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Seasonal Changes in Secondary Metabolites and Antioxidant Activity of *Corylus avellana*

Nasser Zare*, Roghayyeh Hazrati

Plant Production and Genetics, Faculty of Agriculture and Natural Resources, University of Mohaghegh Ardabili, Ardabil, Iran

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ABSTRACT

Taxol is one of the most effective chemotherapy drugs and is effective against a wide range of cancers. Hazelnut is an important nut crop and contains Taxol. Its natural habitats occur in the northern and northwest regions of Iran. The study of hazelnut habitats and seasonal variation in terms of secondary metabolites such as Taxol is important to obtain high-yielding genotypes and conditions. In this study, we investigated the biochemical characteristics and secondary metabolite content of hazel in various hazelnut habitats during the growth season. The results indicated a significant difference in the studied populations, not only between regions but also at different sampling times in terms of biochemical properties and secondary metabolites. So, the samples prepared from the Eshkevarat region had the highest amount of secondary metabolites in September. The highest amount of phenol, flavonoid, Taxol, and Baccatin III was obtained in the Eshkevarat region in the September. The amount of Baccatin III was higher than that of Taxol at all sampling times and regions. These results confirm the role of environmental signals in the production of taxanes in the hazel plants. The significant biochemical diversity among the investigated hazelnut ecotypes opens up possibilities for various applications. It can be utilized in breeding programs to develop hazelnut varieties with higher Taxol content for medical purposes. Additionally, understanding the seasonal variation in secondary metabolite content allows for targeted harvesting and processing, ensuring the highest possible concentration of Taxol in hazelnuts for medical purposes.

Introduction

For a long time, plants have been used to treat various diseases, including cancer, diabetes, and infection. Natural products can be important in drug discovery, new resource creation, and innovation (Newman and Cragg, 2007). Clinical trials have shown that the consumption of foods rich in biologically active molecules with antioxidant capacity can protect the cells against cardiometabolic diseases, neurological disorders, cancer, and age-related frailty (Rusu et al., 2019; Arias-Fernandez et al., 2018). Knowing the biological activities of secondary metabolites and their diversity in different populations and environmental conditions provides an effective approach to producing new drugs, antibiotics, insecticides, and herbicides (Smith, 1996).

Taxol is one of the most popular and effective

*Corresponding author's email: zarenasser@yahoo.com, nzare@uma.ac.ir

drugs in chemotherapy (Siegel and Jemal, 2012) and is effective against a wide range of tumors and cancers (Wang et al., 2006). Cell and tissue culture of yew and hazelnut species are among the most promising methods for mass production of this compound (Wang et al., 2006).

Hazelnut (*Corylus avellana*) has been used in the human diet for a long time and has been recognized by the FDA as a heart-healthy food (Alasavar et al., 2006). Hazelnuts contain all 20 important amino acids required by the human body, and their elevated phosphorus level can enhance and optimize brain function (Chromy et al., 2015). The geographical scattering and main hazelnut habitats are concentrated in the northern hemisphere and areas with temperate winters and cool summers (Muehlbauer, 2017). Hazelnuts are cultivated in a few areas of Iran with high rainfall and relative humidity (Salimi and Hoseinova, 2012). The native habitats of the hazelnut in Iran are mainly located in the northern and northwest regions, including Ardabil, Eshkevarat, Navan, Tarom, Dinochal, Alamut, and Goli Dagh (Ochi-Ardabili et al., 2022). Different factors such as the age of the plant, climatic conditions, soil composition, and the amount of plant access to light can affect the amount of Taxol in different tissues of the hazel. Plants use various biochemical and physiological strategies to adapt to environmental changes (seasonal changes), which usually lead to the production and accumulation of secondary metabolites. On the other hand, environmental changes, along with the physiological state of plants, may affect their secondary metabolite composition (Koricheva and Barton, 2012). It has also been claimed that expression patterns of the gene involved in the secondary biosynthetic pathways can be efficiently exploited under environmental fluctuations (Ncube et al., 2015).

