, equipped with a Zorbax Eclipse XDB-C18 column (4.6 × 5 µm i.d.; × 150 mm film thickness, RP), and a PDA (photodiode array detector). Elution was monitored at 280 and 230 nm. The column temperature was 30 ℃. The injection volume was selected 20µL and it was done automatically using autosampler. The total running time was 30 min. Gradient elution was applied to achieve maximum separation and sensitivity. The elution was performed by varying the proportion of solvent A (formic acid 1% in deionized water) to solvent B (Methanol (v/v)) as follows: Methanol:formic acid 1% (10:90), at 0 min; Methanol: formic acid 1% (25:75), at 10 min; Methanol:formic acid 1% (60:40), at 20 min. And finally, Methanol:formic acid 1% (70:30), at 30 min. Linear calibration graphs were obtained with good correlation for standard solutions .

2.6 Determination of Antioxidant Activity by the DPPH Radical Scavenging Method

The antioxidant activity of plants extract and the standard antioxidant (gallic acid) were assessed on the basis of radical scavenging effect of the stable DPPH (1, 1-Diphenyl-2-picrylhydrazyl) free radical, according to a modified method [32]. The DPPH radical inhibition was measured at 515 nm by using a micro-plate reader model biotek ELx808. The IC_{50} of

each sample (concentration in μg/mL required to inhibit DPPH radical formation by 50%) was calculated by Matlab software (Table 1).

2.7 Statistical Analysis

Statistical analyses were done using SAS software. Means were separated with a *t*-test for comparison of oil content (Table 1) and main classes (Table 2) of leaves from the *L. nobilis* among the seasons. A value of $P < 0.01$ was considered statistically significant.

3. Results

The oils isolated from the leaves of *L. nobilis* were achieved by hydrodistillation method. An analysis of variance indicated significant differences in oil content among the seasons ($P < 0.01$; Table 1) and between the main classes ($P < 0.01$; Table 2). The best harvesting time for high oil levels was winter (Fig. 1). From 71 compounds identified in the oil of *L. nobilis*, included 1, 8-cineole (5.7%-42.6%), α-terpinyl acetate (3.2%-13.1), sabinene (2.3%-12.0%), β-elemene (0.2%-17.7%) and (E)-caryophyllene (0.2%-16.9%; Table 2) as major components. The maximum percentage of 1, 8-cineole was recorded in winter, with an increase in the cineole content from spring to summer, a decrease to autumn and an increase in winter.

For leaves of *L. nobilis*, the minimum percentage of -terpinyl acetate and sabinene occurred as same as cineole content in the spring (3.2% and 2.3%) and the maximum one achieved in the summer (13.1% and 12.0%). The other major oil components such as

Table 2 Essential oils composition and statistical analysis of main constituents of *L. nobilis* in different seasons.

			Spring	Summer	Autumn	Winter
No.	**Compound**	**RI** [a]		(%)b		
1	Tricyclene	919	t	t	-	-
2	α-Thujene	923	0.2	0.9	0.8	0.4
3	α-Pinene	930	2.5	5.2	3.9	4.6
4	Camphene	945	0.7	0.3	0.6	0.4
5	Sabinene	970	2.3	12.0	8.2	10.4
6	β-Pinene	974	1.6	4.2	2.7	3.6
7	Myrcene	987	0.6	1.5	2.3	1.5
8	α-Phellandrene	1003	0.6	1.3	3.5	1.6
9	δ-3-Carene	1008	0.08	0.1	0.2	0.1
10	α-Terpinene	1013	0.2	0.7	0.5	0.2
11	p-Cymene	1021	0.09	1.0	0.1	0.6
12	Limonene	1026	0.7	2.9	-	-
13	β-Phellandrene	1030	-	-	0.3	1.7
14	1,8-Cineole	1031	5.7	37.5	20.3	42.6
15	(Z)-β-Ocimene	1033	0.1	t	-	-
16	(E)-β-Ocimene	1043	1.1	0.1	0.2	0.05
17	γ-Terpinene	1054	0.3	1.2	0.8	0.5
18	cis-Sabinene hydrate	1062	t	0.6	0.9	0.4
19	Terpinolene	1084	0.1	0.6	0.4	0.2
20	Linalool	1096	0.6	3.7	2.5	1.9
21	cis-p-Menth-2-en-1-ol	1123	-	-	0.2	0.1
22	trans-p-Menth-2-en-1-ol	1140	-	-	0.1	0.1
23	δ-Terpineol	1163	t	0.4	0.9	0.5
24	Terpinene-4-ol	1173	0.2	1.6	2.0	1.6
25	α-Terpineol	1187	0.3	2.0	5.4	3.0
26	Nerol	1230	-	-	0.3	0.4
27	Linalyl acetate	1252	t	0.2	-	-

