International Journal of Horticultural Science and Technology
Vol. 4, No. 2; December 2017, pp 251-258
Print ISSN: 2322-1461 Online ISSN: 2588-3143
DOI: 10.22059/ijhst.2018.234339.194
Web Page: https:// ijhst.ut.ac.ir, Email: ijhst@ut.ac.ir

Effect of Phenological Stages on Essential Oil Content, Composition and Rosmarinic Acid in *Rosmarinus officinalis* L.

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(Received: 15 March 2017, Accepted: 13 September 2017)

Abstract

Rosemary (*Rosmarinus officinalis* L.) is an important medicinal plant that contained a wide range of secondary metabolites such as rosmarinic acid. In this study, essential oil content and composition and rosmarinc acid were determined at three phenological stages (before flowering, full flowering and fruit set stages). Hydro distillation method was used for essential oil extraction and GC and GC/MS was used for essential oil composition. On the basis of the obtained results rosemary plants in full flowering stage had higher amount of essential oil (1.99 ml/100 g dry weight) than in before flowering and fruit set stages (1.2 and 1.01 ml/100 per g dry weight, respectively). The extracted essential oil was contained 1,8-cineole, α-pinene, verbenone, camphor, geraniol, borneol acetae, camphene and linalool as major constituents. These constituents were affected by phenological stages. Before flowering, 1,8-cineole, α-pinene and verbenone (13.68%), in full flowering, 1,8-cineole (17.90%) and in fruit set α-pinene (21.77%) were the main constituents of the reosemary essential oil. Using HPLC analysis on leaf extract showed that the highest amount of rosmarinic acid (25.92mg/g DW) was observed in the fruit set stage. In conclusion, for essential oil content full flowering stage can be recommended for harvesting of *Rosmarinus officinalis* L. and for rosmarinic acid, fruit set stage can be the best time for harvesting of this medicinal plant.

Keywords: Essential oil constituents, growth stages, phonological stage, Rosemary

Introduction

Rosemary (*Rosmarinus officinalis* L.) is a dense, evergreen, hardy, perennial aromatic herb with 50–150 cm height and small (1.5–4 cm) pointed, sticky and hairy leaves (Omidbaigi, 2010). For long time it has been harvested for: food (El Omri et al., 2010), aromatherapy (Almela et al., 2006; Pintore et al., 2002) due to its antioxidant activity and antimicrobial properties, and fragrance and flavor industry (Wada et al., 2004; Bicchi et al., 2000). It is listed by the Convention on the Conservation of European Wildlife and Natural Habitats as a source of natural food flavorings. Essential oil (1–2.5%) components vary according to chemo type. Essential oil of rosemary is composed of mainly monoterpene hydrocarbons including α- and β-pinenes, camphene and limonene,

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together with 1,8-cineole, borneol, camphor (20–50% of the oil) and also linalool, verbinol, terpineol, 3-octanone and isobornyl acetate. Moreover, it contains flavonoid (e.g. diosmetin, diosmin, genkwanin and derivatives, luteolin and derivatives, hispidulin, nepetin, nepitrin and apigenin) and phenolic (caffeic, chlorogenic, labiatic, neochlorogenic and rosmarinic acid) compounds (Barnes et al., 2007). Rosmarinic acid (α-O-caffeoyl-3,4-dihydroxyphenyllactic acid) (Fig. 1) which is the major component of *R. officinalis*, is a caffeic acid ester of 3,4-dihydroxyphenyllactic acid (Petersen et al., 2009). It is also one of the main constituent of essential oil in Lamiaceae, Boraginaceae, and Apiaceae families; furthermore, it has been detected in at least 12 other plant families (Ramawat and Merillon, 2008).

