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Exploring vitamin D_3 profile of epiphytic lichen forming fungi in forest ecosystems: Influence of habitat-dependent ecological variables

Gülşah Çobanoğlu [a,*], Hilmi Özdemir [b], Mehmet Özdemir [b], Gafura Aylak Özdemir [c], Emrah Özdemir [c], Fatma Ebru Koç [d], Ahmet Özcan [b]

[a] Department of Biology, Faculty of Science, Marmara University, Göztepe Campus, Kadıköy, Istanbul, Türkiye
[b] Marmara Forestry Research Institute Directorate, Fatih Forest Campus, Maslak-Sarıyer, Istanbul, Türkiye
[c] Department of Forest Yield and Biometry, Istanbul University Cerrahpaşa Forest Engineering, Bahçeköy-Sarıyer, Istanbul, Türkiye
[d] Vocational School of Health Services, Istanbul Gelisim University, Avcılar, Istanbul, Türkiye

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ABSTRACT

Vitamin D is critically important for sustainable human health, and the rising prevalence of deficiency-related diseases has increased interest in natural sources. This study explores the potential of epiphytic lichen-forming fungi, known for their unique metabolites, as a novel biosource of vitamin D_3 for pharmaceutical and nutraceutical applications. Fourteen epiphytic lichen species were collected using a stratified sampling method from four mountainous forests in the Marmara Region of Türkiye. Vitamin D_3 contents of the samples were analysed in relation to six ecological variables: study area layer, stand type, tree species, altitude, aspect, and air humidity and temperature. Extraction methods including maceration, Soxhlet, and supercritical CO_2 techniques were applied, followed by HPLC analysis. Olive oil-based maceration was identified as the most efficient extraction method. One-way ANOVA showed significant associations between vitamin D_3 content and lichen species ($p = 0.024$), layer, stand, and tree species. Concentrations ranged from 0.6 to 20.33 μg/g, with *Bryoria fuscescens*, *Evernia prunastri*, and *Pseudevernia furfuracea* yielding the highest values. The highest vitamin D_3 levels were detected in Uludağ, especially in coniferous forests dominated by *Pinus nigra*. The results indicated that forest layer, stand, altitude, and tree species significantly influence vitamin D_3 amounts, while aspect and humidity do not. This is the first study to comprehensively report natural vitamin D_3 content in lichens, filling an important scientific gap and demonstrating their potential as an eco-sustainable resource for vitamin D_3 production

1. Introduction

Vitamin D has been recognized as an important requirement for sustainable human health for many years. Due to its role in maintaining calcium levels (Grant et al., 2015), vitamin D has a critical function in protecting against autoimmune, cardiovascular, neurological and infectious diseases, especially in the endocrine system, phosphorus and bone metabolism (Dusso et al., 2005; Holick, 2009). Vitamin D deficiency, which is still a global health problem today, has been associated with the treatment of various disorders and chronic diseases including cancer, hypertension, multiple sclerosis, rheumatoid arthritis, osteoporosis, muscle weakness and diabetes (Zhang and Naughton, 2010; Wacker and Holick, 2013). It is defined as vitamin D deficiency when the 25(OH)D level is below 20 ng/mL, and vitamin D insufficiency when it is 21–29 ng/mL (Fidan et al., 2014; Pludowski et al., 2018). Vitamin D is mostly synthesized in the skin by ultraviolet B (UVB) rays or taken with food and converted to the biologically active form, D_3 (1,25-dihydroxyvitamin D) in the liver and kidneys. Since it is difficult to provide adequate vitamin D intake (15 μg/day as determined by the European Food Safety Authority) through diet alone, appropriate doses of food supplements containing vitamin D are generally recommended (Benedik, 2022). Vitamin D is found in two main forms as D_3 (cholecalciferol) and D_2 (ergocalciferol) in many animal-derived products (Dusso et al., 2005). The system and ability of organisms to produce vitamin D differ from each other (Göring, 2018). Potentially rich biological sources of vitamin D in nature include algae, some plants, fungi and lichens, but the literature on this subject is not yet sufficient (Çobanoğlu Özyiğitoğlu, 2020).

* Corresponding author.
E-mail address: gcoban@marmara.edu.tr (G. Çobanoğlu).

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Lichens are unique examples of a symbiotic lifestyle based on the principle of mutual benefit through the association of certain terrestrial fungi (mostly Ascomycota) with photosynthetic species (Chlorophyta and/or Cyanobacteria) (Honegger, 2023). Lichen-forming fungi can protect themselves against biotic (insect bites, danger of being eaten by animals, etc.) (Dayan and Romagni, 2001; Lawrey, 2009), and abiotic (climatic factors such as temperature, drought, UV radiation, etc.) stress by synthesizing their own metabolites in order to survive in the ecosystem (Al-Amoody et al., 2020). The majority of the more than 1050 known lichen-specific secondary metabolites are formed as a result of fungal metabolism (Stocker-Wörgötter, 2008), the number and types of which vary depending on the species (Olivier-Jimenez et al., 2019) and environmental changes (Deduke et al., 2012). In this way, many lichens have antibiotic activity and become resistant to microorganisms in nature (Özyiğitoğlu et al., 2017). Some of the lichen compounds are pigments that protect them from harmful UV rays (Solhaug et al., 2003), such as parietin (Comini et al., 2017), and probably have an important role in the synthesis and storage of vitamin D in lichens (Al-Amoody et al., 2020). Göring (2018) noted that lichens accumulate vitamin D_3 in their structures due to the lack of metabolic enzymes. It was stated many years ago that the reindeer lichen *Cladonia rangiferina* contains small amounts of vitamins A, C and D (Karamanoğlu, 1971) and that high amounts of vitamins D_2 and D_3 are found in *Cladina* species (Björn and Wang, 2000). It has been reported that 100 g of dry material of *Cladonia arbuscula*, one of the lichens that is the food of reindeer, contains 67–204 μg of vitamin D_3 and 22–55 μg of D_2 and high ergosterol (Wang et al., 2001). A positive correlation was found between D_3 content and UV-B radiation in *C. arbuscula* populations growing at different latitudes from Finland to Greece. In their study on lichen-based vitamin D supplements in India, Gangwar (2023) reported that *Bulbothrix setschwanensis* lichen extracts were the most potent and absorbable source, containing 23,000 IU/g (~580 μg/g) of vitamin D.

