Determining the Taxol Contents of Yew Tree Populations in Western Black Sea and Marmara Regions and Analyzing Some Forest Stand Characteristics

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Yew tree (*Taxus baccata* L.) is mainly populated in Türkiye, Europe, and Caucasia regions. It has natural anticancer compounds and is a source of raw materials used in modern medicine. The present study aimed to examine the taxol contents of yew trees naturally grown in Marmara and Western Black Sea regions by subjecting needle samples taken from 17 yew populations to extraction and liquid chromatography tandem mass / mass spectrometer system (LC-MS/MS) analysis. It was also examined whether a relationship exists between some stand characteristics and taxoid contents of the needles. From these analyses, the highest taxol contents were found in Bartın-Gölderesi, Yığılca-Kurtkayası, İnebolu-Karagöl, and Yenice-Kızılkaya populations. The statistical analyses showed that there were significant relationships between compound contents and slope, humus content, total nitrogen content (%), and potassium (K). It is recommended to use these derivatives obtained from natural forests in sapling nursing and tissue culture studies, to produce pharmaceutical materials from leaves and protect and improve the current gene sources.

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INTRODUCTION

Taxus is a needle-leaved species from the Taxaceae family of Gymnospermae, and it is called "porsuk" in Turkish, "eibe" in German, and "yew" in English. The word *Taxus* originates from the Greek word "toxon", which was the origin of the words "toxin" and "bow". Druids blessed this tree and considered it as "the tree of immortality". Christians planted this tree in the yards of churches (Yuksek 2009). As stated by Benham *et al.* (2016), among the tree species in Europe, yew is a species being subjected to the sharpest decrease. Saatçioğlu (1976) reported that the current decrease in the population of yew tree, which is considered to have had a wide distribution in Europe and Britain in the past, was related to the damages made in the previous periods (Oakley *et al*. 1977; Yuksek 2009).

Yew trees are evergreens that can reach 6 to 20 m in length; they may be in the form of a tree or multi-stem bush (Benham *et al*. 2016). Among the needle-leaved trees, this tree species can sprout a new shoot from some part of the tree (Saatçioğlu 1976). It has a long lifetime and lives 800 to 1000 years (Efe *et al.* 2013); some studies reported up to 5000 years of a lifetime (Milner 1992).

Except for the outer part (orange-red-green) of yew's red cone, there is a poisonous alkaloid called "taxine" everywhere on the tree. The core (seed) of the cone is extremely poisonous (Kayacık 1980; Efe *et al*. 2013). The amount of taxine alkaloids depends on the variety of yew trees, and the highest amounts are observed in *Taxus baccata* L. and *Taxus cuspidata* Siebold & Zucc. (Wilson 2001). The taxol and other taxane derivatives that can be isolated from yew trees are used as chemotherapy medications (Le Roux 2016). Taxol, which is one of the natural anti-cancer compounds, is used solely or in combination with other chemotherapeutic compounds in treatments of ovarium, breast, prostate, and non-small cell lung cancer (Rowinsky 1997; Eisenhauer and Vermorken 1998; Erdemoğlu and Şener 1999; FDA 2010; Kutlutürk 2019).

Thus, various researchers have conducted studies on obtaining taxol in semi-synthetic, synthetic, and biological ways (genetic, tissue and cell culture, or isolation from microorganisms). The search for new methods and sources has continued since the demand for taxol medication increased (Cragg *et al*. 1993; Croom 1995; Suffness and Wall 1995; Wall and Wani 1998; Erdemoğlu 1999; Erdemoğlu and Şener 1999; Gallego *et al*. 2017; Kutlutürk 2019; Linhares *et al*. 2022).

The idea of producing taxol and derivatives from needles originated from the necessity of not harming the barks, wood, or chunky parts because, considering that the production of 1.0 kg taxol requires -15 tonnes yew bark (Alternatif Tıp 2020), producing taxol from yew needles for the sustainability of forests seems more rational in comparison to barks and branches. Given the fact that taxol concentration in *Taxus brevifolia* Nutt. needles was reported to be 0.00003% to 0.0030% (Erdemoğlu 1999), the results achieved here show that *Taxus baccata* L. needles contain equal or more taxol in comparison to *Taxus brevifolia* Nutt. (approximately 0.00150%). This finding suggests that this material used in cancer research and treatments could be easily obtained without damaging the forests and that its pharmaceutical potential is high. For this reason, it is thought that, through yew sampling nursing or tissue culture procedures, the production of taxol from its needles could be increased. Furthermore, there exist methods allowing synthetic production in laboratories after obtaining it once.

