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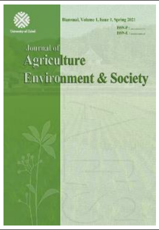

Investigation of the phytochemicals produced by the eucalyptus (*Eucalyptus viminalis*) throughout several growth seasons

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ABSTRACT

Medicinal plants contain flavonoids and phenolic compounds, which are the primary antioxidants produced by plants. Antioxidants are chemical compounds that protect the body from damage by counteracting free radicals. The Myrtaceae family comprises eucalyptus, a medicinal plant. At the Lorestan Research Center, this study investigated how harvesting times affected the phytochemical composition of eucalyptus (Eucalyptus viminalis) essential oil. Three replications of a randomized complete block design were utilized to conduct the experiment. The concentrations of flavonoids and total phenol were ascertained by employing Folin-Ciocalteu's regent and aluminum chloride, respectively. By means of GC/MS analysis, the hydrodistilled essential oils of eucalyptus leaves were evaluated. In general, the results indicated that the highest concentrations of phenolic and flavonoid compounds were detected during the spring season. During the spring, summer, and fall, leaf essential oil contained 43, 14, and 17 components, respectively. 1,8-Cineol, alpha-pinene, and trans-pinocarveol exhibited the highest concentrations across all three seasons. Collectively, the results indicate that the duration of the harvest significantly influences the concentration of bioactive compounds in plants.

Highlights

- The study investigates the effects of harvesting time on the phytochemical composition of eucalyptus essential oil
- The study uses Folin-Ciocalteu's regent and aluminum chloride to measure the concentrations of phenolic and flavonoid compounds in the oil
- The study finds that the spring season yields the highest concentrations of these compounds, followed by the fall and summer seasons
- The study identifies 1,8-Cineol, alpha-pinene, and trans-pinocarveol as the major components of the oil across all seasons

1. Introduction

Eucalyptus trees are among the fastest-growing forest species. This plant is native to Oceania, especially Australia. They have been cultivated in countries other than Australia (their natural habitat) for about 200 years. So far, nearly 65 million hectares of different species of Eucalyptus have been cultivated in more than 200 countries, including Iran (Anonymous, 1979; Tewari, 1992). The genus Eucalyptus, with more than 700 species, belongs to the family Mirtaceae. Eucalyptus was first cultivated about 100 years ago. However, it has been almost 35 years since the study of its various species in the different provinces of Iran was started by executive bodies in arable areas and domestic research stations (Assareh and Sardabi, 2007).

Today, most uses of Eucalyptus essential oil are in the cosmetics and perfumery industries. Also, the essential oil of this plant is used in medicine as an antimicrobial and

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antiseptic to improve the taste of medicines. In addition, the components of the essential oil can be used as a flavoring in food. As mentioned, essential oils have antimicrobial properties that are used in a variety of ways, such as dental fillings, disinfectants, and feed supplements for livestock. In addition, today's few preservatives contain essential oils that are commercially available (Burt, 2004). Phenols and flavonoids play different roles in plants, for example, anthocyanins. Anthocyanins in the petals are responsible for creating different colors, which are very important in attracting pollinators and plant survival. Flavonoids in leaves have a protective role against ultraviolet rays. Accordingly, flowers and leaves are the two main storage organs for these compounds. On the other hand, due to photosynthesis in leaves, the precursors of the flavonoid biosynthesis pathway are more abundant in leaves than in other organs. This is another factor in the higher flavonoid composition of leaves than flowers and other organs (Davis et al., 2004).