Morphological and physiological analysis of plant cultivars and genotypes is one of the most commonly utilized methods in plant breeding programs for evaluating and comparing plant cultivars and genotypes. Little research has been conducted in Iran to identify and collect native hazelnut genotypes, and there is no report concerning the seasonal and regional changes in secondary metabolites and biochemical properties in hazelnut natural habitats. This information can then be used in plant breeding programs to develop improved cultivars that are better suited to the specific environmental conditions and challenges faced in Iran. Additionally, studying the seasonal and regional changes in secondary metabolites and biochemical properties can help in understanding the nutritional and medicinal value of native hazelnut genotypes, opening up opportunities for the development of new products and industries. In the present study, we studied the seasonal variation in the biochemical traits, antioxidant content, and secondary metabolites, including taxol and baccatin III during the growing season in four natural hazelnut habitats.

Material and Methods

Plant materials

Plant materials used in this research were the leaves of hazelnuts, which were collected in May, July, and September from several natural habitats (Table 1). Also, several climate characters were obtained from the nearest weather station to investigate seasonal and environmental changes (Table 2).

Preparation of methanolic extract

A methanol extract was prepared by homogenizing 0.1 g of powdered leaf in 10 mL of 95% methanol and shaking it at 110 rpm for 24 h at room temperature. The resulting mixture was filtered and concentrated at 30 °C in an oven. To measure antioxidant activity and total phenol content, the concentrated extract was dissolved in 1 mL methanol (Chung et al., 2019).

Total phenol assay

Total phenolic content was measured using the FolinCiocalteu reagent according to Al-Farsi et al. (2005). In brief, 3 mL of diluted Folin-Ciocalteu reagent (10:1) was added to 400 µL of extract and kept at 25 °C in a water bath for 5 min. Then, 3 mL sodium bicarbonate solution (7%) was added to the samples and incubated at 25 °C in a water bath for 90 min. The sample absorbance was recorded at 760 nm using a spectrophotometer (SP-UV 200 Series UV–Vis Spectrophotometer). A gallic acid standard calibration curve was used to quantify the total phenolic content in the samples.

Flavonoid assay

The total flavonoids of the samples were measured according to the methods described by Kumar and Sharma (2017). Briefly, 250 µL of 10% aluminum chloride solution and 250 µL of potassium acetate (1 M) were added to one mL of the plant extracts, and their absorbance was recorded at 415 nm. A quercetin standard calibration curve was used to quantify the flavonoid content in the samples.

Antioxidant activity assay

The antioxidant activity of the extracts was measured according to the Chung et al. (2019) method. In brief, 200 µL methanolic extracts were

blended with 2.5 mL sodium phosphate buffer (200 mM) and 2.5 mL potassium-free cyanide (1%) was added to the samples. The samples were incubated for 20 min at 50 °C. Afterward, 2.5 mL TCA (10%) was added to the samples and centrifuged for 10 min at 4000 rpm. Finally, 2.5 mL supernatant was mixed with 2.5 mL distilled water and 0.5 mL ferric chloride (0.1%), and after thorough mixing, the absorbance of the mixture was recorded at 700 nm.

Table 1. Geographical characteristics of collected *Corylua avellana* populations from various regions.

Region Name	Latitude	Longitude	Altitude
Eshkevarat- Gilan	37° 04′ 12.1″ N	50° 21′ 34.8″ E	-
Fandoghloo (Arpatape)- Ardabil	38° 23′ 53.21″ N	48° 32′ 55.75″ E	4806.4 FT
Fandoghloo (Niaraq)- Ardabil	38° 17′ 44.30″ N	48° 38′ 33.25″ E	4950.8 FT

Table 2. Meteorological data utilized in the current study.

