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Growth, herb and essential oil of *Marrubium vulgare* as affected by phenological stages and planting dates

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ABSTRACT

The objective of this study was to investigate the effect of planting dates and harvesting at different developmental stages on growth, herb, essential oil content and its composition of Marrubium vulgare grown under Egyptian conditions. Marrubium vulgare seeds were sown in the nursery in three different dates (March 15, April 1, and April 15). After two months, seedlings were transplanted into the field. Plants from each planting date were harvested in three developing stages, i.e. pre-flowering/vegetative stage, full-flowering and post-flowering/seed forming growth. Essential oil was obtained by hydro distillation and essential oil % was expressed as ml 100 g^{-1} fresh herb. Chemical composition of the essential oil was studied using GC-MS. Marrubium plants cultivated early on March 15th gave the highest plant height, number of branches and fresh and dry weights as compared to the plants that was cultivated later on April 1st or April 15th. On the other hand, harvesting plants at the late flowering stage gave the highest values of these parameters. Regarding the essential oil production, Marrubium plants cultivated early on March 15th or on April 1st and cut at the full flowering stage gave the highest essential oil % and yield as compared to cutting early before flowering stage. About 31 compounds were identified in the essential oil of Marrubium vulgare. Carvacrol (31.80- 40.75%), β-phellandrene (10.99-15.89%) and carvyl acetate (7.57-12.16%) were the major compounds. Whereas β-thujene, α-pinene, 1-octen-3-ol, β-pinene, α-terpinene, 1,8-cineol, linalool, borneol, γ-cadinene and trans-caryophyllene considered minor compounds.

Keywords: *Marrubium vulgare*, Cultivation date, Phonological stages, Essential oil, GC-MS

INTRODUCTION

Marrubium vulgare L. (horehound, white horehound) belongs to the *Lamiaceae* family is a perennial, herbaceous medicinal plant native to temperate regions. This plant was frequently employed as a folk medicine to treat a variety of ailments related to upper respiratory tract infections. Nowadays, the plant is widely used as a herbal medicine to treat liver diseases, biliary tract disorders, bronchial asthma and nonproductive cough [1, 2]. It possesses tonic, stimulant, expectorant, antispasmodic, antidiabetic, diaphoretic, diuretic properties [3-6]. Essential oil is appreciated for their bioactive efficacy as fungicides, bactericides [7], antioxidant [5] and other biological activities.

The essential oil and the extract obtained from the aerial parts of *Marrubium vulgare* have been shown to have strong antimicrobial and antioxidant activities [8]. The main active ingredient that is produced and accumulated in the aerial parts of the plant is a diterpenoid known as marrubiin [9]. A substantial antioxidant, anticoagulant, antiplatelet and antiinflamatory effects have been attributed to marrubiin [10]. Extensive pharmacological studies have demonstrated that marrubiin displays a suite of activities including cardioprotective [11], vasorelaxant [12],

gastroprotective [13], antispasmodic [14], immunomodulating [15], antioedematogenic [16], analgesic [17] and antidiabetic properties [18]. *Marrubium vulgare*, also contains marrubenol and phenylpropanoid esters which have been shown to exhibit L-type calcium channel blocking and cyclooxigenase (COX) inhibitor activities [12, 19]. The crude extract of *Marrubium vulgare* has been shown to decrease systolic blood pressure in spontaneously hypertensive rats and to inhibit KCl-induced contraction in rat aorta [20].

Variation in the composition of essential oil is influenced by three major factors: (1) individual genetic variability; (2) variation among different plant parts and their different stages of development; and (3) modifications due to the environment Franz [21]. These factors influence the plant's biosynthetic pathways and, consequently, the relative proportion of the main constituents. Planting date is an important factor in the crop production and influences growth, biomass partitioning and oil accumulation as a result of the change in the environmental conditions, and therefore related to plant performance. Temperature, sunlight and other meteorological factors may individually or collectively limit or enhance the plant growth and production [22, 23]. For instance, in ajowan plants, plant height, number of branches per plant, number of umbels/plant, essential oil content and composition of seeds were significantly affected by planting dates [22].