Several studies have been conducted on vitamin D biosynthesis in source organisms such as plants (Ferguson et al., 2005; Jäpelt and Jakobsen, 2013; Black et al., 2017) and fungi (Phillips et al., 2012; Keegan Raphael-John et al., 2013; Cardwell et al., 2018), while information is rather limited for lichens. In particular, there is a significant gap regarding the effect of environmental factors on the vitamin D production of lichens. Most research shows that vitamin D production in humans and other organisms is related with UV doses (sun exposure), but the triggering factors are probably not limited to this. It can be predicted that vitamin D biosynthesis in lichens, as well as secondary metabolite and pigment levels, change in response to environmental stress. Further research is needed on what kind of ecological variables affect vitamin D accumulation in lichens in different habitat conditions.

This study pursues two separate objectives together; to determine the vitamin D_3 content profile of various lichen-forming fungi species densely distributed in high mountain forest ecosystems in the Marmara Region of Türkiye, and to determine the extent to which ecological variables are related to vitamin D_3 levels of these lichens. In this way, the ecological parameters that should be taken into consideration when collecting lichen to obtain a vitamin D source will be revealed and an environmentally friendly harvest will be ensured. This study is the first report on the investigation of new natural sources of vitamin D_3 for most of the lichen species examined and the evaluation of their relationships with habitat-dependent ecological factors, and it has contributed new data to the literature in this field. It is anticipated that our study will contribute basic information to future studies on the development and production of lichen-derived vitamin D food supplements and therapeutic drugs.

2. Materials and methods

2.1. Study area and sampling

Uludağ, Kartepe, Yıldız Mountains and Kaz Mountains located in the Marmara Region in north-western Türkiye were determined as study area layers (Fig. 1), and each of them is divided into 3 homogeneous area types, including deciduous and coniferous pure stands and open areas. The management plan, sub-division layer GIS (Geographic Information Systems) data obtained from the General Directorate of Forestry belonging to the study areas were uploaded to Google Earth, and after the field road network and pure stand types were determined, the study points were finalized by considering the lichen density. In order to adequately represent each area type and to reach the targeted highest level of lichen species, 5 points were selected from each of the pure stand types regardless of the area size and all lichen samples within the area were collected by scanning an area of 800 m^2 starting from the centre of these points. During the collection of lichen samples, the temperature and humidity of the environment were measured by setting up a humidity and temperature measuring device at the centre of the sampling point. These data are detailed in Supplementary Table 1.

Lichens were collected from a total of 60 points, 4 study areas (layers) x 3 pure stands (coniferous, deciduous, open area) x 5 points (Figs. 1 and 2), using the stratified sampling method between June 2021 and 2022.

A sufficient amount of fruticose and foliose lichen material for analysis, not affecting the continuity of the species, was collected in paper or plastic bags, mostly from tree trunks and branches, and some from the soil. They were left to dry at room temperature in the laboratory on the same day. The dried lichen samples were cleaned from foreign materials such as bark, dust and soil, then placed in envelopes and labelled.

2.2. Taxonomic identification

The species identification of lichens was done by the first author using various flora books and identification keys (Wirth, 1995; Brodo et al., 2001; Smith et al., 2009). Index Fungorum (https://www.indexfungorum.org) was taken as basis for current names in the nomenclature of taxa. The identified species, together with habitat and locality information, were recorded in the Marmara University Faculty of Science Herbarium (MUFE)-Lichenarium database with herbarium numbers between 2807 and 2900.

A total of 72 lichen materials belonging to 14 different species, *Bryoria fuscescens* (Gyeln.) Brodo and D. Hawksw., *Cladonia furcata* (Huds.) Baumg., *Cladonia rangiformis* Hoffm., *Evernia divaricata* (L.) Ach., *Evernia prunastri* (L.) Ach., *Platismatia glauca* (L.) W.L. Culb. and C. F. Culb., *Pseudevernia furfuracea* (L.) Zopf, *Ramalina calicaris* (L.) Röhl., *Ramalina farinacea* (L.) Ach., *Ramalina fastigiata* (Pers.) Ach., *Ramalina fraxinea* (L.) Ach., *Usnea filipendula* Stirt., *Usnea florida* (L.) F.H. Wigg., *Usnea glabrescens* (Nyl. ex Vain.) Vain., were separated for research and one sample from each was delivered to AksuVital Natural Products Company for specific analyses. Detailed information about lichen samples, study area and geographical sampling points are provided in Supplementary Table 1.

2.3. Chemical extraction and HPLC analysis

Firstly, in order to test the effectiveness of the extraction method, three different extraction methods, namely Soxhlet, Maceration and Supercritical CO_2, specified in the following subsections (2.3.1-2-3), were applied with various solvents (olive oil, acetone, hexane, ethanol and chloroform) to 10 lichen samples determined to represent the sampling points in terms of distribution. Among these, maceration in olive oil yielded the highest and most consistent vitamin D_3 levels. Based on this comparative pre-testing, we standardized the olive oil maceration protocol for all remaining samples in order to ensure methodological consistency and to focus on the most promising approach. After these preliminary trials for optimization, all of the collected lichen samples (72) were extracted with the most efficient method determined. In the final stage, the presence and amounts of vitamin D_3 in these