As a result of the analyses conducted on different parts of *T. baccata* L., which is the only *Taxus* species growing in Anatolia, the highest amount of taxol was found in the wood part and chunky barks of thick branches (0.0084% to 0.0092%) (Şener and Küçükboyacı 1994). However, there are only few studies on this subject, and it was thought that there might be chemical difference potential because the yew populations in Türkiye are at different locations and because of the difference in altitudinal, climatic, and edaphic conditions.

Thus, within the scope of this study, the taxol contents of populations in Marmara and Western Black Sea regions of Türkiye were determined, and it was examined if there was a relationship between some stand characteristics and taxoid contents. Using the information and scientific samples obtained from the cores in these stands, the present study is preliminary research aiming to develop new production methods and production of the derivatives (raw material) without damaging the forests (through sapling nursing or tissue culture, synthetically).

EXPERIMENTAL

Materials

The study materials consisted of the yew needles taken from 17 natural yew populations located in the Western Black Sea and Marmara regions.

Study Universe and Sampling Frame Definition

Avcı (2014) divided Türkiye into 7 geographical regions and 21 ecological subregions. The study area consisted of, among these ecological regions, 1a-Marmara Istranca Zone, 1b- Marmara Çatalca- Kocaeli Zone, 1ç- Southern Marmara zone, and 2a-Western Black Sea Zone (Fig. 1). For 1c subzone, only 2 yew individuals were found in Tekirdağ Ganosdağı area and no sample was taken from this area because no other individual yew tree could be found.

In these geographical regions, the populations from which the samples were taken exist as individuals, clusters, or stands generally together with other tree species in forest locations (Fig. 1).

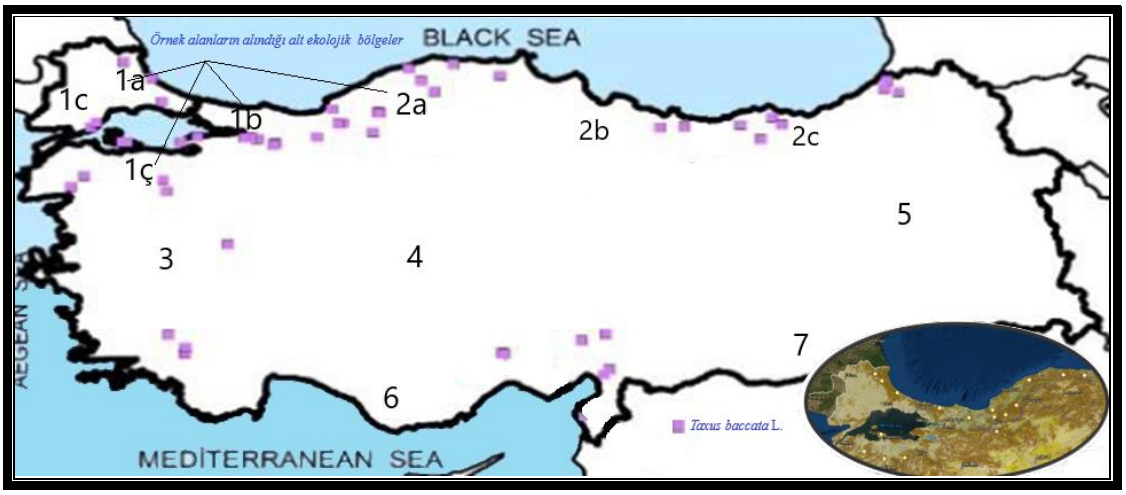


Fig. 1. *Taxus baccata* L. populations, from which the samples were taken

Sampling Area

After determining the sample areas representing the populations, 500 m^2 (50 m × 10 m) areas were established, and the samples were taken from the yew individuals in these areas. Measurements and observations regarding the stand were performed in these areas.