Developmental stage of the plant (ontogeny), affects the yield and composition of essential oil and therefore harvesting time is one of the most important factors influencing essential oil production [24, 25]. Numerous studies in various aromatic plants have demonstrated that essential oil accumulation and its composition were affected by different development stages [26, 27]. Currently, there is an increasing demand on the essential oils for their use in different industries, such as cosmetics, food additives, and pharmaceuticals. Therefore, searching for new aromatic plants that provide high quality essential oil is important to meet this demand. In this concern, *Marrubium vulagre* is one of the promising aromatic plants that showed successful cultivation under the Egyptian conditions. However, there is no data about the best planting date and /or development stage for optimizing the yield of *Marrubium vulgare*. So, the objective of the current study was to study the effect of planting dates and different development stages on growth, aerial herb productivity and essential oil content as well as its composition of *Marrubium vulgare* under conditions of Egypt.

EXPERIMENTAL SECTION

Plant material

Seeds of *Marrubium vulgare* were obtained from the HEM ZADEN B.V - P.O. Box 4 - 1606 ZG Venhuizen - The Netherlands. Seeds were sown in the nursery on March 15, April 1, and April 15 throughout the two successive seasons. Two months after seed sowing, the seedlings were subsequently transplanted into the field in May 15, June 1, and June 15, respectively into plots 3x3.5m. on rows, with 60cm a part and 20 cm between the seedlings. The experimental layout was a complete randomized block design with three replications. This study was carried out during 2013 and 2014 growing seasons at the experimental farm of the Faculty of Pharmacy, Cairo University, Giza governorate, Egypt. Before sowing, physical and chemical properties of the soil of the experiment were determined by standard methods [28]. The soil texture was sandy loam, having a physical composition as follows: 50.8% sand, 26% silt, 23.2% clay and 0.58% organic matter. The results of soil chemical analysis were as follows: pH= 8.4; total nitrogen= 0.05%; available phosphorus= 0.65 mg 100 g^{-1} and potassium=18.92 mg 100 kg^{-1}.

Growth parameters (plant height, number of branches, herb fresh and dry weights/plant or fed) and essential oil content of the fresh herb of each planting date were determined. Plants from each planting date were harvested in three developing stages, i.e. pre-flowering/vegetative stage, full-flowering and post-flowering/seed forming stage. Plants were cut at 10 cm above soil levels, and the herb was weighed and used for essential oil extraction

Extraction and analysis of essential oil

Representative plant samples, separated by planting dates and developing stages, were used for extraction of essential oil by hydro distillation using a Clevenger apparatus according to the method described in the British Pharmacopoeia [29]. Essential oil % was expressed as (ml 100 g^{-1} fresh herb), while essential oil yield per plant was expressed as ml $plant^{-1}$. The resulted essential oil of each treatment was collected and dehydrated over anhydrous sodium sulphate and kept in refrigerator until GC-MS analyses.

GC-MS analyses and identification of components

The analysis of the essential oils were performed using GC/MS system consisted of a HP 5890 series II gas chromatograph, HP 5973 mass detector.TR-FAME (Thermo 260 M142 P) capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) was used with helium as the carrier gas, at a flow rate of 1.5 ml/min. GC oven temperature was programmed at an initial temperature of 40°C for 5 min, then heated up to 140°C at 5°C/min and held at 140°C for 5 min, then heated to

280°C at 10°C/min and held for 5 additional minutes. Injector and detector temperatures were 250°C. Diluted sample (1/100, v/v in heptane) of 1.0 μl were injected automatically. Mass spectrometry was run in the electron impact mode (EI) at 70 eV. The components were identified based on the comparison of their GC retention times, interpretation of their mass spectra and confirmed by mass spectral library search using the National Institute of Standards and Technology (NIST) database [30,31].

Statistical analysis

Except for the constituents of the essential oil, the data in this study were analyzed with the analysis of variance (Two-way ANOVA) using JMP 10 program (SAS Institute, NC, USA). The mean values of treatments were compared using Tukey's HSD test. Values accompanied by different letters are significantly different at $p \leq 0.05$.