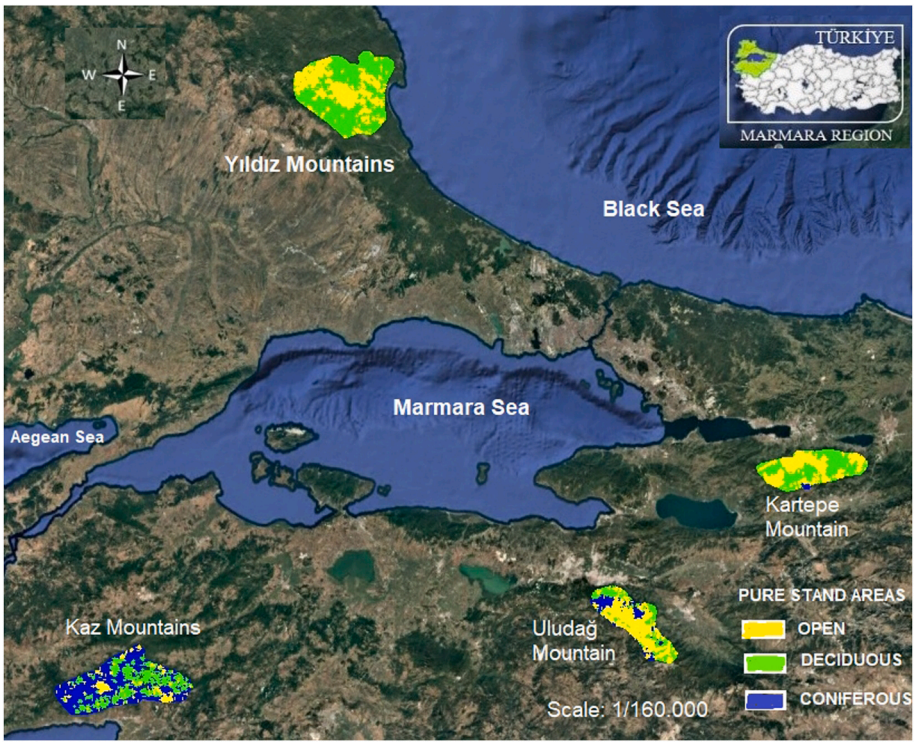

Fig. 1. Satellite image of the study area covering 4 separate layers in the Marmara Region.

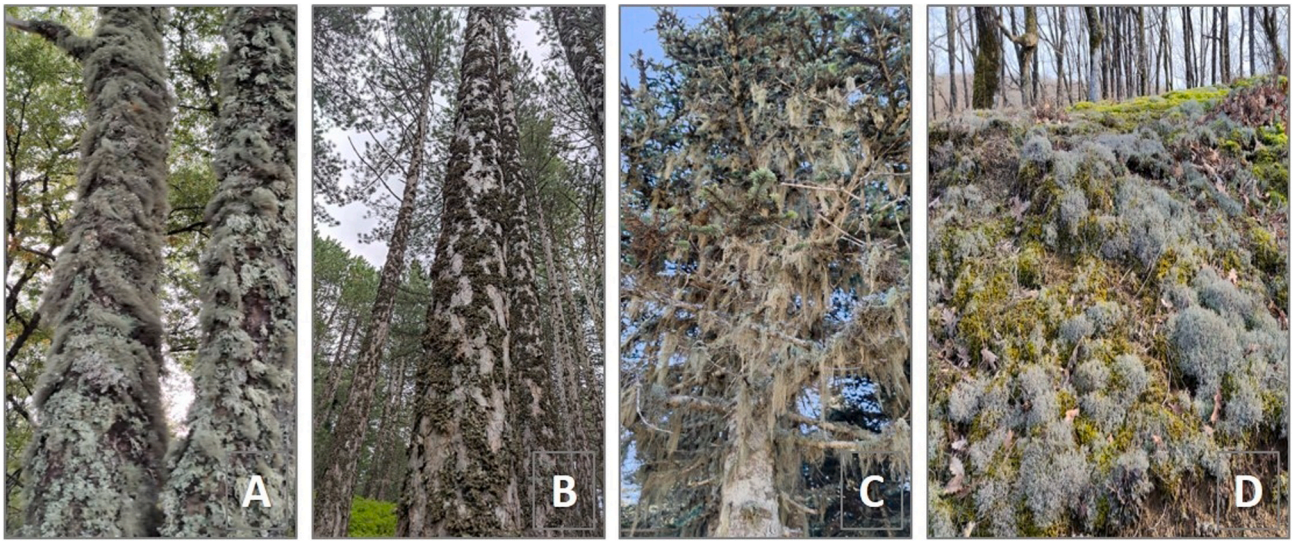

Fig. 2. Lichen-covered forest stands from 4 different study area layers; A. Kartepe oak, B. Kaz Mountains black pine, C. Uludağ fir, D. Yıldız Mountains oak.

extracts were determined by High Performance Liquid Chromatography (HPLC) analysis. The parameters for the HPLC analysis are given in Supplementary Table 2. The analysis was carried out with an isocratic system and a constant flow. Since vitamin D is light sensitive, the procedures were carried out with the light of the automatic sampling device turned off.

Extraction and chemical analysis procedures for the determination of vitamin D_3 in lichens were carried out in the laboratories of AksuVital Natural Products Company. Since the lichen samples were delivered dried, they were passed through a high-speed shredder (Biobase) in the laboratory without being exposed to any heat beforehand and were made ready for extraction.

Extraction pre-experiments were optimized using a response surface methodology (RSM) to identify the most efficient extraction parameters. Based on this optimization, a standard maceration protocol using olive oil was applied uniformly to all lichen samples to minimize methodological variation and ensure comparability of results. The measured vitamin D_3 concentrations are given in Supplementary Table 3.

2.3.1. Soxhlet extraction

8 g of lichen sample was weighed on filter paper and placed in the Soxhlet apparatus. 100 mL of solvent was added to the flask and reflux was performed at 120 °C for 8 cycles and 4 h. After evaporating the solvent in a rotary evaporator at 50 °C under 200 bar vacuums, it was dissolved with 5 mL of DMSO: Methanol (3:7). Then, it was filtered with RC 0.45 (De Castro and Garcia-Ayuso, 1998; Mishra et al., 2010; Alara

et al., 2018).