Collection of *T. baccata* L. Needle Samples

Needle samples were collected from 17 populations in Marmara Region and Western Black Sea subregion. The sampling process was completed during the period, in which taxane derivatives in needle composition did not start changing and the level of taxane derivatives in the composition was static (Alternatif Tıp 2020), which was before the vegetation year of April 2021. Needle samples were taken from similar branches of yew trees and from all four directions. The trees, where the needle samples were taken, were examined in terms of forest pests and the samples were taken from healthy individuals. For each sampling area, approximately 1.0 kg needle sample was taken and labeled. Then, the samples were immediately taken to the laboratory and the drying process was initiated. The drying process was completed hygienically on the drying grids providing equal distribution of air from every direction.

Determination of Taxane Derivatives of Samples

Given the results reported in different studies, approximately 350 taxane-derivative diterpenes were isolated from *Taxus* species. Some of the taxane-derived diterpenes draw attention to the amounts of content. Taxol (paclitaxel), which is the raw material of "Taxol®" that is the most preferred chemotherapy medication, is among the prominent diterpenoids (Gallego *et al*. 2017; Kutlutürk 2019).

Chromatography is the generic name for the methods requiring distinguishing the components of a mixture and in which qualitative and quantitative analyses are performed. The methods, such as high-performance liquid chromatography with UV absorption detection (HPLC) (Çetinkaya 2015) and liquid chromatography–coupled mass spectrometry (LC-MS/MS), are used in identifying the compounds and detecting the amounts. In the present study, LC-MS/MS method was used in determining which population found in Marmara and Western Black Sea regions incorporating yew individuals yielded the highest amount of taxol content. Extraction and chromatographic procedures were conducted in the laboratory of Hitit University's scientific technical practice and research center (HÜBTUAM).

Extraction of *T. baccata* L. Samples

Each of 17 yew samples taken to the laboratory in dried form was ground using a grinder and about 2.0 g of powder was put into experimental tubes. Then, they were mixed with 10 mL solvent (methanol), and extraction was performed using KUDOS (Shanghai KUDOS Ultrasonic Instrument Co., Ltd., Shanghai, China) ultrasonic bath for 2 h at 40 °C. The extract was filtered using a 0.45-µm syringe filter and put into LC-MS/MS vials.

Liquid Chromatography-Coupled Mass Spectrometry

The study of Li *et al.* (2009) using the LC-MS/MS method in determining taxol and 6 taxoid contents of Japanese yew (*Taxus cuspidata* Siebold & Zucc.), *Taxus media* (*Taxus baccata* L. and *Taxus cuspidata* Siebold & Zucc. hybrid), and Chinese yew (*Taxus chinensis* (Rehder & E.H.Wilson) Rehder *var. mairiei* species was taken as a reference for this study. LC-MS/MS is a method offering advanced sensitivity, selectivity, and authenticity and has advantages proven in various implementations including bioanalytic, environmental, and pharmaceutical studies. Moreover, this method is used for the identification of taxol and also for determining its amount (Li *et al*. 2009).

Determination of the amount of taxol content in the yew plant was performed using Thermo Scientific-TSQ Quantum Access MAX brand's (Thermo Fisher Scientific, San Jose, CA, USA) LC-MS/MS instrument (Fig. 2.) in selected reaction monitoring (SRM) mode. First, without passing through a column, the pure standards of taxol molecules were directly injected into the electrospray ionization (ESI) source of the LC-MS/MS device and positive ion MS and MS/MS scanning modes were used in determining the main ion and collision ions. Detected ion and collision ions were used for collision energy optimization in SRM mode. After preparing the gradient flow program (Li *et al.* 2009) and determining the retention time for pure standards in SRM ion mode, the calibration diagram required for quantitative analysis was obtained by analyzing different concentrations of the standards within the range of 6 to 20 ppm. The calibration diagram was drawn as concentration *vs*. peak area. Then, the samples were fed to the device for analysis with the same gradient program as the standards and were analyzed. Using the peak areas obtained from the analysis and the equation in the calibration diagram, concentrations of compounds were determined.

Küçükboyacı (1993), citing Auriola *et al*. (1992), reported that taxol content in *Taxus brevifolia* Nutt. needle ranged between 0.00003% and 0.0030%. Taxol content of *Taxus baccata* L. needle was 0.0030%. Accordingly, the results obtained in mg/kg from 17 populations were expressed as a percentage (%). Then, the taxol contents were classified as low for 0.00003% to 0.00050%, medium for 0.00051% to 0.0015%, medium-high for 0.0016% to 0.0030%, and high for > 0.0030%.