RESULTS AND DISCUSSION

Growth parameters

Plant height, number of branches, herb fresh and dry weights (g/plant or ton/fed.) of *Marrubium vulgare* were significantly affected by different planting dates (table 1-3), where the first date of planting (15 March) gave the best values of these features followed by the second date of planting (1 April) and finally the third date of planting (15 April), which gave lower values of all these traits. These results are in agreement with some reports in the literature. For instance, the study done by Ramesh and Sing[32] clearly indicated that planting dates had profound influence on growth and development of *Tagetes minuta* as reflected from the significant variation on growth phase duration, and aerial biomass partitioning. Jadczak [33] reported that sowing date (10 April, 25 April and 10 May) had a significant influence on the yield quantity of summer savory. Higher yield was obtained when seed were sown on 25 April, but the highest participation of leaves in the yield was noted for sowing on 10 April. In another study, Ziombra and Frąszczak [34] studied the effects of sowing date (7, 14, 21 and 28 April) on summer savory. The highest fresh herbage mass yield was recorded in plants sown on 14 and 21 April. Also, Soleiman et al. [22] studied the effect of different planting dates from October to March on the ajowan productivity. It was indicated that delayed planting date significantly reduced plant height, number of branches per plant, and yield. At first planting date, plants had sufficient time for of the interaction with the environmental factors leading to higher growth. On the other hand, by delaying planting date, time duration for plant growth was decreased leading to a decrease in plant height, the number of branches per plant and fresh and dry weights. Also D'Antuono et al. [35] stated that earlier planting of *Nigella* species resulted in higher plant height and dry biomass. Delayed planting date decreased plant height of milk thistle [36] as well as plant height, number of branches/ plant, number of capsules/plant and number of capsules/branch in sesame plant [37].

Table 1. Plant height (cm) and number of branches of *Marrubium vulgare* as a function of cultivation dates and phenological stages in two successive seasons

Cultivation date	Phenological stage	Plant height (cm)		Number of branches	
		1st season	2nd season	1st season	2nd season
15th March	Before flowering	38.1±1.6 d	36.7±1.4 d	12.5±1.2 cd	11.8±0.6 c
	Full flowering	53.3±1.5 b	53.4±1.7 b	18.8±0.7 b	19.1±0.8 b
	Late flowering	62.7±2.0 a	59.9±0.7 a	27.0±0.6 a	25.3±1.5 a
1st April	Before flowering	37.4±1.4 d	37.2±0.2 d	7.3±0.4 ef	6.67±0.2 de
	Full flowering	45.9±2.1 c	44.1±0.6 c	11.6±0.7 cd	10.8±0.4 c
	Late flowering	53.3±2.2 b	52.7±1.2 b	15.3±0.5 bc	16.5±0.3 b
15th April	Before flowering	28.9±1.68 e	26.3±0.4 e	5.2±0.39 f	5.6±0.4 e
	Full flowering	43.2±3.1 cd	40.2±0.7 cd	9.9±0.5 de	9.53±0.7 cd
	Late flowering	50.2±0.7 b	50.7±0.9 b	13.0±1.2 cd	12.2±0.4 c

**Different letters within the same column denote significant differences at the level≤0.05*

Table 2. Fresh weight and dry weights (g/plant) of *Marrubium vulgare* as a function of cultivation dates and phenological stages in two successive seasons

Cultivation date	Phenological stage	Fresh weight (g)		Dry weight (g)	
		1st season	2nd season	1st season	2nd season
15th March	Before flowering	95.6±6.2 bc	101±0.2 bc	18.7±1.2 c	18.0±0.1 b
	Full flowering	109±5.9 ab	118±6.3 ab	23.0±0.4 b	23.3±0.5 a
	Late flowering	133±7.5 a	131±5.3 a	27.0±1.7 a	25.6±0.8 a
1st April	Before flowering	60.4±3.5 de	57.0±1.5 de	12.3±0.1 de	11.9±0.2 cd
	Full flowering	74.6±4.9 cd	83.5±1.8 c	13.8±0.4 d	13.5±0.8 c
	Late flowering	93.2±6.9 bc	114.3±7.0 ab	17.6±0.1 c	18.0±0.1 b
15th April	Before flowering	36.4±4.35 e	31.0±2.18 f	7.10±0.36 f	7.2±0.28 e
	Full flowering	45.4±3.4 e	39.0±1.5 ef	9.27±0.1 ef	9.67±0.8 de
	Late flowering	73.7±1.9 cd	59.9±3.0 d	12.8±0.3 de	12.6±0.5 c

**Different letters within the same column denote significant differences at the level≤0.05*

Mohammadpour et al. [38] found that on summer savory, the highest plant height, lateral shoot, number of nod, shoot diameter were recorded for first sowing date (11 April). The highest fresh and dry shoot yield was obtained at third and second sowing time (22 April and 3 May). Ahmadi and Hadipanah [39] reported that the highest plant height of dragonhead was recorded for first sowing date, but the highest herb fresh and dry weights were obtained at third and second sowing time. Similarly, planting dates affected growth and vegetative traits of *Mentha arvensis* [40] Moldavian balm [41, 42] and cumin [43].