			T Max (°C)	T Min (°C)	T Mean (°C)	HS (h)	RH Max (%)	RH Min (%)	RH Mean (%)	rainfall
Day of sampling	May	Eshkevarat	24.8	16.2	20.5	11.5	98	79	89	0.0
		Arpatape	13.5	9.0	11.3	8.31	68	41	56	2.2
		Niaraq	19.1	15.8	17.45	8.31	96	20	58	0.8
	July	Eshkevarat	27.1	18.2	22.7	5.6	92	65	79	0.0
		Arpatape	19.8	9.5	14.6	10.72	82	35	61	0.0
		Niaraq	23.4	11.3	17.4	10.72	72	40	54	0.0
	September	Eshkevarat	31.8	20.1	26.0	11.2	90	63	77	0.0
		Arpatape	23.6	16.6	20.1	6.61	74	23	44	0.0
		Niaraq	23.6	16.6	20.1	6.61	74	23	44	0.0
Monthly mean	May	Eshkevarat	20.99	13.68	17.64	201.8	94.4	70.4	82.4	51.8
		Arpatape	17.4	7.2	12.3	257.8	73	43	58	45.1
		Niaraq	17.4	7.2	12.3	257.8	73	43	58	45.1
	July	Eshkevarat	28.18	22.12	26.23	237.3	85.7	61.8	73.8	213.9
		Arpatape	23.6	14.3	19.0	332.5	71	46	59	1.6
		Niaraq	23.6	14.3	19.0	332.5	71	46	59	1.6
	September	Eshkevarat	26.86	19.59	23.22	133.1	92.7	69.7	81.2	137.4
		Arpatape	20.1	11.4	15.8	205.1	88	56	72	21.8
		Niaraq	20.1	11.4	15.8	205.1	88	56	72	21.8

T Max: temperature maximum, T Min: temperature minimum, HS: hours of sunshine, RH Max: relative humidity maximum; RH Min: relative humidity minimum.

Taxol and Baccatin III assay

Analysis of Paclitaxel and Baccatin III in the leaf extract were performed using an HPLC system (YL9100) and C18 column (Eclipse XDB-C18, 5µm, 15× 0.46). Identification and quantification of these compounds were done through comparing their retention times with those of standards (Baccatin III: 0.9-1 min, Taxol: 7.6 min) (Sigma-Aldrich) and using their calibration curves according to the method described by Hazrati et al. (2022a) at 227 nm. The mobile phase consisted of water and acetonitrile at a flow rate of 1.5 mL min^{-1}. The injection volume was 20 µL.

Hydrogen peroxide content

The amount of hydrogen peroxide (H_2O_2) was assayed according to Loreto and Velikova (2001)

and quantified using the H_2O_2 standard curve. For this, 0.1 g of the powdered sample was blended in 1.5 mL TCA (0.1%). The samples were centrifuged at 12,000 rpm and 4 °C for 15 min. Five hundred microliter of PBS (10 mM, PH = 7) and 1 mL of potassium iodide (1 M) were added to the 500 µL supernatant. After thorough mixing, the absorbance of the mixture was recorded at 390 nm.

Lipid peroxidation

The level of lipid peroxidation in the cells was determined by measuring the amount of malondialdehyde (MDA) (Heath and Packer, 1968). Briefly, 1 mL reaction solution (20% TCA and 0.5% TBA) was added to 500 µL of the cell extract. The mixtures were incubated for 30 min at 95 °C, immediately placed on ice, and centrifuged for 5 min at 10,000 rpm and 4 °C. The absorbance of the supernatant was recorded at 532 and 600 nm using a spectrophotometer.

Statistical analysis

All experiments were performed in triplicate. Data variance analysis (ANOVA) was performed, and Duncan's Multiple Range Test (DMRT) (at $P < 0.05$) was used to compare the means. Statistical analysis was performed using IBM SPSS Ver.16 software (IBM Corporation and Others, Armonk, NY, USA). The results were presented as mean ± standard error (SE).

Results

Total phenol

The highest amount of phenol was found in the Eshkevarat region in September with an average of 2590.6 µg g^{-1}, and the lowest amount was found in the Niaraq region in May with an average of 2161.9 µg g^{-1}. There were significant differences in total phenol content between plants in each region at different sampling times. Generally, in all regions, plants in September had the highest phenol content (Fig. 1).