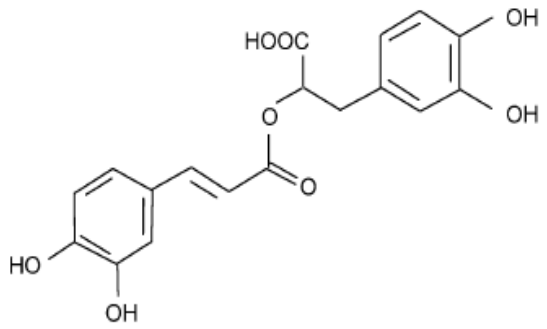

Fig. 1. Rosmarinic acid chemical structure

The pharmacological and antioxidant applications of Rosmarinic acid have been previously reported (Swarup et al., 2007; Tepe, 2008; McCue and Shetty, 2009; Lamien-Meda et al., 2010; Anusuya and Manoharan, 2011; Hassanzadeh et al., 2014). The composition and yield of essential oil of rosemary can be influenced by ecological, physiological, phenological and agronomical factors. In medicinal plants many of these factors are interdependent which can be influenced by seasonal and growth stages, geographical origin, maturity variation, genetic variation, utilized part of plant, postharvest drying and storage conditions (Anwar, 2009). Although previous studies have shown that different growth stages can influence the essential oil compositions in some medicinal plant species such as *Melissa officinalis* (Saeb and Gholamrezaei, 2012), however, no information is available about influence of phonological stages on essential oil attributes of rosemary plants. Therefore, the aim of the current study was to investigate the effects of different growth stages on essential oil content and composition and rosmarinc acid content in rosemary plants.

Materials and Methods

Rosemary plants cultured in Gorgan University of Agricultural Sciences and Natural Resources research station (Table 1) were manually harvested at three different stages including: before flowering, full flowering and fruit set. *Rosmarinus officinalis* L. fresh leaves were collected and carefully separated from shoots then were shade-dried at room temperature for 7 days. The drying process was continued until the mass of the samples reduced to a moisture content of about 0.10 on a dry weight basis or 10% on a wet weight basis (Martinov et al., 2007). The dried samples were powdered and used for essential oil extraction. Dried powdered leaves (40 g) were placed in Erlenmeyer flasks (500 ml) and 250 ml tap water was added to the flasks. The essential oil of samples was extracted via distilled water method for three hours using Clevenger then extracted oil was stored in refrigerator until analysis, according to methods recommended by the Hungarian pharmacopoeia (Hungarian pharmacopoeia, 1984).

Essential oil was analyzed by 5975 C, MODE EI, mass selective detector, connected with a 7890N, AGILENT gas chromatograph. Data processing system was conducted with NIST 14 mass spectral library (National Institute of Standards and Technology), which is an evaluated collection of Electron Ionization (EI) and

Table 1. Soil (a) and climatic (b) characteristics of experimental station

Soil analysis (a)	pH	CEC	EC	Organic matter%	N%	P mg/kg	K mg/kg	Texture
	7.1	13.5	0.71	1.01	0.14	4.5	310	Silty Clay Loam
Climatic Condition (b)	Elevation MSL*	Latitude	Altitude	Lowest Temp.**	Maximum Temp.**	Mean year Precipitation**		Relative Humidity**
	7	36°33´ North	54°23´ East	3.2°C	32.8°C	583.8 mm		76.5%

* Meter above sea level. ** Mean of 30 recent years.

mass spectral. Separation was achieved by a fused- silica capillary, HP 5 MS, column (30 m × 0.25 mm) and film thickness was 0.25 µm. Helium was used as a carrier gas at a flow rate of 1 ml/min. The injection volume was 1 µl and the injection temperature was 260 °C and split (50.1) technique was used during injection. The MS used a mass quadrupole detector temperature at (270 °C) and had an ionization voltage of 70 eV and temperature program was 60 °C for 4 min, then 3 °C/min to 100 °C, then 4 °C/min to 225 °C.

Rosmarinic acid

Rosmarinic acid was determined according to Angelov, et al. (2007) with slight modification. Around 0.5 g of dried rosemary leaves were crushed in a mortar mixing (Belgium) using (1:10) HPLC grade methanol. The obtained solution was sonicated for 10 min at room temperature. Then the mixture was shaken for 12 h. The mixture was centrifuged for 10 min in 3500 rpm. Finally, the supernatant was filtered through 0.45 µm syringe filter and stored at -5 °C until further analysis. The analyses of samples were repeated three times using HPLC, Merck-Hitachi-L7100, consisting of an UV detector Hitachi system and column oven L-2300 Hitachi. The instrument was equipped with a reverse phase column (RP) C-18: (250 × 4.6 mm, 5 µm particle size) using 80:20 HPLC methanol and deionized distilled as a mobile phase. The flow rate was set at 0.4 ml/min throughout the isocratic phase. The system was set on 280 nm. The column temperature was maintained at 25 °C and the injection volume was 10 µl. Quantification was performed by calibration curve, using the available standards. Rosmarinus acid (RA) standard (97%, Sigma Aldrich) curve was prepared using 25, 50, 100, 200 mg/ml concentrations of rosmarinic acid.