From table (1-3), we found that plant height, number of branches, herb fresh and dry weights (g/plant or ton/fed.) of *Marrubium vulgare* were affected by the different dates of harvest (different development stages). An increase in plant height, number of branches, herb fresh and dry weights of *Marrubium vulgare* was associated with the harvest of plants in late flowering stage followed by harvesting in the full flowering stage and then harvest at before-flowering stage, which gave lower values of all these traits.

Table 3. Fresh weight and dry weight (ton/fed) of *Marrubium vulgare* as a function of cultivation dates and phenological stages in two successive seasons

Cultivation date	Phenological stage	Fresh weight (ton/fed)		Dry weight (ton/fed)	
		1st season	2nd season	1st season	2nd season
15th March	Before flowering	3.10±0.20 bc	3.27±0.01 bc	0.61±0.04 c	0.58±0.00 b
	Full flowering	3.52±0.19 ab	3.81±0.20 ab	0.75±0.01 b	0.76±0.02 a
	Late flowering	4.32±0.24 a	4.23±0.17 a	0.88±0.06 a	0.83±0.03 a
1st April	Before flowering	1.96±0.11 de	1.85±0.05 de	0.4±0.00 de	0.38±0.01 cd
	Full flowering	2.42±0.16 cd	2.71±0.06 c	0.45±0.01 d	0.44±0.02 c
	Late flowering	3.02±0.22 bc	3.70±0.23 ab	0.57±0.0 c	0.58±0.0 b
15th April	Before flowering	1.18±0.14 e	1.0±0.07 f	0.23±0.01 f	0.23±0.01 e
	Full flowering	1.47±0.11 e	1.26±0.05 ef	0.30±0.0 ef	0.31±0.03 de
	Late flowering	2.39±0.06 cd	1.94±0.10 d	0.42±0.01 de	0.41±0.02 c

**Different letters within the same column denote significant differences at the level≤0.05*

These results are in agreement with those of Golparvar et al. [44], where they collected the aerial parts of *Thymus daenensis* in five stages of plant growth, i.e. the before blooming, beginning of blooming, 50% blooming, full blooming and fruit set. Results indicated that different stages of plant growth had significant effect on plant height, diameter, fresh and dry herbage weights and the highest yields of fresh and dry herb were obtained at the stage of fruit set. Randhawa and Gill [45] found that a delay in harvesting basil from vegetative to complete flowering stage increased plant height and number of branches per plant. Herb yield were maximum at complete flowering. Studies reported by Omidbaigi et al. [46] indicated that maximum essential oil production of lemon thyme (*Thymus×citriodorus* (Pers.) Schreb) was obtained at the fruit-set stage, Ozguven and Tansi [26] showed that various harvesting times have significant effect on dry biomass weight and the highest dry matter was obtained at the stage of fruit set. Ziombra and Frąszczak [34] harvested summer savory at three different developmental phases of the plant: at flower bud formation period, at the beginning of flowering and in full flowering. Variation in herbage yield depended on the developmental stage. The highest fresh herbage mass yield was recorded for the plants harvested at the beginning of flowering and in full flowering. For the peppermint harvested at flowering, biomass yields were greater than that harvested at bud formation stage [47]. Chauhan et al. [48] reported that the plant height of *Origanum vulgare* significantly increased with the advancement in the crop age; number of branches increased up to flowering initiation stage and reduced at the time of maturity, i.e. full bloom and fruit set stages. The fresh and

dry herbage yield was significantly increased from early vegetative to full bloom stage, whereas in fruit set stage, the herbage yield was reduced.

Essential oil content

There were significant differences in the essential oil percentages depending on planting dates and phonological stages and their interaction (Table 4). Also, date of planting on the 1st of April gave the highest percentage of volatile oil and oil yield, followed by the date of planting on the 15th of March, while the date of planting on the 15th of April gave the lowest percentage of volatile oil and oil yield.