In 2005 and 2006, a study was conducted on seasonal changes in the quantity and quality of essential oils of three species of Eucalyptus in southern Iran in the tropical regions of Shushtar and Dezful (Esfahanianfard et al., 2010). The results of this study showed that the highest essential oil yield was in *E. melliodora* species and was about 1.3-3.9%. Also, the highest amount of 1,8-cineole compound was measured in the Shushtar region in winter, at about 62-73%. According to other results from the above research, the highest yield of essential oil in *E. kingsmilli* in the Shushtar region in winter is about 2.8–3.1%, and the highest 1,8-cineol composition is about 68.4–77.3 %. In another study, the effect of different seasons on the composition and amount of essential oils of *E. maculate* leaves was investigated. The results of their research showed that the amount of essential oil of this species is different in different seasons. The highest production of 1,8-cineole was obtained in late spring and early summer (Assareh et al., 2010). This condition can be due to seasonal changes in location. In one study, the essential oil compounds of *E. globulus* were investigated by the gas chromatography-mass spectrometry (GC / MS) technique. The results showed that of the 32 compounds identified in the essential oil of the plant leaves (including 95.51% of the total essential oil), the compounds 1,8-cineole (39.59%) and alpha- phellandrene (15.83%) contained the highest percentage. Also, 25 compounds were identified in the fruit of the plant (including 94.68% of the total essential oil), among which alpha-pinene (57.82%), alpha-terpineol (6.76%), alpha-camphollenal (4.80), and ortho-cement (3.82%) were identified as the main compounds (Pashazanousi et al., 2012). In another study, by studying the constituents of the essential oils of the leaves of four species of Eucalyptus in Kashan, it was found that the compound 1,8-cineole is the main component of the essential oils of the leaves of all studied species (Batooli et al., 2012).

In other words, the main chemical composition in the essential oils of the leaves of all species of the genus Eucalyptus is 1,8-cineole. It should be noted that the amount of this compound is different in different species of Eucalyptus. Since Eucalyptus grows in different arid and semi-arid regions of Iran and environmental factors affect the amount of essential oil in this plant, it is very important to study the composition of active ingredients in different seasons to select the appropriate time for leaf harvest. This study was conducted to evaluate the phytochemical compositions and essential oils of Eucalyptus leaves in different seasons in Lorestan climatic conditions.

2. Materials and Methods

The leaves *of E. viminalis* were harvested from the Lorestan Agricultural Research Station in different growing seasons. The leaves were dried under shade and without moisture. For this purpose, first the waste was removed from the leaves, and then, after washing with water, they were dried in laboratory conditions. Then the leaves of each season were powdered separately by the mill and then used for extraction (Naznin and Hasan, 2009).

2.1. Measurement of total phenol

The amount of total phenol compounds was measured by the Folin-Ciocalteu's regent, and the results were expressed in terms of mg of gallic acid per gram of extract (Slinkard and Singleton, 1977). The basis of this method is the reduction of folate reagent by phenolic compounds in an alkaline environment and the formation of a blue complex. In this method, the following compounds were first added to the test tube and mixed:

- 200 µl of extract (1 mg/ml) or standard ethanolic solution of gallic acid or another phenolic acid
- 400 microliters of fullene cyclate reagent (diluted with distilled water) in a ratio of 1 to 10
- 400 microliters of 7% sodium carbonate

In the next step, after 30 minutes of storage at laboratory temperature, its light absorption was read by a spectrophotometer at 765 nm. It should be noted that the blank was also prepared by the above method, but 70% methanol was used instead of the extract.

2.2. Measurement of total flavonoids

In this study, the aluminum chloride colorimetric method was used to determine the amount of flavonoids (Chang et al., 2002). For this purpose, first, each of the plant methanolic extracts (0.5 ml) was separately combined with 1.5 ml of methanol, 0.1 ml of aluminum chloride (10% methanol), 0.1 ml of potassium acetate (1 M), and 2.8 ml of distilled water. Then, after storing the solutions at room temperature for 40 minutes, the adsorption of each reaction compound was measured at 415 nm relative to the blank with a spectrophotometer. It should be noted that blank contained all of the above ingredients, but instead of the extract, the same volume of 80% methanol was added.

2.3. Extraction and analysis of essential oils

In this study, essential oils were extracted by distillation with water (Clevenger) for 2 hours. The essential oils were then identified by gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS) with the following characteristics. For this purpose, the essential oil extracted from the studied plant was injected into the GC/MS device by water distillation. The constituents were separated based on boiling point and polarity along a 30 m long column. In all given GC/MS spectra, the inhibition index for each peak was calculated from the normal alkane exit pattern and the spectrum inhibition index. By comparing the obtained indexes with the information available in the computer library and other sources, the spectra related to each object were interpreted. With the help of these results, the constituents of essential oils and their chemical formulas were identified. The relative percentage of each of the constituents of the essential oils was obtained according to the area under its curve in the chromatogram spectrum. The results were compared with the values published in different sources (taking into account the inhibition index).