28	Bornyl acetate	1282	0.6	0.3	0.8	0.3
29	2-Undecanone	1289	0.1	0.2	0.1	0.07

(Table 2 continued)

No.	Compound	RI [a]	Spring	Summer	Autumn	Winter
				(%)b		
30	neo iso-Isopulegyl acetate	1319	-	-	1.0	0.6
31	δ-elemene	1333	0.8	t	-	-
32	α-terpinyl acetate	1347	3.2	13.1	13.0	10.6
33	Eugenol	1353	0.1	0.8	6.9	3.4
34	Neryl acetate	1361	0.2	0.3	0.6	-
35	α-ylangene	1367	0.4	t	0.2	-
36	α-copaene	1372	0.1	t	-	-
37	Geranyl acetate	1387	-	-	0.3	0.08
38	β-elemene	1394	17.7	0.2	2.0	0.8
39	Methyl eugenol	1404	0.5	3.3	5.5	2.7
40	α-gurjunene	1407	0.2	-	-	-
41	(E)-caryophyllene	1422	16.9	0.2	1.7	0.9
42	β-copaene	1426	0.1	-	-	-
43	α-guaiene	1435	1.0	-	0.2	0.09
44	Aromadendrene	1441	-	-	0.3	-
45	(E)-cinamyl acetate	1447	-	-	0.3	-
46	(E)-isoeugenol	1451	-	-	0.8	0.1
47	α-humulene	1454	2.8	t	0.3	0.1
48	(E)-β-farnesene	1457	0.8	t	-	-
49	allo-aromadendrene	1460	-	-	0.2	-
50	Germacrene D	1479	4.8	0.08	0.9	0.2
51	β-selinene	1490	0.7	0.07	0.4	0.2
52	(E)-methyl isoeugenol	1492		0.1	-	-
53	α-selinene	1498	4.8	-	-	-
54	Bicyclogermacrene	1500	2.3	-	0.7	0.06
55	Cuparene	1505	-	-	0.2	0.1
56	α-bulnesene	1508	-	-	0.6	0.2
57	γ-cadinene	1511	0.9	0.07	0.5	0.1
58	δ-cadinene	1520	1.4	0.1	0.4	-
59	(E)-γ-bisabolene	1532	13.1	0.08	-	-
60	10-epi-cubebol	1538	-	-	0.2	0.3
61	Elemol	1549	0.8	0.1	0.6	0.1
62	Elemicin	1555	-	t	0.3	0.08
63	Germacrene D-4-ol	1573	0.6	t	-	-
64	Spathulenol	1575	-	0.3	0.9	0.4
65	Caryophyllene oxide	1580	0.8	0.3	0.4	0.6
66	Viridiflorol	1588	0.2	0.06	-	-
67	Humulane-1,6-dien-3-ol	1619	-	-	0.4	0.2
68	1,10-di-epi-cubenol	1621	-	-	0.5	-
69	β-eudesmol	1648	1.3	0.4	0.6	0.3
70	α-eudesmol	1650	-	0.2	0.6	0.1
71	α-cadinol	1652	2.2	0.2	-	-
	Monoterpene hydrocarbons		8.6[c] C[d]	11.5 D	68.8 A	5.9 A
	Oxygenated monoterpenes		32 A	64.1 B	0.8 D	1.6 D
	Sesquiterpene hydrocarbons		24.5 B	61.9 C	8.6 B	4.2 B
	Oxygenated sesquiterpenes		25.8 B	68.5 A	2.7 C	2.0 C

[a] RI = Retention indices in elution order from HP-5 column.

[b] t = trace (< 0.05); nd: not detected.
[c] % of main constituents within season followed by the same letter are not significantly different at $P < 0.01$.
[d] Duncan's mean separation.