Table 4. Essential oil (%) and yield (L/fed) of *Marrubium vulgare* as a function of cultivation dates and phenological stages in two successive seasons

Cultivation date	Phenological stage	Essential oil (%)		Essential oil yield (L/fed)	
		1st season	2nd season	1st season	2nd season
15th March	Before flowering	0.01±0.001 e	0.01±0.0 f	6.67±0.60 c	5.84±0.0 c
	Full flowering	0.04±0.002 ab	0.04±0.002 ab	28.6±1.6 a	31.5±0.6 a
	Late flowering	0.03±0.002 b-d	0.03±0.002 b-d	23.4±2.0 ab	23.5±1.6 b
1st April	Before flowering	0.02±0.002 de	0.02±0.003 d-f	7.3±0.7 c	5.8±1.2 c
	Full flowering	0.05±0.004 a	0.05±0.004 a	21.6±2.1 b	22.6±2.4 b
	Late flowering	0.03±0.002 a-c	0.03±0.002 bc	19.0±1.0 b	18.5±1.1 b
15th April	Before flowering	0.02±0.004 de	0.01±0.002 ef	3.84±1.0 c	2.68±0.26 c
	Full flowering	0.03±0.002 b-d	0.03±0.003 c-e	9.52±0.6 c	7.97±1.6 c
	Late flowering	0.02±0.002 c-e	0.02±0.003 d-f	9.02±0.8 c	6.13±1.1 c

**Different letters within the same column denote significant differences at the level≤0.05*

These results are in agreement with those of Bourna et al. [41]; Okhchlar et al. [42]; Ahmadi and Hadipanah [39]. Ramesh and Sing [32] found significant variations in essential oil yield of *Tagetes minuta* as a result of planting dates. Soleimani et al. [22] studied the effect of planting date (in 30-day intervals from October to March) on ajowan. Essential oil percentage was significantly influenced by planting time. The highest oil value was obtained from plants cultivated in October, while the lowest essential oil was obtained from plants cultivated in March. Omidbaigi et al. [49] stated that essential oil content of dragonhead was reduced by delaying planting dates.

In the current study with regard to the harvest dates, it was found that there was a significant effect of the harvest dates on the volatile oil percentages and oil yield. Harvesting in full flowering stage was the best in accumulating the volatile oil of *Marrubium vulgare* plants followed by plants harvested at the late flowering stage, and then plants harvested at before- flowering stage, which gave the lowest percentages and yield of volatile oil in both seasons. Golparvar et al. [44] harvested *Thymus daenensis* on (before blooming; beginning of blooming; 50% blooming; full blooming and fruit set) and found that the highest oil percentage was obtained at the stage of 50% blooming and the lowest oil percentage was obtained at the stage of before- blooming. In the meantime, the highest oil yield was obtained at the stage of fruit set. In peppermint, the essential oil accumulated until the beginning of flowering and a significant decrease was observed afterwards [47, 50-52]. Other authors found the essential oil content was the highest at full flowering time [53, 54]. In hyssop, the essential oil increased during the development of the shoots. The highest oil content was measured at full flowering, after which the oil content tend to decrease [55]. The essential oil of Salvia officinalis cultivated in Italy and harvested in spring at the flowering stage produced the highest fresh and dry yields of herb. Moreover, the oil composition was greatly differed by the developmental stage (vegetative stage versus the flowering stage) and by time of cut (spring versus autumn) [56]. Chauhan et al. [48] studied the effect of five stages of development (phenological stages), i.e. early vegetative, late vegetative, flower initiation, full bloom, and fruit set stages on *Origanum vulgare* and found that, the oil yield increased from early vegetative to reach its maximum level at full bloom stage, whereas in fruit set stage, the oil yield reduced. Verma et al. [57] also reported that the oil content in *O. vulgare* was increased with the advancement of crop age to reach its maximum at full bloom stage, and afterwards it started to decrease. The oil content was increased at the time of full flowering in *O. majorana* [58, 59]. In fennel, Chung and Nemeth [60] and Bernath et al. [61] found that essential oil content increased from the appearance of buds until the stage of green fruits, then at ripening of the fruits, an opposite trend was observed. However, Cavaleiro et al. [62] and Gupta et al. [63] found the highest essential oil content at the ripe fruit stage.