2.4. Statistical analysis

The data obtained from the above sections was analyzed based on a one-way analysis of variance (ANOVA) using SPSS 26 and Excel software. The means were compared using Duncan's multiple range test at the statistical level of P 0.05.

3. Results and Discussion

3.1. Investigation of phenolic and flavonoid content:

The total amount of total phenolic compounds in *E. viminalis* leaves is shown in Figure 1. The results of comparing the total phenol content of Eucalyptus leaves in three different seasons showed that the highest phenol content is related to spring and the lowest phenol content is related to autumn (with an average of 30.05 mg GA/ g D.W.). It can be said that the amount of phenolic compounds under the influence of climatic factors in the region has been studied. These results indicate the effect of climatic factors on the total phenol content of Eucalyptus leaves.

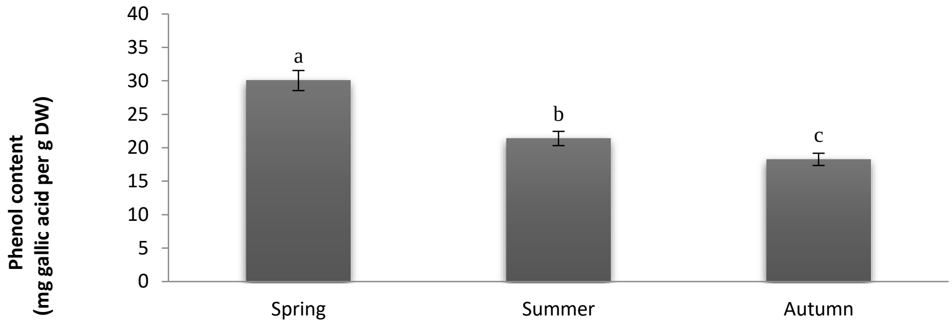

Figure 1. Total phenol content of E. viminalis leave

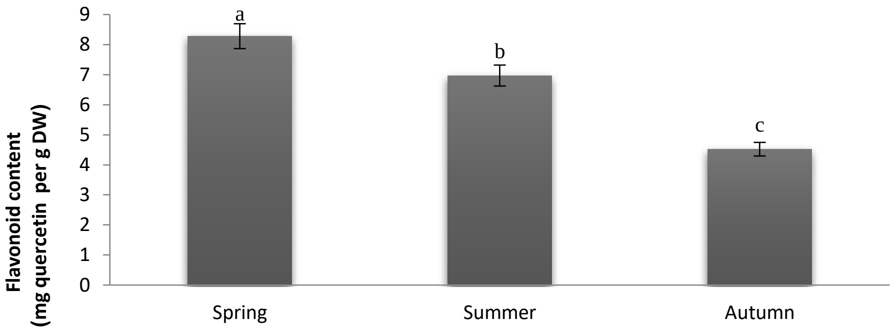

Figure 2. Total flavonoid content of *E. viminalis* leave

The total amount of total flavonoid compounds in *E. viminalis leaves* is shown in Figure 2. Analysis of the data indicates that the amount of total flavonoid compounds of this Eucalyptus leaf in spring has the highest amount (8 mg quercetin per g DW), and the lowest amount of flavonoids (about 4 mg quercetin per g DW) is related to autumn.

3.2. Chemical composition of Eucalyptus essential oil

The results of the constituents of *E. viminalis* essential oil during the three seasons of spring, summer, and autumn are given in Tables 1, 2, and 3. The results obtained from the analysis of Eucalyptus leaves collected from the Lorestan region in spring showed that this season, with 43 compounds, has the highest number of essential oil compounds compared to the other two seasons. The highest percentages of compounds in this season are related to 1, 8-cineole (54.51%), paracetamol (6.31%), and alpha-pinene (4.61%), respectively (Table 1). As shown in Table 2, there are 14 compounds in the summer, of which the three main compounds of this season are 1,8-cineole (59.58%), alpha-pinene (19.54%), and trans-pinocarol (13.2%). The results of Table 3 show the essential oil compositions in autumn. In this season, 17 compounds were observed, of which 1,8-cineole (51.42%), alpha-pinene (14.76%), and trans-pinocarol (6.01%) were the major compounds.