Chemical Characteristics of Soil

In every sampling area representing the population, a soil profile with 75 cm width and 100 cm depth was prepared, and 2 kg disturbed soil samples were taken from each of zero to 30 cm, 31 to 60 cm, and 61 to 90 cm depths (3 × 17 = 51 samples). Using the samples, soil type, and texture (Irmak 1954; Gülçur 1974; Karaöz 1989), organic carbon-organic matter (Irmak 1954; Gülçur 1974; Chapman and Pratt 1982; Çepel 1988), electrical conductivity (Jackson 1962; Eruz 1979), soil reaction (pH) (Irmak 1954; Jackson 1962; Gülçur 1974), total nitrogen (N) (Irmak 1954; Jackson 1962; Gülçur 1974), available phosphorus (P) (Nielsen 1972; Ülgen and Ateşalp 1972), and potassium contents (FAO 1990) were determined.

As a result of the measurements, observations, and analyses completed in 17 sampling areas in Marmara and Western Black Sea regions, it was determined that yew trees grew in north-facing shoulders on limy calcareous and mica shist bedrocks and in deep soils with good drainage. According to the analyses, the soil characters in all the sampling areas were "loamy", "sandy-loamy", "slimy loamy", and "sandy-loamy and slimy", they were saltless and acidic, and climate types were moist. These were noteworthy findings and establishing similar conditions in pharmaceutical cultivations would be important.

Data Analyses

For the data, fitness to normal distribution was tested using Shapiro-Wilk's test, and comparison of normally distributed characteristics in two independent groups was performed using the Student t-test, whereas the Mann-Whitney U test was used in the comparison between non-normally distributed characteristics in 2 independent groups. The relationships between variables in categorical measurement level were analyzed using the exact Chi-Square test. The relationship between quantitative variables was analyzed using Pearson's correlation coefficient. As descriptive statistics, categorical variables were expressed as numbers of percentage values. Statistical analyses were performed using SPSS (SPSS Inc., Chicago, IL, USA) for Windows version 24.0 and statistical significance was set at $p < 0.05$. Sampling areas were numbered between 1 and 17.

RESULTS

Results on Taxane Derivatives in Samples

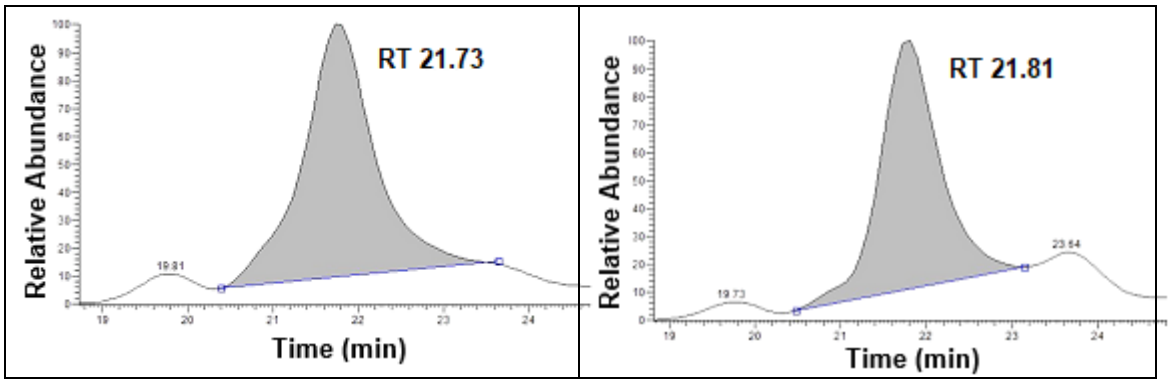
Taxol analysis of yew plant was performed using LC-MS/MS instrument quantitatively. The taxol concentrations found are presented in Table 1, while chromatograms are presented in Fig. 2.

The total nitrogen content of soils having a normal level of taxol compound (4.94 ± 0.24%) was significantly higher in comparison to the soils with a medium level of taxol compound (4.42 ± 1.00%) ($p = 0.036$). The K concentration in soils with a "normal" level of taxol content (3.11 ± 0.68 ppm) was significantly higher than that of soils with a "medium" level of taxol compound (2.61 ± 0.66 ppm) ($p = 0.016$) statistically. The slope of soils having a "medium" level of taxol concentration (41.25 ± 14.72%) was found to be significantly higher in comparison to the soils having a "normal" level of taxol concentration (26.07 ± 17.97%) ($p = 0.025$).

