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Variation in Essential Oil Content and Chemical Composition of Rosemary (*Rosmarinus officinalis* L.) at Various Growth Stages in the Mediterranean Region

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A R T I C L E   I N F O

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A B S T R A C T

Rosemary is an aromatic shrub native to the coasts of the Mediterranean region. Understanding the changes of chemical composition of essential oil is critical for more targeted rosemary harvesting, which can lead to higher-quality essential oils for agro-food, medicinal, and cosmetics uses. Therefore, rosemary plants were harvested at pre-flowering, full-flowering, and post-flowering stage grown in the experimental area of the Department of Field Crops at Çukurova University in order to determine the most suitable harvest time for the highest amount of essential oils and their important compounds. Essential oil content decreased slightly from the pre-flowering to post-flowering. The highest essential oil content (1.68%) was determined at the pre-flowering stage. Twenty-eight compounds were found representing 95.74%-96.74% of the total essential oil. The major compounds for rosemary were camphor (28.43%-32.74%), 1,8-cineol (20.80%-22.61%), isoborneol (6.05%-7.28%), verbenol (5.17%-6.98%), and limonene (5.71%-6.23%), respectively. Consequently, the optimal harvest time in terms of essential oil content, as well as camphor, 1,8-cineole and limonene content, may be considered as pre-flowering stage.

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Introduction

Rosemary (*Rosmarinus officinalis* L.) is an evergreen shrub that belonging to the Lamiaceae family. The name of rosemary is derived from the Latin word "Rosmarinus" which means "dew of the sea" (Santos et al., 2015). From the past to the present, rosemary has been of paramount importance for both therapeutic and cultural purposes. For instance, Egyptians buried rosemary sprigs in Pharaoh's tombs (Hanson, 2016). Furthermore, one of the common believed legends is that rosemary placed under the pillow protects the sleeper from evil spirits at night (Sasikumar, 2012).

Rosemary is used in many different industries, such as the food and food packaging, pharmaceutical, perfumery, and cosmetics industries (Santos et al., 2015). Rosemary has been used for alternative and traditional medicine as a sedative, antiasthmatic, relieving, digestive, headache, anti-rheumatic, visual acuity, circulatory disorders, and irritant effects (Sales and Pashazadeh, 2020). It is cultivated widely for the important valuable essential oil currently and its essential oil is found in flowering tops, stems, and leaves. The essential oil has an intense and spicy aroma and is a colorless or light-yellow liquid. Rosemary and also its essential oil has been used as a bath essence, hair lotions, shampoos, soaps, perfumes, room sprays, and deodorants (Stefanovits-Bányai, 2003; Özcan and Chalchat, 2008; Yıldırım, 2018). Furthermore, its essential oil is used for its antimicrobial (Jiang et al., 2011), antibacterial (Stojiljkovic et al., 2018), antioxidant (Ojeda-Sana et al., 2013), and cytotoxic (Al Zuhairi et al., 2020) activities. Studies show that rosemary extracts have preventive effects for cancer and depression, which are very common today (Machado et al., 2013; González-Vallinas et al., 2015). Thanks to all these properties, rosemary has been reported as a cosmetic product that can be used as a fragrance and skincare cream at safe concentrations in the cosmetic industry, helps maintain skin homeostasis, and prevents the occurrence of some skin diseases (González-Minero et al., 2020).

Previous research on rosemary essential oils has revealed that there are primarily four chemotypes: camphor, 1.8-cineole, α-pinene, and myrcene (Elhassan et al., 2014). These differences in the chemical composition of essential oils and phenols are crucial for identifying superior chemotypes for prospective industrial uses in the food, pharmaceutical, perfumery, and cosmetic areas (Bellumori et al., 2021). Moreover, understanding the changes of chemical composition of essential oil is critical for more targeted rosemary harvesting, which can lead to higher-quality essential oils for agro-food, medicinal, and cosmetics uses. The differences in the essential oil composition of rosemary due to seasonal and geographic fluctuations may allow for the quality assessment of rosemary oils and, consequently, to determine the best period for processing rosemary (Salido et al., 2003). Since rosemary is an evergreen plant in the Mediterranean region, it is a plant suitable for leaf harvest throughout the year. However, it is important for industrial use to determine the most appropriate harvest time by determining the development stage in which the highest essential oil content and compounds are obtained. Therefore, the purpose of this study was to evaluate the quality characteristics of rosemary by comparing it to different growth stages in the Mediterranean region, where the effects of global warming and climate change are intensively felt.

Material and Method

Plant Material

Rosmarinus officinalis L. plants grown in the experimental area of the Department of Field Crops at Çukurova University (37°00′55.9″ N, 35°21′26.5″ E) were harvested at pre-flowering, full-flowering and post-flowering stage with a sickle. The materials were collected in March-May 2021 at various growth stages (pre-flowering (March 31st), mid-flowering (April 14th), and post-flowering (May 25th)). In Figure 1, the meteorological data that was recorded both throughout the harvesting year and over the long term (1961-2020) was given.