Table 1. Essential oil content of *E. viminalis* leave in spring

Row	Compounds	Retention time (RT)	Percentage of compounds
1	1,8-cineole	8.24	54.51
2	p-Cymene	5.93	6.31
3	α –pinene	5.63	4.61
4	trans-Pinocarveol	11.40	3.61
5	*β*- Pinene	6.72	2.93
6	L-Alloaromadendrene	23.64	2.80
7	Crypton	12.87	2.19
8	Naphthalene, 1,2,3,4,4a,7-hexahydro	24.59	1.75
9	α-Phellandrene	7.38	1.59
10	Terpinene-4-OL	12.47	1.54
11	Cembrene	23.56	1.23
12	δ –cadinene	21.66	1.13
13	Spathulenol	23.41	1.02
14	Tert-butydimethylsilyl ether	28.54	1.01
15	Isoledene	24.94	0.84
16	p-Cumic aldehyde	14.41	0.71
17	γ –terpinene	8.76	0.52
18	α-terpinene	15.75	0.48
19	α-4-trimethylbenzyl carbar	12.73	0.48
20	Torreyol	21.18	0.47
21	(+)-Ledene	23.86	0.35
22	(-)-Calamenene	21.83	0.34
23	β –myrcene	6.82	0.33
24	β –selinene	25.36	0.33
25	γ –Muurolene	25.05	0.32
26	Phellandral	15.43	0.32
27	Myrtenal	13.04	0.29
28	α-Terpinene	7.65	0.29
29	Trans-Sabinene hydroxide	10.80	0.28
30	Isovaleric acid	9.98	0.27
31	B-Phelladrene	6.56	0.25
32	α- Cadinen	25.01	0.25
33	Carvomenthol	13.94	0.24
34	Epi-Bicyclosquiphell	12.10	0.22
35	Myrtenol	12.98	0.22
36	Butanal, 3-methyl	2.17	0.21
37	Terpinolene	9.52	0.20
38	Trans-Carveol	11.47	0.15
39	Caryophyllene	19.12	0.14
40	Cis-carvyl acetate	13.61	0.13
41	Carvacrol	15.92	0.12
42	Cyclohexene, 1-butyl	11.62	0.11
43	α-Campholene aldehyd	10.89	0.10

Table 2. Essential oil content of *E. viminalis* leaves in summer

Row	Compounds	Retention time (RT)	Percentage of compounds
1	1,8-cineole	17.23	59.58
2	α –pinene	12.50	19.54
3	trans-Pinocarveol	21.98	2.13
4	Borneol	23.13	0.64
5	Fenchol	21.07	0.47
6	Pinocarvon	22.89	0.39
7	4-Terpineol	23.51	0.33
8	γ-Terpinene	18.19	0.26
9	*β*- Pinene	14.51	0.25
10	Camphene	13.20	0.16
11	p-Cymene	16.75	0.18
12	Myrcene	15.07	0.18
13	Campholenal	21.37	0.12
14	α -Pinene oxide	20.11	0.10

Table 3. Essential oil content of *E. viminalis* leave in autumn

Row	Compounds	Retention time (RT)	Percentage of compounds (%)
1	1,8-cineole	17.33	51.42
2	α –pinene	12.47	14.76
3	trans-Pinocarveol	22	6.01
4	α –terpineole	24.15	1.48
5	Pinocarvon	22.87	1.40
6	Cis-Carveol	25.54	1.15
7	Borneol	23.26	0.92
8	Fenchol	21.03	0.69
9	Camphene	13.19	0.46
10	p-Cymene	16.74	0.23
11	4-Terpineol	23.47	0.19
12	Campholenal	21.32	0.17
13	*β*- Pinene	14.49	0.14
14	Trans-Carveal	23.85	0.12
15	Verbenone	23.84	0.12
16	E-2-Hexenal	9.24	0.11
17	α -Pinene oxide	19.72	0.08