2.3.2. Maceration extraction

For olive oil maceration, 20 g of sample was weighed in a beaker and 100 mL of olive oil was added. The beaker was closed and macerated in olive oil for 24–48 h at 40 °C in a water bath. 20 mL of sample was taken from the macerate and placed in a separating funnel with 50 mL of hexane and shaken vigorously. The separated lower phase was separated and shaken vigorously again with 50 mL of hexane. Hexane was completely removed in a 40 °C rotary evaporator under 200 bar vacuums. The obtained extract samples were dissolved with 5 mL of DMSO: Methanol (3:7). The upper phase was filtered through RC 0.45 filter, and analysed on HPLC device (Zeytinoglu et al., 2008; Ren et al., 2009; Triggiani et al., 2009).

For solvent macerations, 10 g of sample was weighed into a beaker and 100 ml of solvent was added. It was macerated in a water bath at 40 °C for 24–48 h. The sample was filtered through coarse filter paper. The filtrate was evaporated in a rotary evaporator under 200 bar vacuums at 60 °C for chloroform, 25 °C for acetone, and 40 °C for ethanol and hexane. 5 mL of DMSO: Methanol (3:7) was dissolved in the obtained extract sample and then passed through the RC 0.45 filter.

2.3.3. Supercritical CO_2 extraction

For studies on this technique, services were purchased from the laboratory of LUK Oil. For samples that were not in sufficient quantity to fill the chamber of the device used at the desired rate (2 L), adjustments were made; 100 g of sample was mixed with 1 kg of high-oil ground sesame seeds and added to the machine chamber. The studies were carried out at 295 bar pressure, 40 °C and 2.3 mL/min flow rate. After weighing 1 g of sample from the obtained extraction products, 10 mL of isooctane was added and centrifuged for 10 min (Sihvonen et al., 1999; Zizovic et al., 2012; Zugic et al., 2016).

2.4. Data processing and statistical analysis

The study involved a comparative evaluation of vitamin D_3 content among lichen species, and across the layers and tree species where these lichens were distributed. For this purpose, one-way analysis of variance (ANOVA) was conducted to determine whether significant differences existed among groups. Before the analysis, assumptions of normality and homogeneity of variances were tested to ensure the appropriateness of the ANOVA model. When the results indicated statistically significant differences ($p < 0.05$), Duncan's test was employed as a post hoc procedure to identify homogeneous subgroups. In addition, the t-test was used to compare forest stand types (coniferous and deciduous) in which lichen species occur in terms of their vitamin D_3 content, while the Mann–Whitney U test was employed to assess differences among aspect, elevation, and humidity classes. The relationships between the vitamin D_3 content of lichen species and three continuous environmental variables: the altitude of the locality, the temperature and the air humidity at the time of collection were examined using Pearson correlation analysis. In addition to these analyses, since the data were measured on a categorical scale, the Chi–Square test of independence was applied to assess whether there was a statistical dependence between the distribution of lichen species and six ecological variables: "Study area layer, pure stand, tree species, aspect, altitude and humidity at the time of collection." Data analysis was evaluated using the IBM SPSS Statistics 29.0.2.0 for Windows program. Tables and visualizing plots were created with Microsoft Office Excel 2016.

3. Results

3.1. Vitamin D_3 profiles of lichen species

Since the most efficient results detected in the preliminary experiments were obtained with the maceration method with olive oil, the amount of vitamin D_3 per unit weight (μg/g) was determined with this maceration extraction in a total of 72 lichen samples belonging to 14 different lichen species included in the study. 8 of the lichen species that provided statistically sufficient data were compared with one-way variance analysis in terms of vitamin D_3 they contain. Data for species with insufficient sample sizes were also shown in the data table as preliminary data, but they were not included in the statistical evaluations. The results of variance analysis showed that there was a statistically significant difference ($p = 0.024 < 0.05$) among lichen species in terms of vitamin D_3 content (Table 1). As a result of the Duncan test, which is one of the post-hoc tests we applied to reveal the differences between lichen species, *C. furcata*, *R. farinacea* and *R. fastigiata* differed only from *B. fuscescens* in terms of vitamin D_3 content. No significant difference was found among other lichen species. Vitamin D_3 concentrations ranged from 0.6 to 20.33 μg/g, with *B. fuscescens* (11.31 μg/g), *Evernia prunastri* (7.63 μg/g) and *P. furfuracea* (5.82 μg/g) showing the highest levels. The average value of all samples was calculated as 5.62 μg/g. *B. fuscescens* had the highest mean, while *C. furcata* had the lowest (0.76 μg/g) (Fig. 3).

When the vitamin D_3 contents of 8 lichen species with sufficient data are compared with the genus-based grouping, excluding species with limited data, the results show a decreasing order of *Bryoria* > *Evernia* > *Pseudevernia* > *Usnea* > *Ramalina* > *Cladonia*. The fact that the content levels of the two species belonging to the same genus observed in *Ramalina* and *Usnea* are not similar indicates that the differences are more pronounced at the species level. In addition, the terricolous *Cladonia* genus was separated from the epiphytes by having the lowest vitamin D storage of all.

3.2. Distribution of lichen taxa in relation to ecological variables

Firstly, some ecological variables that are likely to affect the change in vitamin D_3 levels were checked in terms of their relationships with the

Table 1
Statistical differences in vitamin D_3 levels (μg/g) among 14 lichen species.