Essential Oil Extraction

For essential oil hydrodistillation, 35 g of dried leaves from each treatment were weighed and placed in a glass balloon with 350 mL distilled water. The glass balloon was then placed in the Clevenger apparatus for 2 hours. The amount of extracted essential oil was recorded. The samples were stored at 4°C until further analysis. The essential oil content was represented as a percentage of dry tissue weight. The chemical analyses were carried out in triplicate.

Gas Chromatography ± Mass Spectrometry (GC MS) Analysis

10 µl of essential oil was mixed with 0.25 mL of dichloromethane at the Department of Biology at Kahramanmaras Sutcu Imam University and 1 µl of the mixture was injected into the column for GC MS analysis. The analysis of essential oil compounds was performed using an Agilent 5975C mass spectrometer in conjunction with an Agilent GC 6890 II series. The HP-88 capillary column (film thickness of 100 m × 250 m × 0.20 m) was used in the GC, and the carrier gas flow rate was 1.0 mL/min. After maintaining a temperature of 70°C for 1 minute, the oven temperature was gradually increased to 220°C at a rate of 10°C/min and waited for 10 minutes. Temperature of the injection part was 250°C. The mass spectrometer was set to EI mode at 70 eV. The split ratio was 20:1. The mass range was 35−400 m/z and scan speed (amu/s) were 1000. The compounds were identified by mass spectra using Flavor2 and W10N14 libraries as reference compounds.

Statistical Analysis

The experimental data relevant to the outcomes of the chemical analysis were analyzed using analysis of variance (one-way). Significantly different means were separated at P=0.05 using the least significant difference (LSD) test. ANOVA and principal components analysis on correlations was performed using JMP 14.0® statistical software (SAS Institute Inc., Cary, NC, 1989-2019). The 'corrplot' package in R Studio was used to compute correlations between the characteristics by Pearson's coefficient. Flourish studio was used to create the heat map.

Results and Discussion

Chemical Composition of Essential Oil

The chemical compounds of essential oils for *R. officinalis* were analyzed by GC/MS, and obtained results were summarized (Table 1). The essential oil content obtained at three different harvest times showed statistically significant differences between the percentages calculated on a dry matter basis. Essential oil content decreased from the pre-flowering to post-flowering. Pre-flowering resulted in the highest essential oil content (1.68%) which was 16% and 20% higher than mid-flowering and post-flowering, respectively (Figure 2). When comparing these essential oil content findings with the earlier studies, diverse results were found, such as 2.73%-3.30% (Dzięcioł, 2021), 0.72%-1.73% (Afshar et al., 2021), 1.25%-1.38% (Oualdi et al., 2021), 1.5% (Aouadi et al., 2021), 2.8% (Ouknin et al., 2021), 0.5% (Karadağ et al., 2022), 1.35% (Elyemni et al., 2022), 1.35%-1.60% (Xylia et al., 2022). In Southern Iran, Afshar et al. (2021) reported that rosemary essential oil content began to increase in August and peaked (1.73%) at the beginning of the cold season (November), and following that the accumulation of essential oil production gradually decreased, reaching its lowest point in February (0.72%). On the other hand, Salido et al. (2003) revealed that the highest essential oil production (1.60%-1.80%) was observed in rosemary during the summer. However, in our study, a decrease in essential oil content was observed from spring to summer. Contrarily, Elamrani et al. (1998) reported that essential oil content was particularly high in full flowering (1.6%) and much lower in early or late flowering.

Table 1. Essential oil compounds of rosemary (*Rosmarinus officinalis* L.) from different growth stages

Growth stages		Pre–flowering	Mid–flowering	Post–flowering	Mean	LSD (%5)
Essential oil content (%)**		1.68a	1.45b	1.40b	1.51	0.13
Compounds	RT (min)	Relative Peak Area (%)				
Myrcene	11.140	1.93	1.51	1.56	1.67	
α–phellandrene	11.378	1.60	1.07	1.02	1.23	
Limonene**	11.615	6.23a	5.71b	5.81b	5.91	0.18
γ–Terpinene	12.137	2.04	2.79	2.87	2.56	
Terpinolene	12.517		0.72	0.77	0.74	
1,8–cineol**	12.660	22.61^{a}	20.80^{b}	20.82^{b}	21.41	1.08
p–Cymene	13.069	5.31	3.01	3.22	3.85	
3–Octanone	13.948	1.74	0.26	0.32	0.77	
Ylangene	14.791		0.12	0.14	0.13	
Copaene	14.909	0.36	0.51	0.55	0.47	
Linalol	16.286	1.51	2.36	2.33	2.07	
β–Caryophyllene	17.230	2.42	1.75	1.69	1.95	
Menthol	17.639	0.56	0.44	0.45	0.48	
Bornyl acetate	17.889	2.85	6.18	6.21	5.08	
α–humulene	18.245	0.37	0.53	0.52	0.47	
Isopinocamphone	18.423	0.51	0.98	0.98	0.82	
Camphor**	18.832	32.74^{a}	29.58^{b}	28.43^{c}	30.25	0.65
Isoborneol**	19.444	6.05b	7.24a	7.28a	6.86	0.33
Homomyretenol	20.008	0.55	1.38	1.40	1.11	
Myrtenol	20.203	0.56	0.76	0.73	0.68	
α–Calacorene	21.088		0.80	0.47	0.63	
Verbenol**	22.132	5.17^{c}	6.66^{b}	6.98^{a}	6.27	0.17
Isopiperitone	23.325	0.14	0.18	0.18	0.16	
Thymol	23.848	0.23	0.13	0.12	0.16	
Rosefuran	24.139	0.52	0.53	0.42	0.49	
Carvacrol	24.673	0.36	0.25	0.19	0.27	
γ–Selinene	24.975	0.21	0.10	0.10	0.13	
Caryophylla–4(12),8(13)–dien–5–ol	26.364	0.17	0.18	0.18	0.18	
Total		96.74	96.53	95.74		