Data analysis

Data analysis was done using SAS software (version 9) in complete randomized design and the mean value was compared through LSD test at 95% probability.

Results

The amount and composition of essential oil is strongly influenced by plant phenological stages. Analysis of variance for different parameters is shown in Table 2.

Table 2. Analysis of variance for secondary metabolites of Essential oil content (a) and Rosmarinic acid content (b) of Rosemary according to tukey test

ANOVA table for Essential oil content (a)	SS	DF	MS	F (DFn, DFd)	P value
Treatment	1.6	2	0.82	F (2, 6) = 1073	P<0.0001
Residual	0.0046	6	0.0007		
Total	1.7	8			
ANOVA table for Rosmarinic acid content (b)					
Treatment	11	2	5.3	F (2, 6) = 409	P<0.0001
Residual	0.079	6	0.013		
Total	11	8			

Essential Oil Content

Harvesting of *Rosmarinus officinalis* L. in different phenological stages had significant effects on its essential oil content (Fig. 2). Highest essential oil content was observed in full flowering stage (1.99 ml/100 g dry weight) and the lowest amount was observed in the stage of fruit set (1.01 ml/100 g dry weight). Similarly, the highest and lowest essential oil indices were detected in full flowering and fruit set stages, respectively (Table 3). The essential oil content of full flowering stage was 9% and 50% higher than before flowering and fruit set stages, respectively (Table 3).

- ***Essential oil constituents***

Analysis of essential oil of *R. officinalis* L. showed that not only the content of essential oil but also its constituents were affected by phenological stages. At the before flowering stage, 32 constituents of the rosemary oil were identified by GC and GC/MS analysis (Table 3). At full flowering and fruit set stages 30 and 28 constituents of the rosemary oil were identified, respectively (Table 3). The main constituents of rosemary oil at before flowering stage were 1,8-cineole, α-pinene and verbenone (13.68%), camphor (6.85%), borneol acetae (6.45%), borneol (6.21%), camphene (5.80%), β-pinene (4.15%), geraniol (3.47%) and linalool (3.05%). The main constituents of its oil at full flowering stage were 1,8-cineole (17.90%), verbenone (16.27%), α-pinene (14.76%), borneol (9.04%), camphor (6.48%), geraniol (4.07%), α-terpineol (3.58%), camphene, linalool and borneol acetae (3.34%) and the main constituents of the oil at fruit set stage were α-pinene (21.77%), 1,8-cineole (19.61%), verbenone (13.45%), borneol (8.67%), camphor (6.16%), camphene (4.57%) and borneol acetae (3.43%) (Table 4).

The compounds in essential oil from *R. officinalis* L. leaves can be divided into two main groups: monoterpenes and sesquiterpenes. Monoterpene hydrocarbons were found to be the major group of the compounds. For all three phenological stages, 1,8-cineole, α-pinene and verbenone monoterpenes constituted the major part of the essential oil. The highest level of 1,8-cineole and α-pinene were observed at fruit set stage, while highest level of verbenone was observed in full flowering stage (Fig. 3).

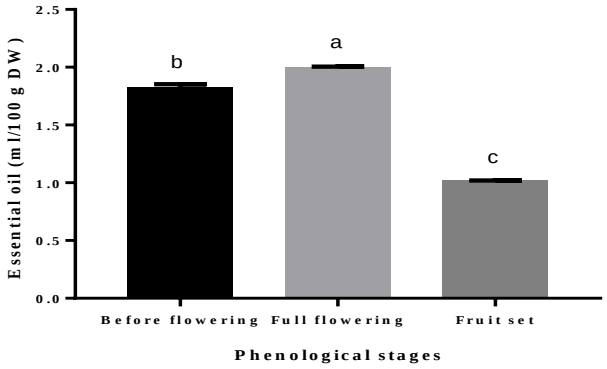

Fig. 2. Effects of phonological stages on essential oil content of *Rosmarinus officinalis* L.

Table 3. Effects of phenological stages on essential oil and rosmarinic acid content of *R. officinalis* L.