The amount of 1,8-cineole monoterpene varies between 51.42 and 59.58% in the studied Eucalyptus species. This monoterpene has been reported as the main compound in *E. globulus* (85.6%) (Barazandeh, 2005), *E. porosa* (6.58%) (Assareh et al., 2005), *E. caesia* (4.69%) (Assareh et al., 2007), *E. spathulata* (72.5%) and *E. torquata* (9/66%) (Sefidkon et al., 2007), *E. sargentii* (7.56%), *E. stricklandii* (71.2%) (Abravesh et al., 2007) and *E. globulus* (60 to 70%) (Pereira et al., 2005). Other studies have been performed on the essential oil components of *E. tereticornis* smith and *E. resinifera* smith (grown in Cuba). The results of their study showed that the composition of 1,8-cineole accounted for 68% of the essential oil of *E. resinifera*. This compound also contained 23.3% of the essential oil of *E. tereticornis*. In addition, paracetamol constitutes 13.8% of the essential oil of the above species, so paracetamol, along with 1,8-cineol, were the main components of the essential oil in this species (Batooli et al., 2012). It seems that the reason for the difference in the amount of monoterpene depends on the geographical conditions, climatic conditions, collection season, and the conditions of essential oil collection and phytochemical decomposition of essential oil. Alpha-pinene is the second major essential oil compound in *E. viminalis*, with the highest amount in summer (19.54%) and the lowest in spring (4.61%). Alpha-pinene is the second major essential oil compound in the species (*E. viminalis*). The highest amount of this compound is related to summer (19.54%) and the lowest amount is related to spring (4.61%). According to research, this monoterpene has been reported as the main component of essential oils in *E. camaldulensis* (12.8%), *E. alba* (20.1%) (Samate et al., 1998), *E. coleziana* (6.46%) (Ogunwande et al., 2005), *E. microtheca* (10.7%), *E. spathulata* (12.7%) (Sefidkon et al., 2007), *E. stricklandii* (9.2%), *E. brockwagii* (14%), *E. kruseana* (15.9%) (Abravesh et al., 2007) and *E. porosa* (12.8%) (Assareh et al., 2005). The main constituents of eucalyptus leaf essential oil studied in this study are consistent with the major constituents of eucalyptus cultivated in Kashan Botanical Garden (Batooli et al., 2012). Jaimand et al. (2012) reported the major constituents of *E. stricklandii* Maiden essential oil from northern Khuzestan as 1,8-cineole (72.7%) and alpha-pinene (12.2%). The third known compound is transpinocarol. This compound has shown the highest (6.01%) in autumn and the lowest (2.13%) in summer. Other compounds, such as para-cement, beta-pinene borneol, and phenolic, also have an acceptable amount of essential oil compounds. In addition, the results show that the amount of 1, 8-cineole and alpha-pinene (the two main constituents of essential oils in the present study) in the summer is greater than in the spring.

The increase in these compounds in the summer may be due to the drought stress of this season. In addition to increasing the amount of essential oil compounds in summer, the highest amounts of flavonoids and antioxidant activity are also observed in this season.

As can be seen in Table 1, spring has a higher number of identified compounds than summer and autumn. These results are probably related to seasonal changes (temperature conditions, water availability, etc.) that have led to the formation of new and more compounds in the essential oil. These findings can also be explained by the fact that in the summer, a high percentage of compounds related to 1, 8-cineole (59.58%) are present.Therefore, this can reduce the percentage of other compounds in the essential oil. On the other hand, in spring, the amount and percentage of compounds in the essential oil are lower and this has caused more compounds in the plant essential oil to be identified in this season.

This result is consistent with the results of Sefidkon et al. (2009), who showed that for obtaining the highest number of compounds of essential oil from *E. porosa* leaves, the best harvesting time is spring. In another study, the effect of region and harvest time on the type and amount of constituents of Eucalyptus essential oil was investigated. The results of that study showed the highest number of essential oil constituents (39 compounds) and the highest amounts of the main constituents of this plant (i.e., 1,8-cineole, alpha-pinene, and alpha terpinene) are in the Qasr Shirin and Gilangharb regions in summer (Gerdakaneh et al., 2018). One of the most important factors influencing the amount of active compounds in medicinal plants is the harvest time of plant organs.In this study, the highest concentration of the main components of Eucalyptus essential oil was obtained in summer. In some species, the best season for essential oils is hot and sunny weather.

These results are consistent with the results of the present study. The results of other researchers also showed that harvest time and seasonal changes also had a significant effect on the components of mint essential oil (Kofidis et al., 2006) and basil (Hussain et al., 2008).

4. Conclusion

One of the most important factors influencing the amount of active ingredients in medicinal plants is harvesting time. The results of the study showed that the highest amounts of essential oils and main compounds of Eucalyptus were obtained in summer.

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