RT: Retention time, *: P<0.05, **: P<0.01

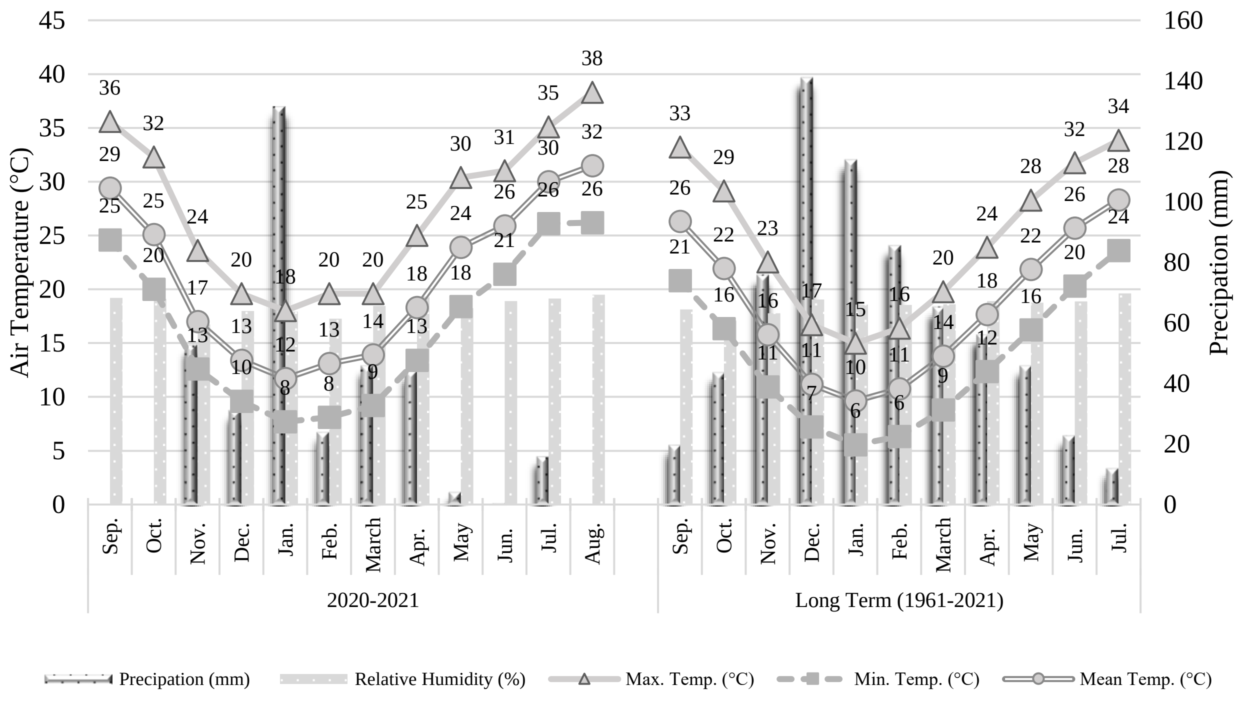

Figure 1. Meteorological data recorded during the field experiment and over the long term (1961-2020). The data was derived from the Meteorology Directorate of Adana, Türkiye.