Harvest time	Essential oil content (ml/100 g DW)	Essential oil index in %	Rosmarinic acid (mg g^{-1} DW)	Rosmarinic acid index in %
Before flowering	1.82±0.026 [b]	180	23.59±0.1 [b]	100
Full flowering	1.99±0.008 [a]	197	23.63±0.02 [b]	100.16
Fruit set	1.01±0.003 [c]	100	25.92±0.0018 [a]	109.87

Mean separation in each column by Tukey test $P \leq 0.05$. The values are the mean±SEM.

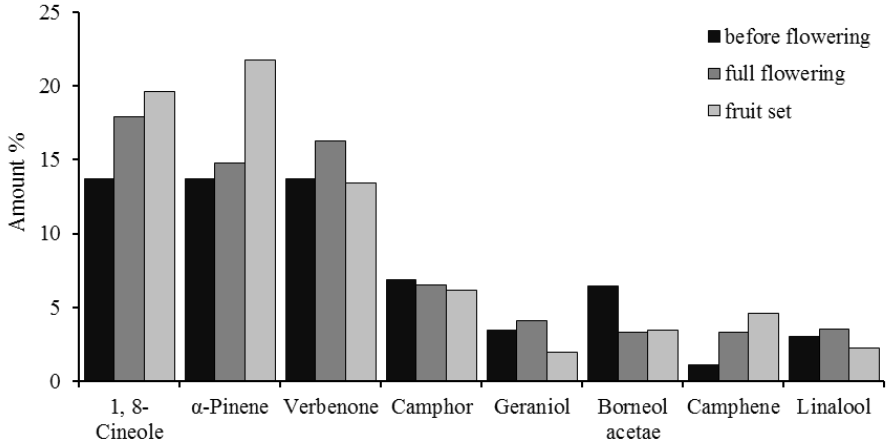

Fig. 3. Major composition of monoterpene in the essential oil of *Rosmarinus officinalis* L. in various phonological stages

Table 4. Essential oil components of *Rosmarinus officinalis* L. at three harvest stages. Before flowering (BF), full flowering (FF) and fruit set (FS)

	Constituents	BF (%)	FF (%)	FS (%)
1	Tricyclene	0.34	0.17	0.22
2	α-Thujene	0.39	0.12	0.17
3	α-Pinene	13.68	14.76	21.77
4	Camphene	5.80	3.34	4.57
5	Verbenene	0.86	0.72	0.76
6	β- Pinene	4.15	1.77	2.17
7	Ethyl amyl ketone	0.65	0.48	0.54
8	β-Myrcene	2.39	1.16	1.32
9	α-Phellanrene	0.42	0.25	0.23
10	1,8-Cineole	13.68	17.90	19.61
11	γ-Terpinene	1.73	0.90	0.70
12	β- Terpineol,Z	0.88	-	-
13	α-Terpinolene	1.42	0.78	0.63
14	Linalool	3.05	3.54	2.27
15	Chrysanthenone	0.95	0.69	1.24
16	Camphor	6.85	6.48	6.16
17	Pinocarvone	0.36	-	0.55
18	Borneol	6.21	9.04	8.67
19	Pinocamphone	1.27	1.57	1.42
20	Terpinene-4-ol	1.23	1.90	1.28
21	α-Terpineol	2.74	3.58	2.50
22	Myrtenol	0.48	0.80	-
23	α-Fenchene	-	-	0.96
24	Verbenone	13.68	16.27	13.45
25	Geraniol	3.47	4.07	1.95
26	Z-Citral	0.22	0.16	-
27	Borneol acetae	6.45	3.34	3.43
28	Geranyl acetate	0.39	0.19	-
29	Methyleugenol	0.40	0.20	-
30	Trans-Caryophyllene	1.36	0.63	0.99
31	α-Humulene	0.27	-	-
32	Caryophyllene oxide	0.81	0.46	0.62
33	Globulol	0.23	0.20	-
34	β-Citronellol	-	0.24	-
35	Benzen,1-methyl-4	-	-	1.01
36	Trans-Sabinene hydrate	-	-	0.34