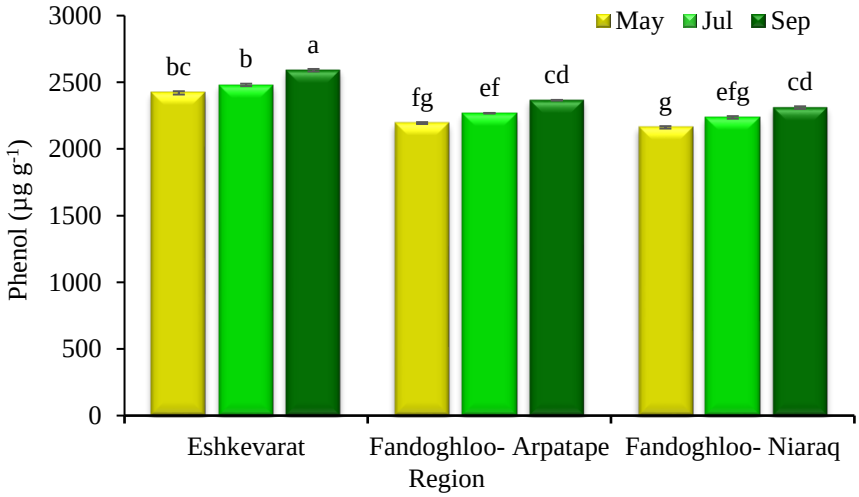

Fig. 1. Total phenol content of various studied *Corylus avellana* growth regions (values with different letters have significant differences at $P \leq 0.05$ According to DMRTs).

Flavonoid content

Analysis of the studied populations showed that flavonoid levels differed significantly between different regions and sampling times. The highest flavonoid content was observed in the Eshkevarat region in September. At all sampling times, the Eshkevarat region had higher flavonoid content than other regions (Fig. 2), and this amount increased in September over July and May.

Antioxidant activity

Among all regions and sampling times, the highest antioxidant capacity was observed in the Arpatape region in September, while the lowest antioxidant activity was observed in the Niaraq region in May (Fig. 3). Furthermore, in terms of antioxidant activity, there were significant differences between sampling times. There was a significant increase in antioxidant activity in September in all regions compared to the other two sampling times.

Paclitaxel and Baccatin III

A significant difference was found between the studied regions and sampling times in terms of Taxol and Baccatin III metabolites content. The amount of Taxol and Baccatin III in the samples collected in September was greater than those in the samples collected in July and May. Moreover, the amount of these metabolites in the July

samples was higher than in the May samples. Generally, the amount of Taxol and Baccatin III in Eshkevarat was higher than in other regions. As a result, the highest amount of Taxol (26.822 µg g^{-1}) was found in Eshkevarat region in September, and the lowest amount (19.971 and 20.524 µg g^{-1}) was found in Niaraq and Arpatape regions in May. Similarly, the highest amount of Baccatin III (69.474 and 66.19 µg g^{-1}) was obtained from Eshkevarat in September and July, and the lowest amount of Baccatin III (28.91 and 30.597 µg g^{-1}) was obtained from Niaraq and Arpatape in May samples. Furthermore, the results showed that Baccatin III level was significantly higher than Taxol level in the studied populations (Fig. 4).

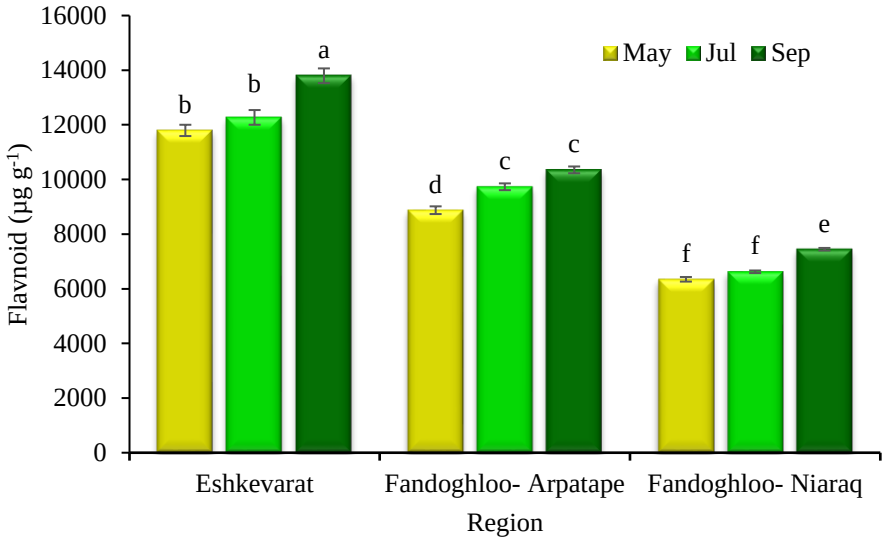

Fig. 2. Flavonoids content of various studied *Corylus avellana* growth regions (values with different letters have significant differences at $P \leq 0.05$ according to DMRTs).