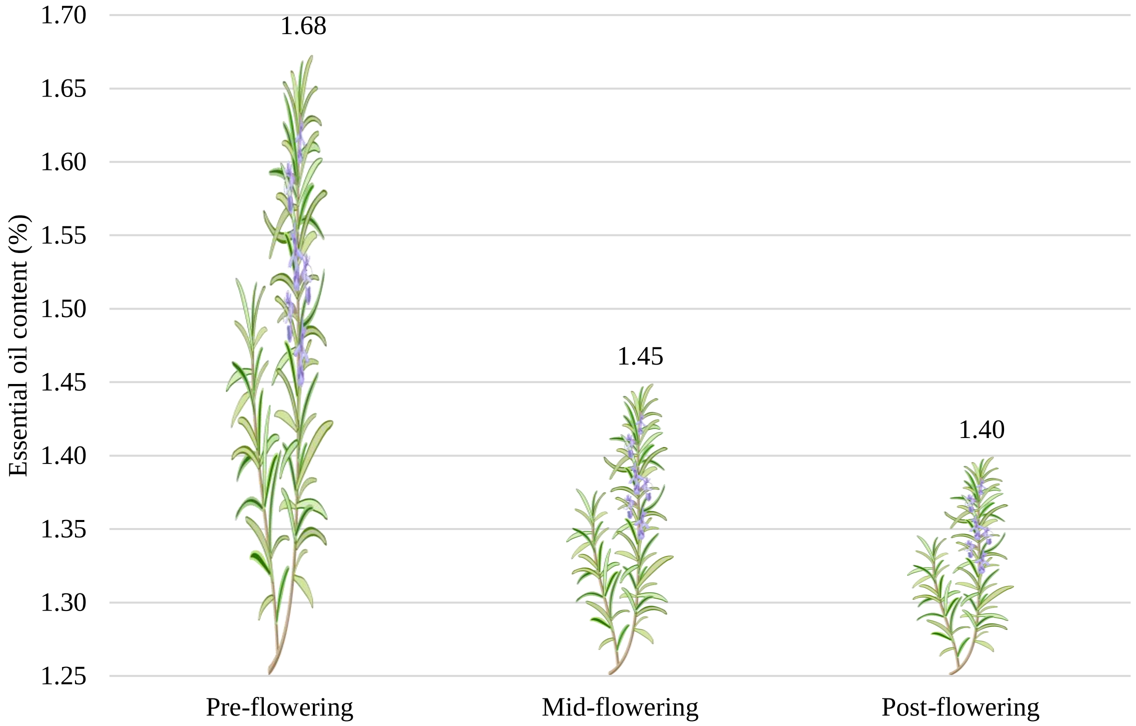

Figure 2. Essential oil content of rosemary (*Rosmarinus officinalis* L.) at various growth stages.