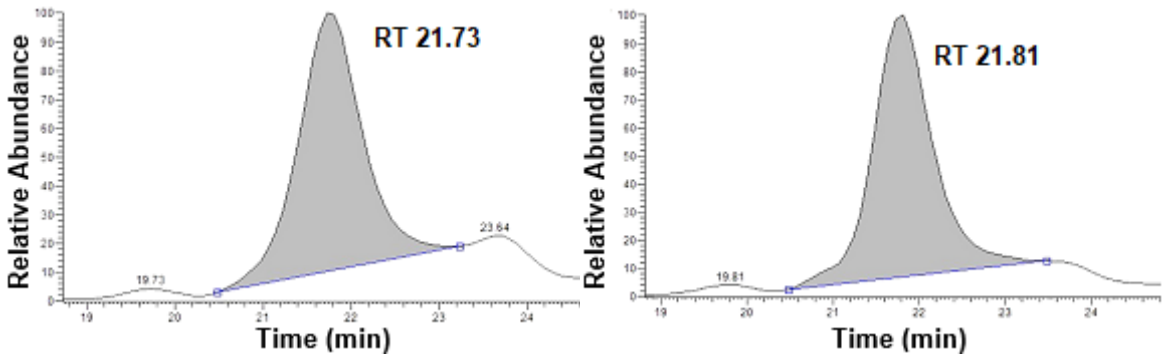
Examining the relationships of soil characteristics with different sampling areas (p exact Chi-Square), it was determined that they were similar in terms of pH, organic matter, P_2O_5, and K ($p > 0.05$). Statistically significant differences were observed in nitrogen concentrations of 17 samples ($p = 0.002$).

Table 1. Taxol Concentrations in *T. baccata* L. Needles

Region	Population	Sample Area	Taxol (mg/kg)	Taxol (%)	Clasification
Çatalca-Kocaeli	Şile-Ağva	1	10.39	0.00103	Medium
Çatalca-Kocaeli	Çataca-Yalıköy	2	18.14	0.00181	Medium-High
Çatalca-Kocaeli	Vize-Sergen	3	10.79	0.00108	Medium
Istranca	Demirköy-Kurudere	4	12.44	0.00124	Medium
South Marmara	Çanakkale-Kalkım	5	13.79	0.00138	Medium
South Marmara	Balıkkesir-İvrindi	6	9.19	0.00091	Medium
South Marmara	Bandırma-Erdek	7	14.25	0.00142	Medium
South Marmara	Mustafakemalpaşa	8	17.25	0.00172	Medium-High
Çatalca-Kocaeli	Hendek-Çamdağı	9	10.76	0.00107	Medium
West karadeniz	Bolu-Aladağ-Kökez	10	13.76	0.00137	Medium
West karadeniz	Yığılca-Kurtkayası	11	20.99	0.00210	Medium-High
West karadeniz	Alaplı -Gümeli	12	11.67	0.00117	Medium
West karadeniz	Dirgine Aksu	13	9.54	0.00095	Medium
West karadeniz	Yenice-Kızılkaya	14	19.29	0.00193	Medium-High
West karadeniz	Bartın-Göldersi	15	25.01	0.00250	Medium-High
West karadeniz	İnebolu-Karagöl	16	19.63	0.00196	Medium-High
West karadeniz	Sinop -Ayancık	17	15.51	0.00155	Medium-High



Şile Ağva Yalıköy



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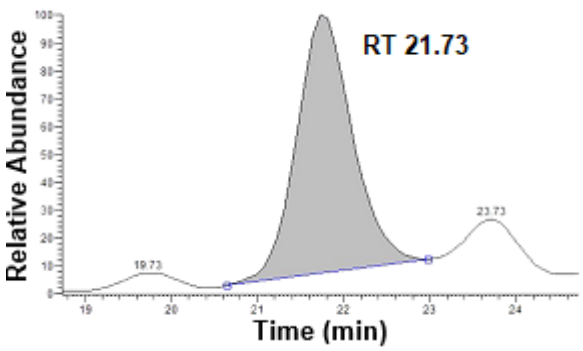