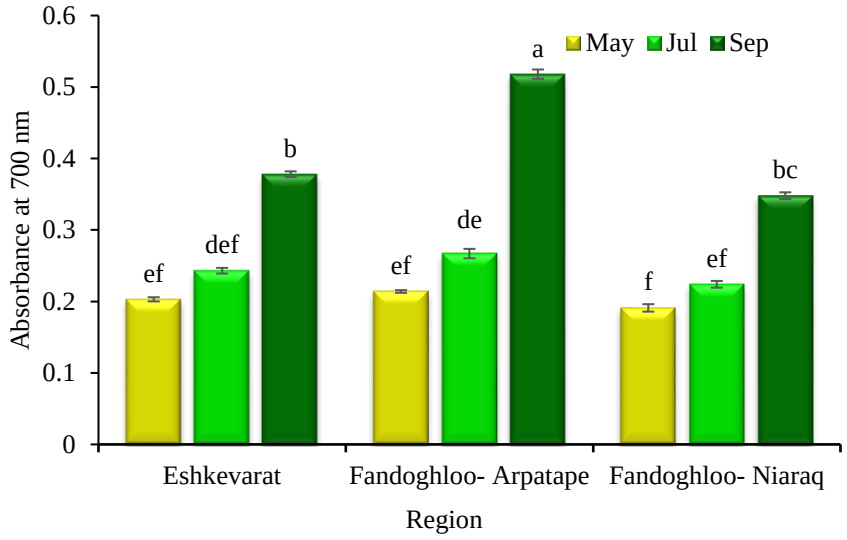

Fig. 3. Antioxidant capacity of various studied *Corylus avellana* growth regions (values with different letters have significant differences at $P \leq 0.05$ according to DMRTs).

Hydrogen peroxide and lipid peroxidation

There were significant differences between the studied regions and sampling times in terms of H_2O_2 and MDA content. The highest H_2O_2 content was observed in the Eshkevarat region in July, with an average of 13.108 µmol g^{-1}, while the lowest H_2O_2 content was observed in the Niaraq and Arpatape regions in May, with an average of 7.801 and 7.927 µmol g^{-1}, respectively. The highest MDA content was found in the Eshkevarat region in July. In contrast, the Arpatape, Niaraq, and Eshkevarat regions had the lowest MDA content in May. In addition, in all studied regions, H_2O_2 and MDA were significantly higher in May than the other two sampling times (Fig. 5).