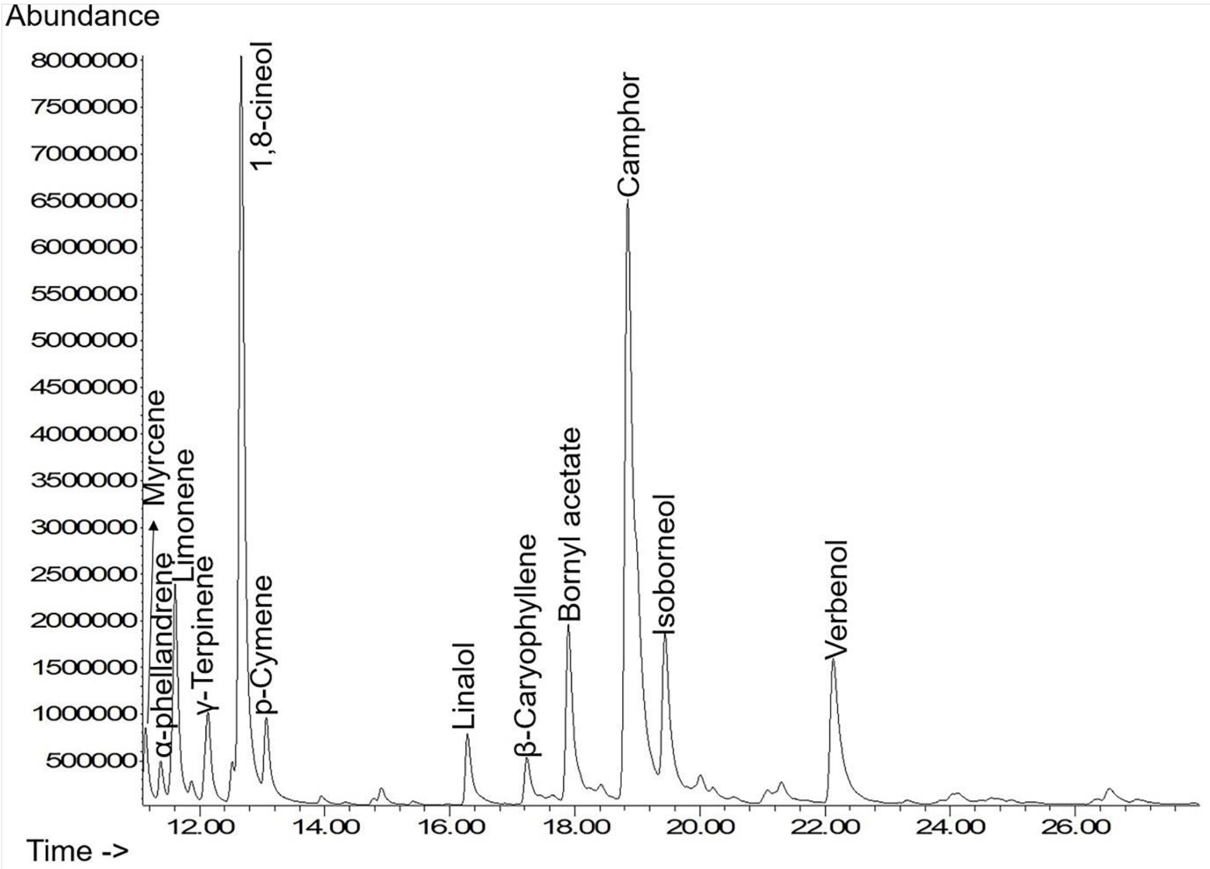

Figure 3. The representative GC MS chromatogram of rosemary (*Rosmarinus officinalis* L.) essential oil.

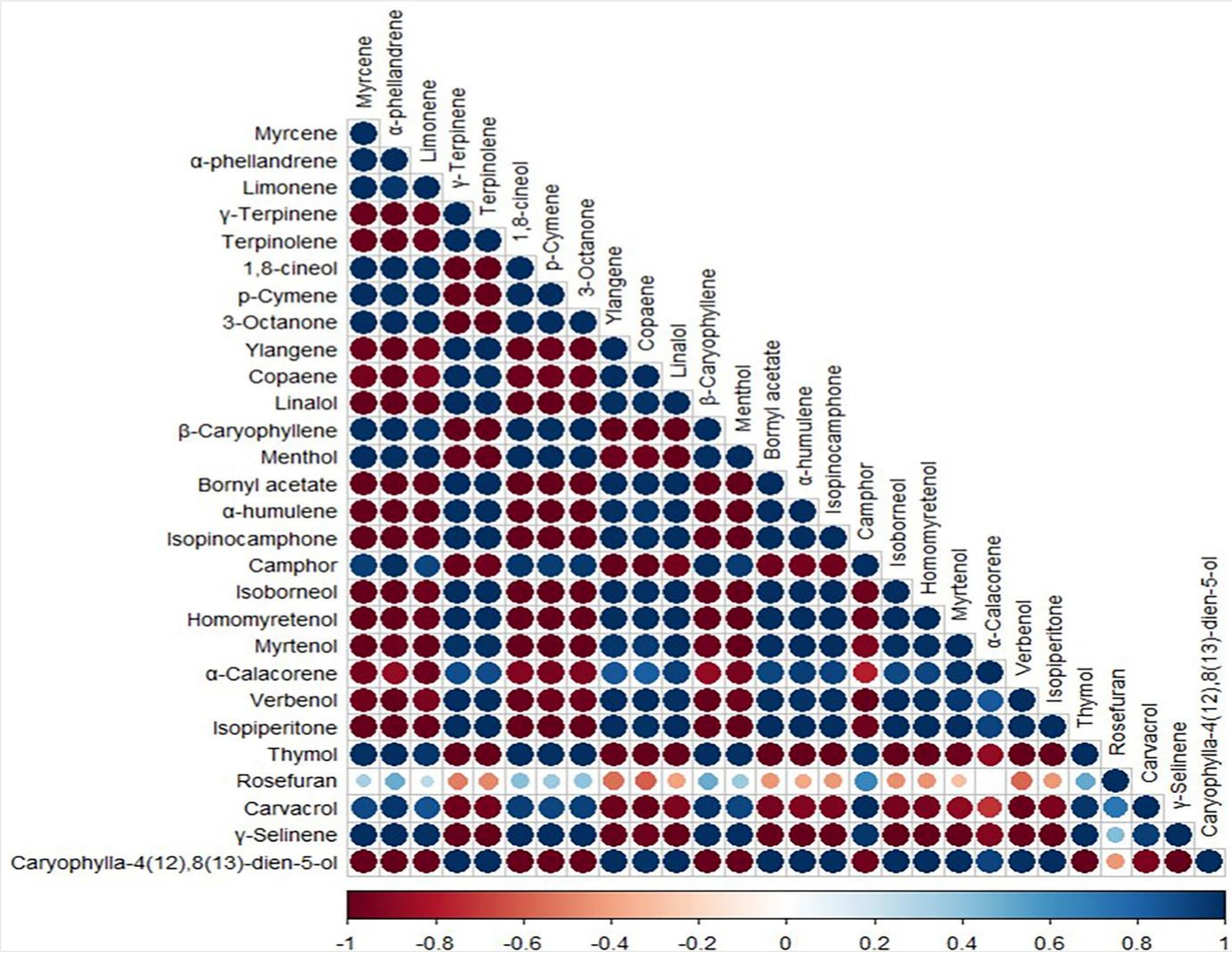

Figure 4. Correlation analysis of essential oil compounds.