Lichen Species	N	Average ± standard deviation	Min.	Max.	Analysis of Variance	
					F	Significance Level (p)
Bryoria fuscescens	5	11.31 ± 8.62[b]	0	20.33	2.54	0.024*
Cladonia furcata	4	0.76 ± 0.95[a]	0	1.98		
Evernia prunastri	14	7.63 ± 5.21[a, b]	0	17.15		
Pseudevernia furfuracea	14	5.82 ± 6.03[a, b]	0	16.35		
Ramalina farinacea	11	1.71 ± 3.963[a]	0	13.25		
Ramalina fastigiata	5	3.63 ± 5.77[a]	0	13.23		
Usnea filipendula	3	5.26 ± 4.76[a, b]	0	9.28		
Usnea florida	7	6.10 ± 3.89[a, b]	0	12.05		
Cladonia rangiformis	2	1.86 ± 0.16	1.75	1.98	No sufficient data	
Evernia divaricata	2	5.50 ± 3.75	2.85	8.15		
Platismatia glauca	2	9.94 ± 5.71	5.90	13.98		
Ramalina calicaris	1	–	7.58	7.58		
Ramalina fraxinea	1	–	0	0		
Usnea glabrescens	1	–	3.53	3.53		

N = number of samples; Min = Minimum; Max = Maximum; * indicates 5 %; a and b show the groups that differ according to the Duncan post-hoc test.

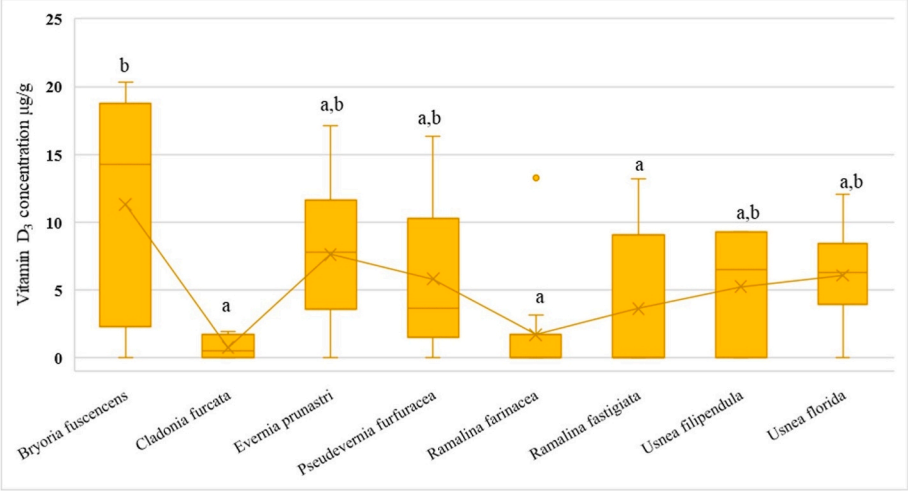

Fig. 3. Box and whisker plot showing analysis of variance data comparing vitamin D_3 concentrations (µg/g) in 8 lichen species with statistically normal data distribution (In histograms, the "x" symbol represents the mean, the line represents the median, a and b represent group differences).

distribution frequency of lichen species (Table 2). The correlation between the distribution of lichen species and 6 ecological variables, including "study areas layers, pure forest stands (coniferous, deciduous), tree species, aspect, elevation levels and humidity rates at the time of collection" were statistically examined by the Chi–Square independence test (Supplementary Table 4). Some groups were combined in order to perform chi-square analysis reliably in calculations in non-data sections. A total of 14 lichen species were reduced to *Cladonia*, *Evernia*, *Ramalina* and *Usnea* based on genera, and *B. fuscescens*, *P. glauca* and *P. furfuracea* were considered as "other lichen species". Since there was data for 2 groups in pure forest areas, altitude levels and air humidity classes, no reduction was made. In tree species, Fir and Oak, which have more lichen species, were considered as separate groups, while Red Pine, Black Pine and Beech species were collectively grouped as "other species". Layers were examined in 2 groups by combining Kartepe and Yıldız Mountains, Uludağ and Kaz Mountains, and aspects were examined in 2 groups as sunny and shady aspects.

Accordingly, it was revealed that there was a dependency between the distribution of lichen taxa and the study areas (layers) with a significance level of $p = 0.007 < 0.05$, between tree species with a significance level of $p = 0.005 < 0.05$, and between elevation levels with a significance level of $p < 0.001$, but since $p = 0.290 > 0.05$, there was no dependency between aspects. Since the expected frequency of more than 20 % of the cells is less than 5, the test statistics and significance level of the Fisher Exact test were taken into consideration.

Dependency was determined between the distribution of lichen taxa and pure stand areas with a significance level of $p = 0.001 < 0.05$. However, there was no dependency between the air humidity at the time of collection and the distribution of lichens with a significance level of $p = 0.078 > 0.05$. Here, since the expected frequency of 20 % of the cells is less than 5, the Pearson' Chi–Square test statistic and significance level were considered.

3.3. Relationships between ecological variables and D_3 vitamin contents

Vitamin D_3 concentration levels of lichen species were compared in relation to differences in study area layers, tree species, pure stands, elevation steps, aspects and air humidity classes. Statistical evaluation results analysed separately for each ecological variable are shown in Table 3, and shown graphically in Fig. 4.

Correlation analysis between the vitamin D_3 content of lichens and the altitude of locality, the temperature at the time of collection and the air humidity (Table 4) revealed that there was no significant relationship between these parameters ($p = 0.05$).

Study area layers: Data on vitamin D_3 content of lichen species in 4 different layers (Yıldız Mountains, Kartepe, Uludağ and Kaz Mountains)

Table 2
Distribution frequency of 14 lichen taxa by study areas, pure stand areas and tree species.