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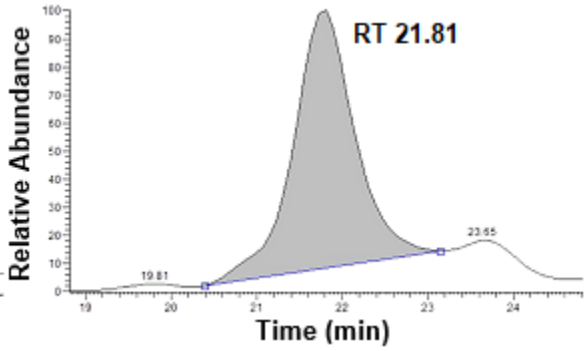

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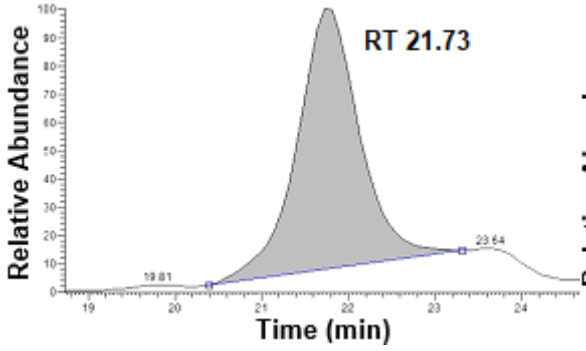

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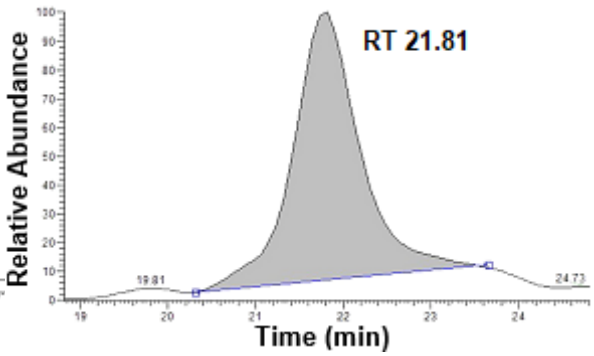

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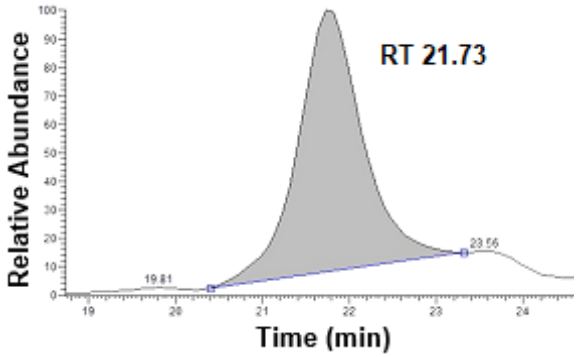

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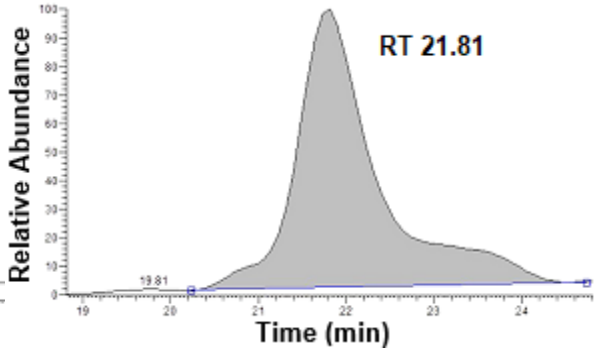
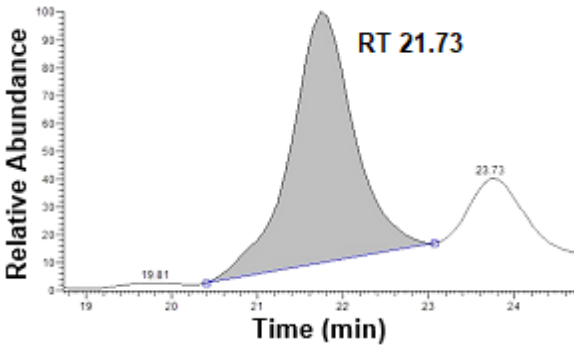
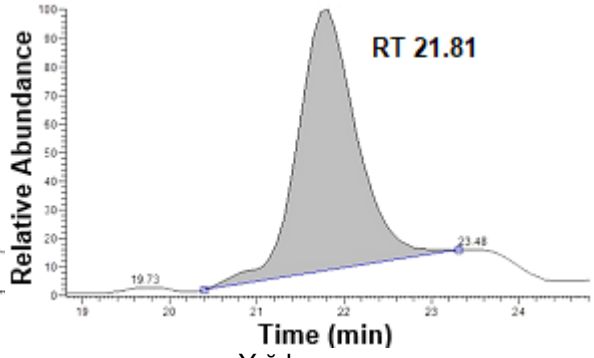