In addition, Tawfik (1998) reported that essential oil content showed the higher values during the flowering stage (1.43%) compared to the vegetative state (1.23%). As a matter of fact, essential oil content may vary depending on the vegetative stage, age, bioclimatic conditions, origin, variety, part harvested, soil, agrochemicals, storage time, preparation and other factors (Borges et al., 2019; Jeevalatha et al., 2022).

Essential oils compositions of rosemary can vary widely depending on the chemotypes. Therefore, the different chemotypes are likely to present different biological activities (Satyal et al., 2017). The biological activities of rosemary are mainly due to terpenes, especially 1,8-cineole, borneol, camphene, camphor, and myrcene (Borges et al., 2019). The chemical composition of essential oils varied according to the growth stages. Representative GC-MS chromatogram of the essential oil was given in Figure 3. Twenty-eight compounds were found, representing 95.74%-96.74% of the total essential oil. The major compounds for rosemary were camphor (28.43%-32.74%), 1,8-cineol (20.80%-22.61%), isoborneol (6.05%-7.28%), verbenol (5.17%-6.98%), and limonene (5.71%-6.23%), respectively. The highest value of camphor (32.74%), 1.8-cineol (22.61%), and limonene (6.23%) were obtained at the pre-flowering stage while the highest value of verbenol (6.98%) and isoborneol (7.24%-7.28%) obtained from mid-flowering and post-flowering stage. Numerous studies have highlighted the variability of the composition and the yield of the essential oil because of intrinsic factors such as genetics, subspecies, and plant age or extrinsic factors such as climate and cultivation conditions (Flamini et al., 2002; Borges et al., 2019).

When camphor content was compared by the growth stages, higher amounts were found in the pre-flowering stage. Differences in the camphor content were also reported in rosemary by other researchers, such as 11.25%-20.41% (Gürbüz et al., 2017), 1.10%-18.80% (Flamini et al., 2021), 12.40% (Christopoulou et al., 2021), 17.30%-19.07% (Dzięcioł, 2021), 14.39%-17.17% (Micić et al., 2021), 10.01%-16.20% (Elyemni et al., 2022), 7.05%-31.50% (Rathore et al., 2022), 29.46%-31.92% (Yeddes et al., 2022), 18.26% (Karakaş and Bekler, 2022). Similar to what we detected in our study, Afshar et al. (2021) reported that camphor content decreased from spring (8.19%) to summer (6.95%). Variations in rosemary terpene and phenol concentrations are typically the result of the interplay between genes and the environment (Yosr et al., 2013; Moore et al., 2014). Despite this terpenoid's wide spectrum of medicinal applications, Chen et al. (2013) warns of its toxicity, indicating that only 2 g can have toxicogenic effects in adults, causing gastrointestinal disturbances and cerebral abnormalities, and a dose of 3.5 g directly leads to death. Numerous biological activities have been claimed to camphor, although it is crucial to remember that bioactivity was determined in many cases utilizing an essential oil rich in camphor rather than pure camphor.