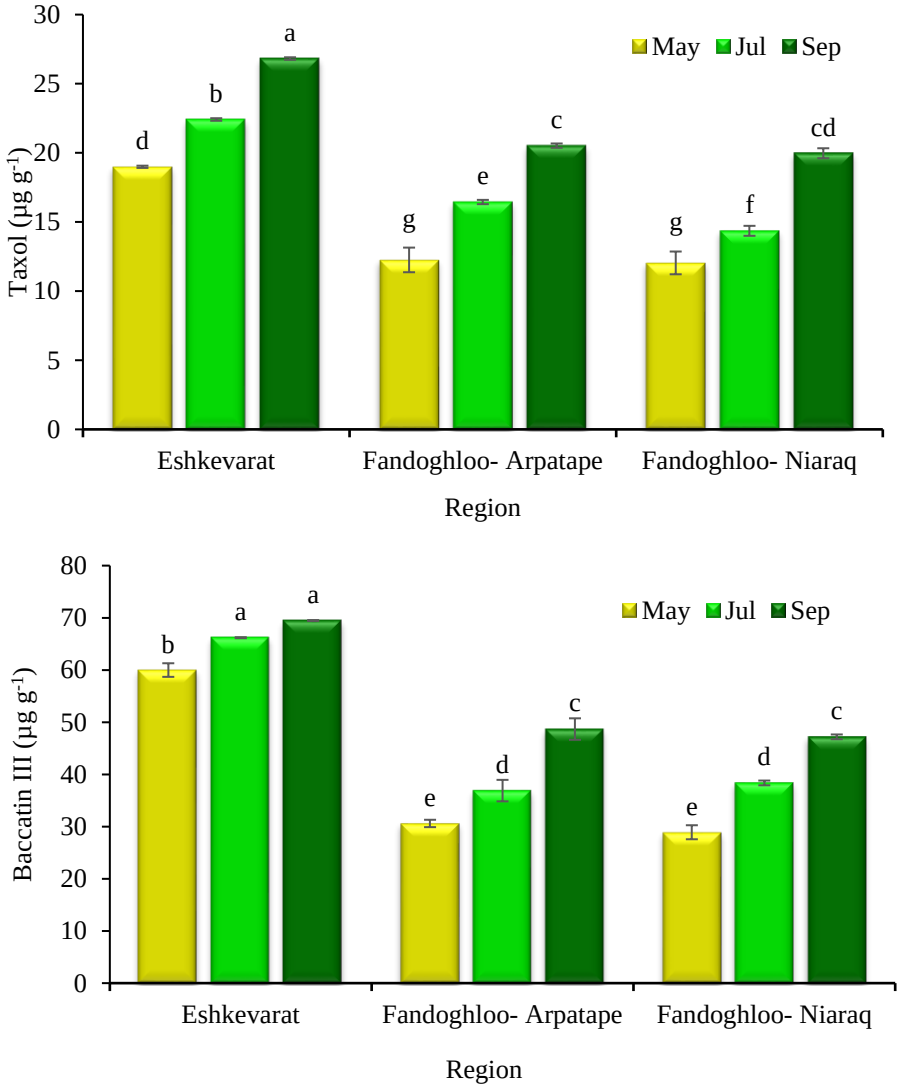

Fig. 4. Taxol and Baccatin III content of various studied *Corylus avellana* growth regions (values with different letters have significant differences at $P \leq 0.05$ according to DMRTs).

Discussion

Previous research has demonstrated that medicinal plants and their metabolites are effective at treating diseases without significant adverse effects, which has led to increased interest in the production and use of these plants. Phytochemicals can also be utilized as models for designing, semi-synthesising, and synthesizing many drugs that can be used to manage ailments in both humans and animals. While several anticancer medications have significant side effects and limited efficacy against various types of cancer, Taxol exhibits a distinct impact on cancer cells with minimal side effects in comparison to other similar substances (Baloglu and Kingston, 1999; Chaachouay and Zidane, 2024). Taxol is the most widely used anticancer drug, and along with Baccatin III and 10-deacetylbaccatin III as precursors of Taxol, they are considered the most important taxanes (Zhao et al., 2015). One of the natural sources that contain Taxol and related taxanes is the hazel plant (Mehlenbache, 2006). Hazel plant and its cell culture have been introduced as a new source of taxol (Zhao et al., 2005) and confirmed by subsequent studies (Hazrati et al., 2017a; Hazrati et al., 2017b; Hazrati et al., 2022a; Hazrati et al., 2022b; Hazrati et al., 2023). So it is crucial to investigate phytochemical content and variation in hazel natural habitats. Most of the hazelnuts growing areas in Iran are limited to the edge of the Caspian Sea and mountainous areas, which include Gilan, Qazvin, Ardabil, Mazandaran, and Qom provinces (Nejatian et al., 2012).