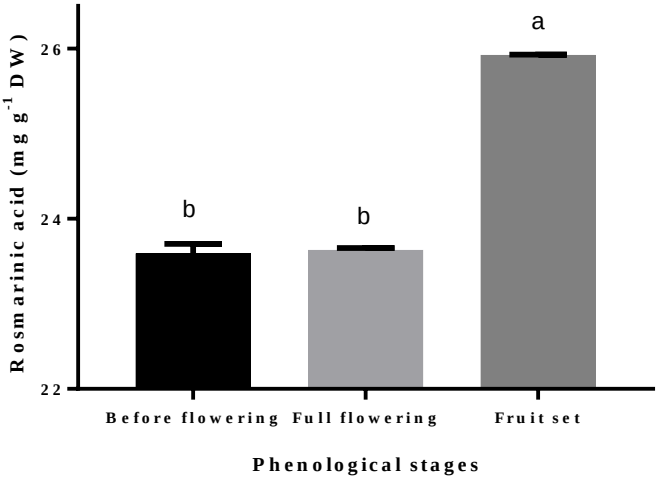

Fig. 4. Effects of phonological stages on rosmarinic acid content of *Rosmarinus officinalis* L.

- ***Rosmarinic acid content***

Phenological stages had significant effect on rosmarinic acid content of *Rosmarinus officinalis* L. (Table 2). Contrary to essential oil content, the highest rosmarinic acid content (25.92 mg/g DW) was observed in the fruit set stage in comparison with the rosmarinic acid content in before flowering and full flowering stages (23.59 and 23.63 mg/g DW, respectively) (Fig. 4). The rosmarinic acid content in fruit set stage was 9.87% and 9.16% higher than before flowering and full flowering stages, respectively (Table 3).

Discussion

The results of the current study showed that the Rosemary oils obtained from the different phenological stages had different compositions. It has been shown that environmental conditions as well as phenological stages can affect the yield of medicinal plants (Anwar, 2009; Aliniaeifard et al., 2010). Early or late harvesting of medicinal plants during their growth period would result in decreased yield of leaves as well as the content of essential oil. Medicinal plants would be immature or over-mature if they are harvested in early or late stages than the optimum time for having the highest vegetative yield or essential oil content. As the season progresses, the specific ontogenic growth stages would influence the harvesting part (Saeb et al., 2012). On the basis of the current research, *Rosmarinus officinalis* L. essential oil content, constituents and rosmarinic acid content were strongly affected by phenological stages. The results showed that full flowering stage is the best time for harvest in order to have the highest essential oil content. With respect to rosmarinic acid, the best time for harvesting of the *Rosmarinus officinalis* L. plants is fruit set stage. Previous studies in other medicinal plants also confirmed that phenological stages can influence the quality of essential oil (Saeb et al., 2012). Emadi et al. (2007) reported that the constituents in leaves of *Rosmarinus officinalis* L. plants collected in three different periods (before, after and during blooming) were α-Pinene (20.08%, 27.65% and 17.82%, before, after and during blooming, respectively), 1, 8-Cineole (7.32%, 7.55% and 9.99%, before, after and during blooming, respectively) and Camphor (9.11%, 8.84% and 15.68%, before, after and during blooming, respectively). In a study on *Hypericum perforatum*, effect of harvest time on essential oil content and composition were investigated and full flowering time was

reported as the most suitable time for collection and best composition of *Hypericum perforatum* essential oil (Azizi, 2008). McGiimpsey et al. (2006) reported seasonal variation in the essential oil composition and yield from naturalized *Thymus vulgaris* collected from New Zealand. The highest essential oil yield from *Thymus vulgaris* was reported in the samples collected in spring time and over the nine month harvesting period, thymol was found to vary from 31.5% to 52.4%. Ramezani et al. (2009) investigated the effect of phenological stages on essential oil content of coriander (*Coriandrum sativum* L.). They reported that green fruits stages possessed the highest essential oil content in coriander. In garden Thyme (*Thymus vulgaris* L.), although beginning of the blooming stage had the highest essence efficiency, but the highest fresh and dry herbages as well as essence and thymol yield were observed in 50% blooming stage. As a result it was reported that 50% blooming stage is the best harvesting times for obtaining the highest essential oil and thymol yield in garden Thyme (Golparvar et al., 2011).

On the basis of the findings of current study, harvesting of rosemary plants at full flowering stage would result in higher essential oil content than those harvested at earlier or later phenological stages. The study more revealed that harvesting of the rosemary plant at fruit set stage is the optimum phenological time to have the highest amount of rosmarinic acid content. Monoterpene hydrocarbons were found to be the major group of the compounds in rosemary plants. For all three phenological stages (before flowering, full flowering and fruit set stages) 1,8-cineole, α-pinene and verbenone monoterpenes constituted the major part of the essential oil.

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