GC/MS analysis

The results of the GC/MS analysis of the essential oils of the *Marrubium vulgare* in the second season are shown in Table (5). About 31 compounds were identified and grouped into three categories ie., major compounds (more than 10%), minor compounds (less than 10% and more than 1%) and trace ones (less than 1%). In this respect, it is evident that, carvacrol (31.80- 40.75%), β-phellandrene (10.99-15.89%) and carvyl acetate (7.57-12.16%) were majors.

From (Table 5), we found that plants that were cultivated late at April 15, and cut early at pre-flowering stage gave the the lowest percentage of carvacrol (31.80%), while plants cultivated on April 1st and cut at full-flowering gave the highest percentage of carvacrol (40.75%) and the lowest percentage of β- phellandrene (10.99%) and carvyl acetate (7.57%). For the other major compounds, plants cultivated on March 15th and cut at pre-flowering gave the highest percentages of carvyl acetate (12.16%). β-phellandrene compound was the highest (15.89%) in plants that were cultivated on March 15th and cut at the full-flowering stage.

Comparing planting dates we found that the early cultivated date on March 15th was the best in the contents of volatile oil three main compounds a combined, followed by planting date on April 1st and then planting date on April 15th. And also we found that the date of harvest at full flowering is optimized for high content of volatile oil three main compounds a combined followed by harvest at late flowering and then harvested plants at pre flowering time. In general, we concluded that the first date of planting March 15th and harvesting at full-flowering was the best in the contents of carvacrol, β- phellandrene and carvyl acetate a combiend.

Our result indicates that the accumulation of essential oil in *Marrubium* is largely influenced by the time of harvest and planting date. The biosynthesis of secondary metabolites, although controlled genetically, is strongly affected by the environmental influences of a particular growing region. In this regard, the differences in the volatile composition of the plants could be attributed to genetic (genus, species, and ecotype), chemotype, distinct environmental and climatic conditions, seasonal sampling periods, geographic origins, plant populations, vegetative plant phases, and extraction and quantification methods.

Table 5. Major compounds (more than 10%) of the essential oils of *Marrubium vulgare* herb

Compound	Planting date								
	15th March			1st April			15th April		
	Phenological stage								
	Before flowering	Full flowering	Late flowering	Before flowering	Full flowering	Late flowering	Before flowering	Full flowering	Late flowering
carvyl acetate	12.16	11.23	10.11	9.66	7.57	10.03	10.20	10.66	10.16
β-phellandrene	15.07	15.89	12.55	12.06	10.99	14.00	13.87	14.01	11.78
carvacrol	35.77	39.06	38.60	36.38	40.75	34.95	31.80	33.63	33.32

The chemical composition of *Marrubium vulgare* essential oil from various origins has been the subject of many studies. The literature shows the occurrence of many chemotypes. In Egypt, Salama et al. [64] reported that thymol and γ-cadinene are major components and also β-caryophyllene and germacrene-D [65]; carvacrol, β-phellandrene, carvyl acetate [66]. In Lithuania, the main constituents of M. vulgare volatile oil were (Z)-β-farnesene, β-caryophyllene, (E)-2-hexenal, α-humulene and germacrene D [67]. In Czech Republic, β-caryophyllene and germacrene D were the main components [68]. In Poland, the main components of the oil of *M. vulgare* were E-caryophyllene, germacrene D and δ-amorphene [69]. In Tunisia, the major constituents of *M. vulgare* volatile oil were β-bisabolene, β-caryophyllene, (E)-β-farnesene and 1,8-cineole [70]. In Libya, carvacrol, E-β-farnesene and thymol [71]. In Algeria, eugenol and β-bisabolene [72]. Also, α-pinene, germacrene D-4-ol and 4,8,12,16-tetramethyl heptadecan-4-olid were three major compononents in some Algerian chemotypes [73]. In Iran, various chemotypes were identified. For example, Khanavi et al. [74] reported β-caryophyllene, β-bisabolene, germacrene-D and (E)-β-farnesene as the major components of *Marrubium vulgare*. Whereas, Saleh and Glombitz [75] found β-pinene,tricyclene, bisabolol, β-elemone and isomenthon-8-thiol were main compounds.