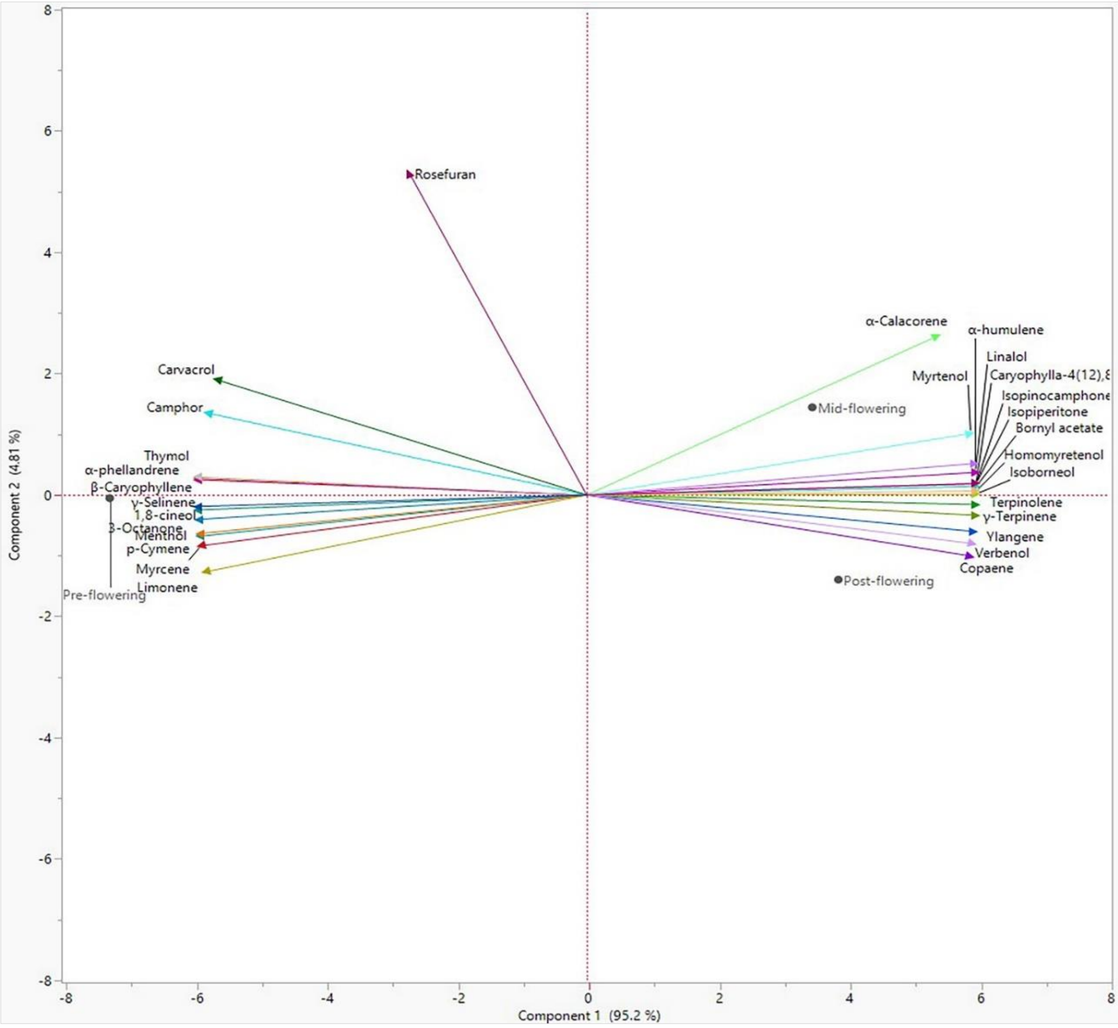

Figure 5. Principal component analysis on correlations of essential oil compounds (pre: pre–flowering, mid: mid–flowering, post: post–flowering).

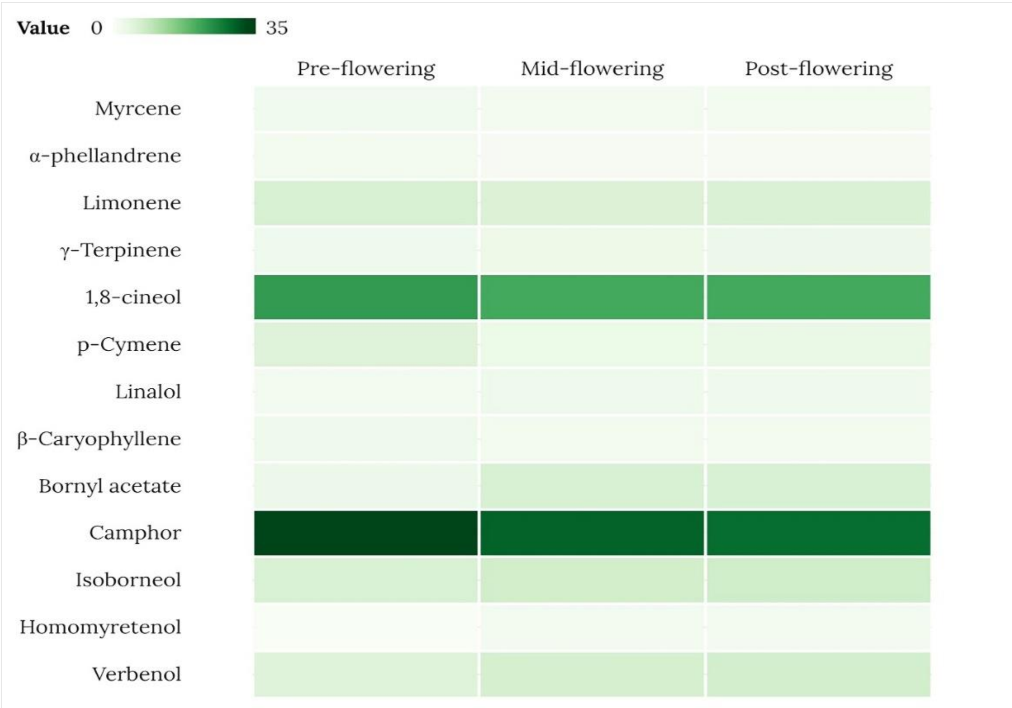

Figure 6. Heatmap based on the growth stages for the main essential oil compounds of rosemary (*Rosmarinus officinalis* L.) (Value=%).

These effects may be mistakenly assigned to camphor because to the large percentage of camphor, although synergism appears to be far more plausible, as shown in the case of 1,8-cineole and camphor. Pure camphor, according to other studies, does not have the same activity as compared to essential oil (Chen et al., 2013).