İnebolu



Vize Sergen



Yığılca

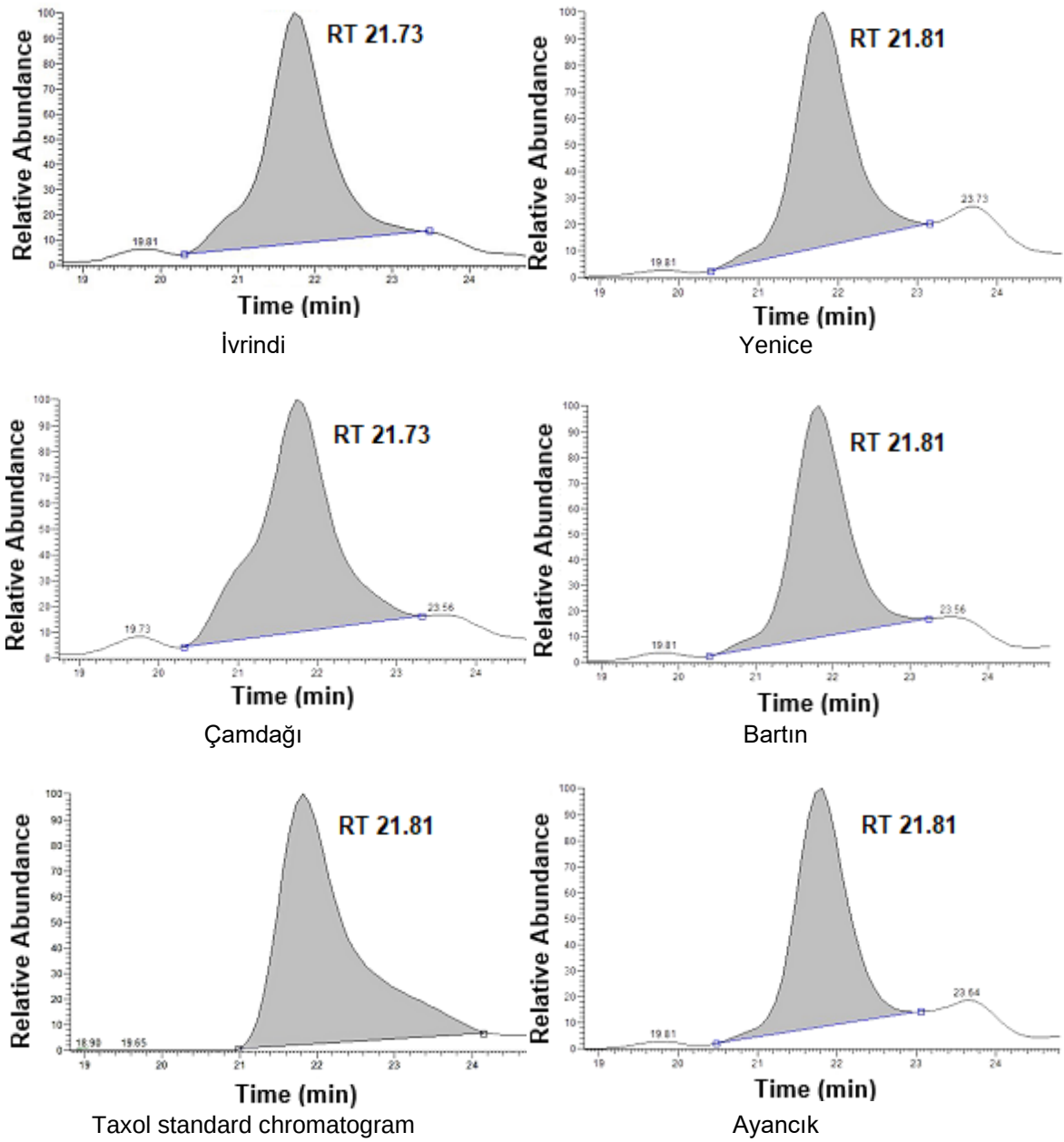


Fig. 2. *Taxus baccata* L. needle extract's LC-MS/MS chromatograms

The concentrations of the taxol compound in comparison to qualitative variables are presented in Table 2.

All the samples taken from sampling areas 4 (Demirköy-Kurudere), 8 to 11 (M. K. Paşa, Hendek-Çamdağı, Bolu-Kökez, Yığılca-Kurtkayası), and 14 to 17 (Yenice-Kızılkaya, Bartın-Gölderesi, İnebolu-Karagöl, Ayancık) had high nitrogen contents. Statistically significant differences were observed in taxol concentrations of 17 samples ($p = 0.001$). All the samples taken from sampling areas 1 (Şile), 8 (M.K.Paşa), 14 (Yenice-Kızılkaya), 15 (Bartın-Gölderesi), 16 (İnebolu-Karagöl), and 17 (Ayancık) had "medium-high" level of taxol concentrations. Taxol concentrations of other sampling areas were found to be medium.

Table 2. Comparison between Taxol Compound Concentrations in Quantitative Variables

Parameters	Taxol Compound Concentrations			
	(n = 7) Average ± SD	Medium (n = 10) Aveage ± SD	z	p
pH	3.11 ± 0.76	3.21 ± 0.65	-0.43	0.665
Organic Matter (%)	3.00 ± 1.28	2.30 ± 1.07	-1.87	0.061
Total Nitrogen (%)	4.94 ± 0.24	4.42 ± 1.00	-2.10	**0.036**
P_2O_5 (ppm)	3.28 ± 0.96	3.18 ± 0.88	-0.47	0.639
K (ppm) in $NH4COOCH_3$ Extract	3.11 ± 0.68	2.61 ± 0.66	-2.41	**0.016**
Elevation (m)	619.00 ± 364.70	707.50 ± 312.16	-0.58	0.601
Slope (%)	26.07 ± 17.97	41.25 ± 14.72	-2.25	**0.025**

z: score of standardization and normalization **p:** significance level; **SD:** Standard deviation

DISCUSSION