Table 6. Minor compounds (less than 10% and more than 1%) of the essential oils of *Marrubium vulgare* herb

Compound	Planting date								
	15th March			1st April			15th April		
	Phenological stage								
	Before flowering	Full flowering	Late flowering	Before flowering	Full flowering	Late flowering	Before flowering	Full flowering	Late flowering
β-thujene	2.18	1.90	2.52	5.03	2.70	2.23	2.90	2.80	1.72
α-pinene	0.44	0.77	1.04	3.06	1.22	1.30	1.36	1.41	1.55
1-octen-3-ol	1.99	1.36	0.89	1.23	2.00	2.12	1.87	3.03	4.56
β-pinene	1.98	0.67	1.45	2.53	2.19	2.22	2.50	3.78	4.66
α-terpinene	1.89	3.11	3.40	1.23	3.89	1.63	3.76	1.80	3.31
1,8-cineol	0.85	0.57	1.04	2.12	3.08	1.80	1.22	1.40	0.09
linalool	4.26	3.45	3.79	1.86	0.99	2.13	2.55	3.13	3.33
borneol	0.72	1.10	1.42	3.18	5.62	5.77	1.78	1.80	3.10
γ-cadinene	1.08	3.01	2.41	0.93	1.23	1.56	3.23	2.00	2.78
trans-caryophyllene	4.34	1.96	3.67	5.56	5.13	4.22	2.40	3.45	4.13

As shown in Table (6), β-thujene (1.72-5.03%), α-pinene (0.44-3.06%), 1-octen-3-ol (0.89-4.56%), β-pinen (0.67-4.66%), α-terpinene (1.23-3.89%), 1,8-cineol (0.09-3.08%), γ-cadinene (0.93-3.23%), linalool (0.99-4.26%), borneol (0.72-5.77%) and trans-caryophyllene (1.96-5.56%) were represented as minors. Plants cultivated on April 15th and cut at late-flowering gave the highest percentages of β-pinene (4.66%), 1-octen-3-ol (4.56%). Plants cultivated on April 1st and cut early at pre-flowering gave the highest percentages of β-thujene (5.03%), α-pinene (3.06%) and trans-caryophyllene (5.56%). While, plants cultivated on April 1st and cut at full-flowering gave the highest percentages of α-terpinene (3.89%), 1,8-cineol (3.08%) and borneol (5.62%). But, plants cultivated on March 15th and April 15th and cut at pre-flowering gave the highest percentages of linalool (4.26%) and γ-cadinene (3.23%), respectively. As well as, the remaining compounds (less than 1%) included in Table (7) were considered as traces.

Table 7. Trace compounds (less than 1%) of the essential oils of *Marrubium vulgare* herb

Compound	Planting date								
	15th March			15th March			15th March		
	Phenological stage								
	Before flowering	Full flowering	Late flowering	Before flowering	Full flowering	Late flowering	Before flowering	Full flowering	Late flowering
camphene	0.60	0.47	-	0.04	0.23	0.44	0.46	0.11	0.31
sabinene	0.18	-	0.33	0.13	0.25	0.05	0.21	0.20	0.33
3-octanol	0.40	0.05	0.44	0.56	-	-	0.57	0.66	0.89
α-phellandrene	0.70	0.40	0.55	0.81	0.66	0.78	0.96	0.90	0.75
limonene	0.74	0.91	0.80	0.65	0.44	0.77	0.80	0.54	0.34
sabinene hydrate	0.57	0.23	0.46	-	0.52	-	0.32	0.50	0.34
camphor	0.25	0.64	-	0.32	-	0.24	0.11	0.80	0.45
terpinen-4-ol	0.97	0.92	0.85	0.83	0.90	0.90	0.50	0.76	0.66
α -terpineol	0.30	0.12	0.23	-	0.03	0.09	0.11	0.14	0.08
thymol	0.30	0.20	0.11	0.24	0.11	0.46	0.19	0.35	0.22
α-humulene	0.08	-	0.13	0.10	0.03	-	0.09	-	0.11
2,7dimethyl-1-octanol	0.12	0.32	0.22	0.10	-	-	0.35	-	-
α-cubebene	0.23	0.41	0.46	0.42	0.16	0.40	-	0.44	-
germacrene D	0.20	0.21	-	-	0.22	0.12	0.06	-	0.21
caryophyllene oxide	0.60	0.55	0.45	0.58	0.77	0.61	0.77	0.67	0.65
cubenol	-	0.15	0.19	-	-	0.13	0.18	-	0.11
α-copaene	-	0.24	0.22	0.13	0.12	0.32	-	0.20	0.23
(E)-β-farnesene	0.24	0.12	0.21	0.20	0.24	0.25	0.27	0.14	0.17