Lichen Species	Study Area Layers					Pure Stands			Tree Species					
	Kartepe	Uludağ	Yıldız	Kaz	Total	Coniferous	Deciduous	Total	*Abies* spp.	*Pinus brutia*	*Pinus nigra*	*Fagus* spp.	*Quercus* spp.	Total
B. fuscescens	1	4	–	–	**5**	5	–	**5**	5	–	–	–	–	**5**
C. furcata[a]	–	–	4	–	**4**	–	4	**4**	–	–	–	–	4	**4**
C. rangiformis[a]	–	–	2	–	**2**	–	2	**2**	–	–	–	–	2	**2**
E. divaricata	–	2	–	–	**2**	2	–	**2**	2	–	–	–	–	**2**
E. prunastri	7	–	1	6	**14**	4	10	**14**	3	1	–	–	10	**14**
P. glauca	–	–	–	2	**2**	2	–	**2**	–	–	2	–	–	**2**
P. furfuracea	7	5	–	2	**14**	11	3	**14**	8	–	3	–	3	**14**
R. calicaris	–	–	–	1	**1**	–	1	**1**	–	–	–	–	1	**1**
R. farinacea	7	1	1	2	**11**	5	6	**11**	5	–	–	1	5	**11**
R. fastigiata	5	–	–	–	**5**	3	2	**5**	3	–	–	1	1	**5**
R. fraxinea	1	–	–	–	**1**	–	1	**1**	–	–	–	1	–	**1**
U. filipendula	1	2	–	–	**3**	2	1	**3**	2	–	–	–	1	**3**
U. florida	7	–	–	–	**7**	5	2	**7**	5	–	–	–	2	**7**
U. glabrescens	1	–	–	–	**1**	–	1	**1**	–	–	–	–	1	**1**
Total	**37**	**14**	**8**	**13**	**72**	**39**	**33**	**72**	**33**	**1**	**5**	**3**	**30**	**72**

[a] On the soil under oak (*Quercus*) stand; spp. species.

Table 3
Statistical results of lichen-vitamin D_3 content (μg/g) dependent on ecological variables.

Ecological Variable	Vitamin D_3 Amounts				Statistics Test	
Layer	N	Average ± standard deviation	Min.	Max.	Analysis of variance	
					F	Significance Level (p)
Yıldız mountain	8	0.85 ± 0.95[a]	0	1.98	5.611	0.002**
Kartepe mountain	37	4.36 ± 4.93[a, b]	0	15.58		
Uludağ mountain	14	7.31 ± 6.14[b, c]	0	20.33		
Kaz mountains	13	9.12 ± 5.73[c]	0	17.15		
Tree species					Analysis of variance	
					F	Significance Level (p)
Quercus spp.	30	4.29 ± 4.22[a]	0	13.25	5.12	0.009**
Abies spp.	33	5.52 ± 5.87[a]	0	20.33		
Pinus nigra	5	12.21 ± 4.78[b]	5.90	16.35		
Pinus brutia	1	–	17.15	17.15	No sufficient data	
Fagus spp.	3	–	–	–		
Pure stand area					t-Test	
					t	Significance Level (p)
Coniferous	39	6.67 ± 6.28	0	20.33	2.232	0.029*
Deciduous	33	3.90 ± 4.21	0	13.25		
Aspect					Mann–Whitney U Test	
					U	Significance Level (p)
Shady Aspects	23	6.46 ± 5.57	0	16.35	462	0.214[NS]
Sunny Aspects	49	4.90 ± 5.57	0	20.33		
Altitude					Mann–Whitney U Test	
					U	Significance Level (p)
<1000 m	27	5.95 ± 5.81	0	17.15		
>1000 m	45	5.07 ± 5.47	0	20.33	560	0.576[NS]
Humidity					Mann–Whitney U Test	
					U	Significance Level (p)
<%50	35	5.21 ± 5.63	0	17.15	610	0.669[NS]
>%50	37	5.58 ± 5.59	0	20.33		

Note. spp. species; a, b, c show the groups that differ according to the Duncan post-hoc test; * %5; ** %1; *** %0.01; NS = not significant.

were compared using one-way analysis of variance (ANOVA). The value of $p = 0.002 < 0.05$ showed that there was a statistically significant difference in terms of vitamin D_3 content of lichen species among the layers of the study areas. According to the Duncan test result, Yıldız Mountains differed from all other study areas except Kartepe in terms of vitamin D_3 content of lichens. Kaz Mountains differed from all other study areas except Uludağ. In other words, the Yıldız Mountains layer, where the average vitamin D_3 concentration in lichens is the lowest (0.85 μg/g), is separated from the Kaz Mountains, where it is the highest (9.12 μg/g). No significant difference was observed between Kartepe and Yıldız Mountains, Uludağ and Kaz Mountains (Fig. 4 and Table 3).

Tree species: It was determined that there was a statistically significant ($p = 0.009 < 0.05$) and important difference between the amounts of vitamin D_3 in lichen species and tree species compared by one-way analysis of variance (Table 3). The Duncan test was then performed and it was determined that the vitamin D_3 content was at the highest level in lichen species found in Black Pine (12.21 μg/g). Vitamin D_3 amounts of lichens on Oak and Fir tree species did not show a significant difference. Vitamin D_3 was detected in only one lichen species in Red Pine and in none of the lichen species in Beech (Fig. 4).

Pure stand areas: The study areas cover coniferous, deciduous and open homogeneous areas, lichen species were detected in the first two. A significant difference at $p = 0.029 < 0.05$ level was found when comparing the vitamin D_3 contents of lichen species in coniferous and deciduous pure stand areas where the data showed normal distribution using the t-test (Table 3). This result reveals that the vitamin D_3 contents of lichen species are affected by the forest form in which they are located. While lichen species in coniferous areas had the highest average vitamin D_3 content with a value of 6.67 (μg/g), the average vitamin D_3 concentration of lichen species in deciduous areas was found to be 3.90 (μg/g) (Fig. 4).

Aspect (sun/shade condition): Since lichen species are distributed to all aspects in the study area, two groups were formed in the examination of aspects as shaded aspects (North, Northeast, Northwest, East) and sunny aspects (South, Southeast, Southwest, West). Vitamin D_3 data of lichens did not show normal distribution among aspects and therefore, they were compared with Mann Whitney U test, which is one of the non-parametric methods. In the obtained result ($p = 0.214 > 0.05$), no statistically significant difference was found in terms of the directions where lichen species were collected (Table 3). The average vitamin D_3 concentration was highest in lichen species in shaded areas with 6.46 μg/g, while it was 4.9 μg/g in sunny areas (Fig. 4).