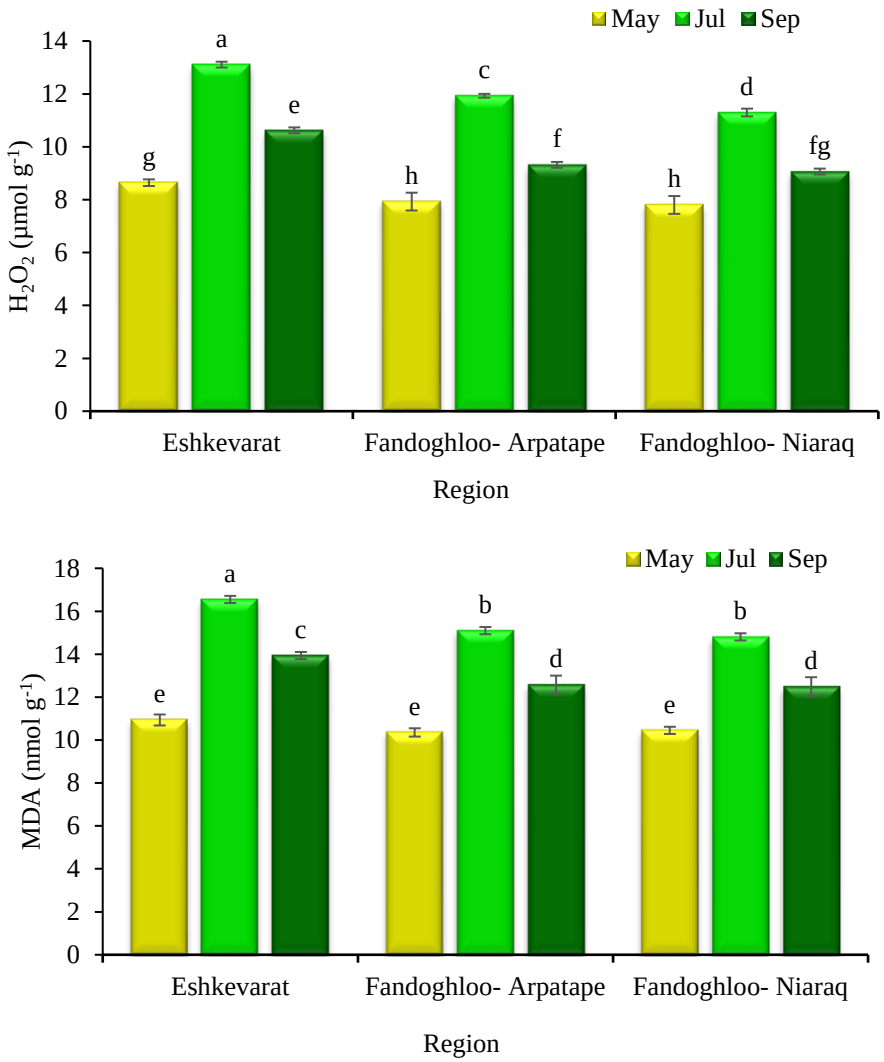

Fig. 5. Amount of MDA (malondialdehyde) and H_2O_2 (hydrogen peroxide) of various studied *Corylus avellana* growth regions (columns with different letters have significant differences at $P \leq 0.05$ according to DMRTs).

Although plant growth and development, the quality and quantity of secondary metabolites in medicinal plants, are controlled by genetic processes, environmental factors also play a critical role (Naghdi Badi et al., 2003). According to the results, the amount of Taxol, Baccatin III, phenol, flavonoid, and antioxidant capacity gradually increased from May to September, so the highest amount of these compounds was observed in September. H_2O_2, MDA showed a significant increase in July compared to May and decreased again in September, but it was still higher than in May. The significant increase in H_2O_2 and MDA in July could be attributed to higher temperatures and increased exposure to sunlight during that period. These environmental factors can induce oxidative stress in plants, leading to the production of reactive oxygen species like H_2O_2 and the accumulation of lipid peroxidation products such as MDA. The results suggest that changes in environmental factors such as temperature and light likely play a role in physiological processes and secondary metabolite production in hazel (Naghdi Badi, 2003). The higher secondary metabolite content in September can be attributed to the increase in temperature at sampling time. Letchamo et al. (1995) reported that plants exposed to increased light exhibit higher concentration of essential oils compared to plants grown in normal light settings. Furthermore, they found that the biosynthesis of secondary metabolites was significantly influenced by specific light regimes. In addition, Yanli et al. (1997) found that *Salvia officinalis* yields the highest amount of essential oil in full sunlight. According to Kelsey and Vance (1992), sunlight also affects the accumulation of Taxol in *Taxus brevifolia*. Additionally, Nasiri et al.