Asadipour et al. [76] identified three major compounds in *Marrubium vulgare*; caryophyllene oxide (18.7%), β-caryophyllene (12.8%) and germacrene-D (10.0%). β-caryophyllene, β-bisabolene, germacrene D and E-β-farnesene (8.3%) [77]; β-bisabolene (20.4%), δ-cadinene (19.1%) and isocaryophyllene (14.1%) [78]; γ-eudesmol, germacrene D, citronelly formate, β-citronellol, geranyl tiglate, geranyl formate [79] were identified as the major components in some Iranian chemotypes.

Previous studies have shown that changes in the oil composition as a result of growth development are dependent on the species and the nature of compounds. Some compounds are increased with the age of plants. For example, the essential oil yield of *Daucus sahariensis* was low during the flower-budding stage, but increased with the development of plants to reach a maximum at the flowering and fruiting stages. Also the essential oil collected from plants during the flower-budding and full-flowering periods contained mainly monoterpene hydrocarbons, while during the fruiting stage; the oil was dominated by the phenylpropanoid compounds. Myrcene in the essential oil decreased significantly from the vegetative stage to the fruiting stage. The opposite trend was observed for myristicin [80]. The essential oil and the polyphenolic extracts of *Rosmarinus officinalis* harvested at the fruit maturation phase possessed higher antioxidant and antimicrobial activities than the oil obtained from plants cut at the full-bloom phase due to the differences in chemical composition. Volatile compounds such as α-terpinene, γ terpinene, terpinolene and caryophyllene oxide in the essential oils and rosmarinic acid, hesperidin, and carnosol in the phenolic extracts were higher at fruit maturation phase [81]. The essential oil percentage in *Origanum majorana* increased from 0.04% at the early vegetative stage to 0.09% at the full-flowering stage. At the late vegetative and budding stages, percentage of essential oil showed intermediate values [59]. In *Valeriana officinalis*, valerenal, valerenic acid, and alpha-humulene contents increased with the age of plants. These differences in the composition did affect the antimicrobial efficacy of the oil [82]. The essential oil % in *Ziziphora clinopodioides* was reduced from 1.8% at the flowering stage to 1.1% at the post flowering stage [83]. In *Mentha pulegium*, the essential oil % increased from 0.3% at the vegetative stage to 1.6% at the full-flowering stage with no differences in the composition of oil as a result of cut date [84]. Chauhan et al. [48] found that thymol recorded its maximum amount in *Origanum. vulgare* at the late vegetative stage, while for γ-terpinene, the maximum % was recorded at the reproductive stages (flower initiating, full blooming, and fruit set). The percentage of p-cymene was slightly higher in full bloom and fruit set stage of the crop as compared with vegetative and flowering initiation stage. On the contrary, the highest essential oil percentage (4.72%) in *Satureja rechingeri* was obtained at the beginning of flowering stage as compared to 4.24% at full flowering stage. While, 53 compounds were identified in the essential oil at the full flowering stage, 23 compounds were detected at the full flowering phase. However, carvacrol, the main component in the oil was higher in the latter flowering stage [85]. On the other hand, the essential oil composition was not affected as a result of harvesting time in wild *Thymus mastichina* (L.) plants, but the oil yield was influenced by harvest time with the highest yield (2.1%) at the time of full flowering [24]. Similarly, M'barek et al. [86] found that the level of major compounds (α-pinene, limonene, camphor, borneol and bornyl acetate) in the essential oil of thuya (*Tetraclinis articulate*) were not influenced by the harvest period.

CONCLUSION

The current study showed that sowing date and harvest time and their interaction influence the growth and essential oil accumulation of *Marrubium* plants. Different phonological stages not only caused quantitative changes in the essential oil components, but also qualitative changes were found, which put more emphasis on the importance of selecting the proper harvesting date of herbage for essential oil usage. It can be concluded that cultivating *Marrubium* plants early on March 15th in combination with the harvest at the full flowering stage is essential for maximizing the production from *Marrubium* herb and essential oil yield. These changes could be relevant to the quality of essential oil and its use in certain food and cosmetic applications.

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