Similar to the camphor content, the highest values for the 1.8-cineole content were obtained in the pre-flowering stage (22.61%). When compared the 1.8-cineole content with the earlier studies, diverse results were found, such as 6.19%-10.52% (Gürbüz et al., 2017), 41.25%-45.96% (Yıldırım, 2018), 37.50% (Khanjani et al., 2021), 10.81% (Xylia et al., 2021), 17.79%-23.40% (Micić et al., 2021), 26.40%-49.10% (Flamini et al., 2021), 40.10% (Christopoulou et al., 2021), 30.89%-31.61% (Dzięcioł, 2021), 28.97%-32.18% (Elyemni et al., 2022), 32.50%-51.79% (Rathore et al., 2022), 30.26% (Mwithiga et al., 2022), 25.26%-29.15% (Yeddes et al., 2022), 11.86% (Karakaş and Bekler, 2022).

The highest values for the isoborneol content were obtained in the mid- and post-flowering stage. Isoborneol content in the previous studies was found different, such as 3.48% (Martínez et al., 2009), 0.20% (Hussain et al., 2010), 5.68% (Asressu and Tesema, 2014), 2.28%-9.8% (Farhat et al., 2017), 6.04%-12.02% (Gürbüz et al., 2017), 8.10% (Caputo et al., 2018), 11.96%-14.89% (Yıldırım, 2018), 0.11% (Micić et al., 2021), 6.00% (Khanjani et al., 2021), 30.29% (Xylia et al., 2021).

The highest values for the verbenol content were obtained in the post-flowering stage (6.98%). Verbenol content in the previous studies was found different; 0.1%-0.7% (Diab et al., 2002), 2.3% (Jamshidi et al., 2009), 5.72%-19.05% (Hosni et al., 2013), 0.1% (Tomi et al., 2016), 8.50% (Caputo et al., 2018). The perfume business benefits greatly from verbenol, which is produced through catalytic oxidation of alpha-pinene (Kulesza et al., 1972). Verbenol has a fresh piney note and it is used for an herbaceous pine note in flavor blends (Anonym, 2021). Moreover, verbenol has significant pharmacological and therapeutic properties as a bicyclic monoterpene. Verbenol stimulates tonic inhibition in dentate gyrus granule cells by acting on subunit-containing GABAA receptors (VanBrederode, 2016). Low levels of GABA are linked to seizures and epilepsy (Treiman, 2001).

The highest values for the limonene content were obtained in the pre-flowering stage (6.23%). Limonene content in the previous studies was found different; 0.19% (Golami et al., 2018), 21.99% (Borges et al., 2018), 2.1-2.8% (Verma et al., 2020), 3.97%-4.44% (Afshar et al., 2021), 0.84% (Amina et al., 2022), 0.37%-0.92% (Elyemni et al., 2022). Different stages of the plant life cycle reveal that rosemary essential oil is important in terms of chemical composition.

Correlation Analysis, Principal Component Analysis (Pcabiplot) on Correlations, and Heatmap According to Essential Oil Compounds

When interpreting the correlation analysis of essential oil compounds, the colors indicated the negative or positive correlation and the size of the circles indicated the level of importance (Figure 4). The correlation analysis showed that camphor was positively correlated with 1,8-cineol and limonene and negatively correlated with isoborneol and verbenol. PCAbiplot is essential for effectively depicting a data source with several components that accurately reflect the data's variation. In order to visualize the influence of growth stages on correlations, PCAbiplot was performed (Figure 5). The experimental groups were separately discriminated using PCAbiplot. As seen in the Figure 5, PCAbiplot indicates that component 1 revealed most of the variance. A heat map was used to illustrate the changes in essential oil compounds (Figure 6). The main essential oil compounds were visualized using the heat map based on the various growth stages. The differences can be observed clearly between growth stages in the essential oil compounds. For instance, during the pre-flowering stage camphor rate was the highest, but as the harvest was delayed, it decreased.

Conclusion

There are numerous applications for the essential oils of rosemary. It is used in many different industries, such as the food and food packaging, pharmaceutical, perfumery, and cosmetics industries. Moreover, rosemary essential oil becomes an exceptionally important and significant gem in phytotherapy. Rosemary cultivation provides economic benefits to growers, but a range of applications necessitate a standardized level of quality. The essential oil content and its compounds varied according to different growth stages. The highest essential oil content was obtained in the pre-flowering period. Although it varies according to different harvest times, the major compounds for rosemary were camphor, 1,8-cineol, isoborneol, verbenol, and limonene, respectively. In terms of essential oil content, as well as high camphor, 1,8-cineol, and limonene content, it is possible to consider pre-flowering as the most appropriate harvest time for rosemary. Furthermore, it is recommended that additional agronomic studies be carried out, with a particular emphasis on studies to obtain higher quality products that are demanded by the market. In future studies, it is recommended to conduct agronomical studies with a particular emphasis on studies to obtain higher quality products demanded by the market in rosemary.

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