Altitude levels: The altitude range of 81–1961 m where lichen species are distributed in the study area was examined as two different altitude levels as lower than 1000 m and higher than 1000 m (Fig. 4). Since the data did not show a normal distribution, the altitude levels in terms of vitamin D_3 amounts found in lichen species were compared with the Mann Whitney U test. No statistically significant difference ($p = 0.576 > 0.05$) was found in terms of vitamin D_3 content between the altitudes where lichen species were collected. The highest average vitamin D_3 content was determined as 5.95 (μg/g) in lichen species below 1000 m, while it was determined as 5.07 (μg/g) in those above 1000 m (Table 3).

Air humidity: The humidity variable, which was between 30 % and 72 % at the time of collection of the species in the study area, was examined in two different humidity classes as lower than 50 % and higher than 50 % (Fig. 4). Humidity classes were compared with the Mann Whitney U test in terms of vitamin D_3 amounts found in lichen species due to the non-normal distribution of the data. It was shown that there was no statistically significant difference ($p = 0.669 > 0.05$) in terms of vitamin D_3 content between the humidity classes (Table 3).

In this comparative study conducted in 4 different montane forests, the possible effects of ecological variables on vitamin D_3 biosynthesis of naturally growing lichens were evaluated. Most of the *Bryoria fuscencens, Evernia prunastri, P. glauca* and *P. furfuracea* samples in Uludağ and Kaz Mountains study areas showed higher vitamin D_3 concentrations. According to the data of the Forest Management and Planning Department of the Ministry of Forestry, the Uludağ and Kaz Mountains layers are a region with very high productivity, and this situation is reflected in the distribution of lichens and has also yielded important results in terms of vitamin D_3 production. Evidence based on statistical analysis revealed that vitamin D_3 profiles of lichens varied depending on the species and these amounts were significantly and positively related to the study area layer, stand type and tree species. It was determined that the altitude, aspect, air humidity and temperature of the sample area, among other habitat variables examined, did not specifically affect vitamin D levels.

4. Discussion

The relationship between vitamin D deficiency and human health is well documented worldwide, but the real need is directed towards natural raw material sources and vitamin D treatments and supplements

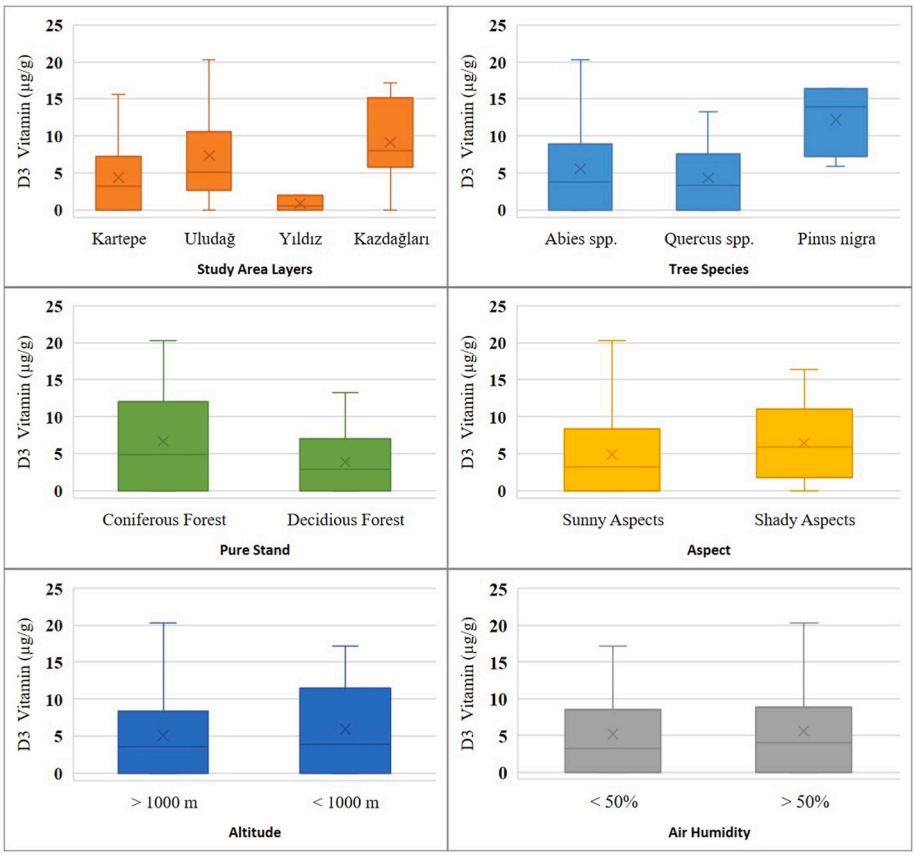

Fig. 4. Boxplots visualizing analysis of variance data for vitamin D_3 concentrations (µg/g) comparing individual ecological variables (The "x" symbol in histograms represents the mean, and the line represents the median).

Table 4
Correlation matrix between vitamin D_3 and some ecological parameters.

Parameters	Vitamin D_3	Altitude	Temperature	Humidity
Vitamin D_3	1			
Altitude	.127^{NS}	1		
Temperature	−.075^{NS}	−0.418**	1	
Humidity	.129^{NS}	0.337**	−0.642**	1

** The correlation is significant at the p = 0.01 level; NS = correlation is not significant at p = 0.05.

made from them. The results of our study revealed that most of the tested lichens have high levels of vitamin D_3 biosynthesis. Differences in vitamin D_3 content of lichen species are due to habitat conditions, which have been determined to have an effective role on the genes responsible for the synthesis of metabolites that are decisive in the phylogenetic distinction of species (Miao et al., 2001; Frisvad et al., 2008; Stocker-Wörgötter, 2008; Deduke et al., 2012). The positive correlation reported between vitamin D_3 content and UV-B radiation is well known (Wang et al., 2001; Al-Amoody et al., 2020).