(2016) investigated the seasonal variations in secondary metabolites in *Taxus baccata*. They found that 10-DAB III and total taxanes production are related to light intensity, and Taxol and 10-DAB III production was higher in September, which agrees with our results. Odabas (2009) investigated the effect of light on *Hypericum perforatum* phytochemical characteristics and reported that increasing light intensity causes a significant increase in the accumulation of hyperforin, hypericin, and pseudohypericin.

According to the results, in the Eshkevarat region, the amount of Taxol, Baccatin III, phenol, and flavonoid metabolites was significantly higher than in the other regions (Arpatape and Niaraq). Regarding H_2O_2 and MDA content, the highest values were observed in the Eshkevarat regions, and Arpatape and Niaraq regions showed lower values. In May and July, antioxidant activity in all regions was the same, but it was higher in Arpatape region in September compared to other regions. These differences in the amount of investigated secondary metabolites may be related to habitat differences, environmental factors, climate and weather conditions, soil conditions, and plant access to light. The higher H_2O_2 and MDA content in the Eshkevarat region suggests that there may be increased oxidative stress and lipid peroxidation occurring in this area. This could be indicative of environmental or physiological factors that are affecting the antioxidant defence system in plants, potentially making them more vulnerable to oxidative damage. These environmental stressors can lead to the production of reactive oxygen species, which can then induce the plant defence system's including the increased activity of secondary metabolite biosynthesis pathways. Some potential environmental factors that could be affecting the antioxidant defence system in plants include geographical characteristics of the habitat, climate and weather conditions, soil conditions and contaminations, and plant access to light, and levels of UV radiation (Qaderi et al., 2023). These factors can disrupt the balance between antioxidant production and reactive oxygen species, leading to increased oxidative stress and lipid peroxidation in plants. The higher amount of compounds in the Eshkevarat region could be attributed to the higher temperature and a longer growing season in the sample collection times compared to the other regions, which could also contribute to the higher concentration of these metabolites. Ranalli et al. (1999) reported that phenolic substances, tocopherols, and fatty acids are affected by different climatic conditions. Salvador et al. (2003) also reported the effect of the production area on the chemical composition and quality of *Cornicabra* olive oil. In another study, Aguilera et al. (2005) investigated the quality of olive oil of Brantoio and Lechino cultivars in two regions with different altitudes from sea level and reported that the oleic acid content of the oil was higher at higher altitudes.

The secondary metabolites biosynthesis is a complex process that can be influenced by various factors, including plant internal and external factors, as well as environmental signals. An accurate understanding of the mutual effects of plant internal factors and environmental signals on the synthesis of secondary metabolites will provide efficient strategies to manipulate these factors and ultimately enhance the production and efficacy of secondary metabolites. Environmental signals play a crucial role in secondary metabolite production by influencing gene expression and enzyme activity. For example, changes in temperature, light intensity, and nutrient availability can trigger specific biochemical pathways leading to the synthesis of desired secondary metabolites. Sampling time and environmental signals as epigenetic factors can have a central role in the production of taxanes and the regulation of gene expression, as well as the genes involved in secondary metabolism. DNA methylation, as a critical epigenetic factor, is stimulated in response to various plant cell growth and developmental stages and environmental signs (Mahfouz, 2010). It can be concluded that plant cells may use different methylation states depending on the type, quantity, and quality of environmental signals. This dynamic response to environmental signals can influence the expression of genes involved in secondary metabolism, including the Taxol biosynthesis pathway, ultimately resulting in different levels of taxane biosynthesis and accumulation in hazel plants throughout the year (Nasiri et al., 2016).

Conclusion

In the present study, in addition to Taxol and Baccatin III, differences in the amount of antioxidant compounds such as total phenol, flavonoid, and antioxidant capacity were also observed among the studied regions and sampling times. The differences in these compounds between *Corylus avellana* populations in different regions can help identify and select the appropriate population for breeding and biotechnological purposes.

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Author contributions
NZ designed the project and performed the sample collection supervised. RH performed the HPLC analysis, antioxidant, TSP, and TF assays. RH and NZ analyzed the data and prepared the manuscript.

Conflict of Interest
The authors indicate no conflict of interest in this work.

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