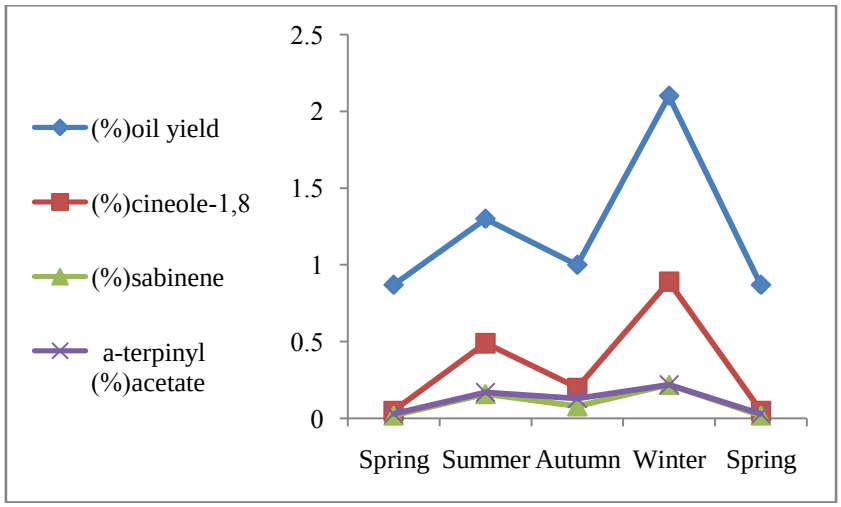

Fig. 1 Percentage of oil and main components of *L. nobilis* in different seasons.

Table 3 Phenolic compound of *L. nobilis* in different seasons.

	Spring	Summer	Autumn	Winter
Polyphenol		**Amount[a]**		
Gallic acid	nd[b]	nd	nd	nd
Catechin	nd	nd	nd	nd
Caffeic acid	nd	nd	nd	nd
Chloregenic acid	nd	nd	nd	nd
Quercetin	0.03 ± 0.006	0.019 ± 0.001	0.004 ± 0.001	0.001 ± 0.0006
Cinnamic acid	3.0 ± 1.00	2.1 ± 0.058	2.9 ± 0.058	2.7 ± 0.100
Coumarin	0.02 ± 0.006	0.002 ± 0.0006	0.02 ± 0.006	0.001 ± 0.0006
p-Comaric acid	nd	nd	nd	nd
Rutin	nd	nd	nd	nd
Carvacrol	0.5 ± 0.058	0.07 ± 0.006	0.4 ± 0.058	0.05 ± 0.006

[a] Calculated mean amount of the polyphenol (mg/g) based on the weight of the ground dry plant in three replicates ± SD.
[b] nd: not detected.

δ-elemene and (E)-caryophyllene differed with the change in seasons. The minimum amount of δ-elemene and (E)-caryophyllene was observed in the summer (0.2% and 0.2%) and the maximum one determined in the spring (17.7% and 16.9%). The predominant phenolic constituents in *L. nobilis* were cinnamic acid, carvacrol, quercetin and coumarin, respectively. Gallic acid, catechin, caffeic acid, chloregenic acid, p-Comaric acid and rutin were not detected in any seasons (Table 3). Investigation of antioxidant activity showed the best activity in spring (268.6 µg/mL) and the worst one was observed in winter (702.1 µg/mL) for leaves extract of *L. nobilis*. Although, the maximum percentage of 1, 8-cineole and oil yield were recorded in winter, but the antioxidant activity followed the reverse trend. The antioxidant activity was varied in the different season (Table 1). Our study showed that there was a correlation between the antioxidant capacity and phenolic compounds cinnamic acid and carvacrol (Fig. 2). With decreasing in cinnamic acid and carvacrol content from spring to summer, the antioxidant activity also decreased. From summer to autumn the amount of the two phenolic compounds and antioxidant capacity increased and then decreased both of them during the period autumn-winter.

All considered EOs were characterized by a clear predominance of oxygenated compounds especially monoterpenes and sesquiterpenes, respectively. Also, the concentration of oxygenated monoterpenes was