The results we achieved in the present study showed that, besides the lichen species, 3 important ecological parameters related to vitamin D metabolism are stronger: "layer, pure stand and tree species". Especially in pure conifer stands and on *Pinus nigra*, the vitamin D_3 content of lichens was higher. Although it is known that the amount of sunlight exposure may have a possible effect on vitamin D production by stimulating the metabolic activities of lichens (Wang et al., 2001), in our study the average vitamin D_3 value of lichens in shaded areas (north) was higher than lichens in sunny areas (south). However, our aspect classification oversimplified actual light exposure conditions, and tree species grouping may have masked important ecological differences. All observed relationships should be interpreted as correlative rather than causal, given the observational study design and interconnected nature of ecological variables. Furthermore, the role of lichen chemotypes and microclimate factors, along with the interactions between the ecological variables assessed independently here, needs further investigation.

The limited number of studies on vitamin D_3 in lichens have generally been conducted on specific species of *Cladonia*, and since no information is found in the literature regarding the lichen species included in our study, it is difficult to make a comparative comment. Björn and Wang (2000) stated that reindeer lichens contain vitamins D_2 and D_3. HPLC analysis of *Cladina arbuscula* chloroform extract samples reported that vitamin D_3 content ranged from 0.67 to 2.04 µg/g dry matter (Wang et al., 2001). Benedik (2022) recorded that 87 µg/100 g vitamin D was determined in the reindeer lichen *C. rangiferina*. *C. furcata* (0.76 µg/g) and *C. rangiformis* (1.86 µg/g), which had the lowest average vitamin D_3 content among the species included in our study, showed similar values when compared to the *Cladonia* species in these publications. In addition, it was determined that the vitamin D_3 profiles of the studied lichens could vary depending on three different extraction methods (Supercritical CO_2, Maceration and Soxhlet processes) supported by HPLC analysis, and the most efficient results were obtained with the maceration method with olive oil.

The amounts of vitamin D in various biological sources (per 100 g) have been reported as 5–25 µg in most fish, 21.1–58.7 µg in mushrooms and 250 µg in fish liver oils (Black et al., 2017), 1.3–2.9 µg in some dietary foods such as cheese, beef liver and eggs, and 4 µg in dark chocolate (Göring, 2018). Among the analysed lichens, the highest value in terms of average vitamin D_3 was found in *B. fuscescens* (11.31 µg/g), while the lowest value was found in *C. furcata* (0.76 µg/g) (Table 3). While the highest vitamin D_3 value based on the study area layer was obtained in the *B. fuscescens* sample of the Uludağ coniferous forest fir

tree (20.33 μg/g), the lowest value was obtained in the *R. farinacea* sample of the Kartepe Mountain fir (0.6 μg/g) (Table 2). It is noteworthy that the results obtained from the lichen species examined, limited by the time and conditions under which our study was conducted, have a considerably high vitamin D_3 content compared to most reported lichen species and other biological organisms. However, it is possible that changes in forest composition, geographical and climatic conditions, and some ecological variables not included in the study may cause a possible difference in vitamin D levels in lichens. One limitation of this study is the unequal number of samples collected for different lichen species due to their natural distribution and habitat specificity. Although standardized collection procedures were followed, complete uniformity in environmental conditions could not be achieved. Therefore, the results should be interpreted with this sampling constraint in mind.

Several limitations should be acknowledged. Temperature and humidity measurements were recorded only at the time of collection, representing instantaneous conditions rather than the long-term climatic regime that influences lichen metabolism. Additionally, sampling was conducted across different seasons and varying canopy conditions, which may have influenced vitamin D_3 content due to differences in light exposure and seasonal metabolic patterns. Future studies should incorporate historical climate data from meteorological stations to better characterize environmental conditions and maintain consistent seasonal timing across all sampling sites. Additionally, unequal sample sizes across lichen species due to natural distribution patterns may have affected statistical comparisons. Seasonal variations in vitamin D_3 content were not assessed, representing an important area for future research. Despite these limitations, this study provides the first comprehensive assessment of vitamin D_3 content in epiphytic lichens and establishes a foundation for future investigations.

5. Conclusions

This study, conducted through stratified sampling in four ecologically different mountainous forest areas in Türkiye, revealed vitamin D_3 levels in the natural lichen species examined, allowing the first association of vitamin D metabolism of lichens with ecological factors. The most efficient extraction of lichen-derived vitamin D_3 was achieved through olive oil maceration. Vitamin D_3 production varied depending on the "lichen species" and some ecological variables in the habitat, especially the forest composition. The "layer", "stand type" and "tree species" parameters came to the forefront in interaction with the vitamin D_3 biosynthesis mechanism of lichens. The differentiation in layers indicated mountains, which have a positive effect on lichen distribution, showing particularly high productivity, and the vitamin D level increased in lichens on conifer stands and black pine. However, it should be kept in mind that the amount of vitamin D in lichens may vary depending on various environmental factors to which the ecosystem is exposed, which may affect lichen chemistry, in addition to the variables examined.

In recent years, as in many areas concerning human health, solutions have been sought from nature to the global vitamin D pandemic problem. Lichens appear to be important candidates among the promising biological resources in this regard with their ability to produce natural compounds. It is anticipated that the production of natural organic vegan vitamin D_3 food and drug supplements from these lichen species will make a great contribution to public health. In order to carry out this production, it is important to conduct studies to determine the yield and harvesting techniques of lichens, as the continuity of raw materials and the sustainability of lichen species must be ensured. Subsequently, conducting R&D studies to determine whether lichens can be produced by tissue culture and propagated with bioreactors will be important to investigate the therapeutic potential of lichens from which vitamin D_3 is obtained in the future.

CRediT authorship contribution statement

Gülşah Çobanoğlu: Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Conceptualization. **Hilmi Özdemir:** Writing – original draft, Visualization, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization. **Mehmet Özdemir:** Writing – review & editing, Visualization, Methodology, Formal analysis, Data curation. **Gafura Aylak Özdemir:** Methodology, Formal analysis, Data curation. **Emrah Özdemir:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Fatma Ebru Koç:** Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Ahmet Özcan:** Visualization, Methodology, Investigation.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2025.101634.

Data availability

The authors affirm that the data supporting the conclusions of this study are included within the article.

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