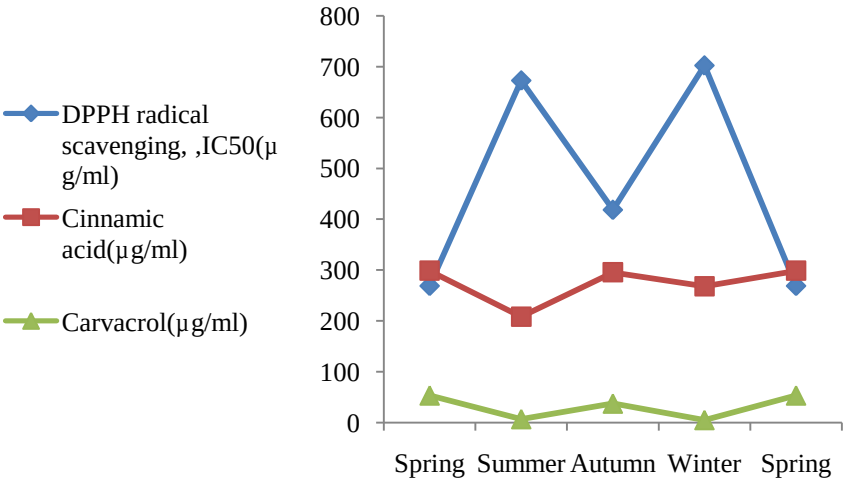

Fig. 2 Correlation between antioxidant activity, cinnamic acid and carvacrol content of *L. nobilis* extract in different seasons.

varied during the seasons (Table 2). The trend of this class was because of cineole content. In general, winter was the season with higher in the EO and 1, 8-cineole. The 1, 8-cineole content was not significant in spring. The highest and lowest amount of monoterpene hydrocarbons (8.6%-32%) found in the spring and summer, oxygenated monoterpenes, occurred in the spring and winter (11.5%-68.5%), sesquiterpene hydrocarbons found in the summer and spring (0.8%-68.8%) and oxygenated sesquiterpenes followed the same trend sesquiterpene hydrocarbons in the summer and spring (1.6%-5.9%), respectively. In the spring, there are a maximum amount of sesquiterpene hydrocarbons because of the high level of β-elemene (17.7%), (E)-caryophyllene (16.9%), (E)-γ-bisabolene (13.1%), Germacrene D (4.8%) and α-selinene (4.8%), respectively. The amount of these compounds was not remarkable in other seasons. Sabinene (12.0%), α-pinene (5.2%), β-pinene (4.2%) and limonene (2.9%) were responsible for the highest amount of monoterpene hydrocarbons in summer.

4. Discussion

Similar results are reported that a seasonal variation both in yield and composition of *L. nobilis* has been revealed [18]. By comparison with our research, all essential oils considered in a previous study have been characterized by a predominance of oxygenated compounds especially monoterpenes, phenylpropanoids and sesquiterpenes, respectively. The concentration of this class (specially oxygenated phenylpropanoids) has been decreased during the period October-July [18]. Another study has been showed that the essential oil recovery increased in early June, reached a maximum in late July, and constantly decreased thereafter [16]. Putievsky et al. detected that the content of essential oil in fresh leaves from cultivated trees of *L. nobilis* reached the highest level in autumn, while the lowest one was observed in late spring [15]. It is difficult to see the accordance of the results in literature on antioxidant activity because of the usage of the different testing methods. The lower free radical scavenging activity values was obtained from *L. nobilis* (1.901, IC_{50}, [mg/mL]), in Konya [33]. The antioxidant activity of the *L. nobilis* floral buds oil collected in Tunisia was more effective than the synthetic antioxidant BHT (butylated hydroxytoluene), at 200 ppm [28]. The antioxidant capacity was determined by a common used and highly accepted method. However, it was clearly noted that, there are a

lot of variables affect on antioxidant activity in the literature [18]. Therefore, the numerous of antioxidant activity methods makes it necessary to use more than one method in such studies.

5. Conclusions

In conclusion, a seasonal variation in yield and composition of *Laurus nobili oil* has been observed. The seasonal variation of 1, 8-cineole in the oil of *L. nobilis* was remarkable. A terpenoid oxide, 1, 8-cineole is present in many plant essential oils and displays an inhibitory effect on some types of experimental inflammation in rats, such as paw edema induced by carrageenan and cotton pellet-induced granuloma. The 1, 8-cineole also has antimicrobial, antiinflammatory and antinociceptive effects [34]. Further research is needed on the determination of the correlation between the antioxidant capacity and the chemical composition of the plants oil. But, the presence of phenolic compounds in the plant extracts contributes significantly to their antioxidant potential. Antioxidant properties of these compounds are directly related to their structure. Indeed, phenolics are composed of one (or more) aromatic rings with one or more hydroxyl groups which are able to quench free radicals by forming resonance-stabilized phenoxyl radicals [35, 36].

In this study, it was concluded that the methanol extract of *L. nobilis* in spring could be have the best antioxidant activity because of the high amount of phenolic compounds. Further investigation of total phenol and flavonoid contents and *in vivo* antioxidant activities is needed.

Acknowledgments

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