The novelty of this study is the examination of taxol concentrations of different yew populations together with several stand characteristics in natural forests in Türkiye for the first time. Needle samples taken from 17 sampling areas, where yew trees have natural distribution as individual, cluster, or stand in Marmara and Western Black Sea regions, were subjected to extraction and LC-MS/MS, and their taxol contents were analyzed. Moreover, some stand characteristics (physiographic characteristics, chemical and physical soil characteristics, and climate type) of each sampling area were determined, and their relationship with taxoid contents was assessed. Considering the taxol contents, extraction and LC-MS/MS analyses showed that the highest concentrations were found in Bartın-Gölderesi, Yığılca-Kurtkayası, İnebolu-Karagöl, and Yenice-Kızılkaya populations. Given the results achieved in this study, the taxol concentration in *Taxus baccata* L. needles obtained from 17 populations ranged between 0.00091% and 0.00250% and 7 populations were classified as "medium-high" and 10 populations were classified as "medium".

As a result of the analyses conducted on different parts of *T baccata* L., which is the only *Taxus* species grown in Türkiye, it was determined that the highest taxol concentrations (0.0084% to 0.0092%) were found in thick branches' chunky barks and wood parts (Şener and Küçükboyacı 1994). Küçükboyacı (1993), citing Auriola *et al.* (1992), carried out analyses on determining the concentrations of taxol and other taxane derivatives in *Taxus* extracts and reported that the taxol content of *Taxus brevifolia* Nutt. needle ranged between 0.00003% and 0.0030%. In that study carried out with the material obtained from Rize-Çamlıhemşin Palovit Brook (Eastern Black Sea region) and Ankara Park, the author reported the taxol concentration in *Taxus baccata* L. needle to be 0.0030%. However, it was also stated that this pathway could not be used because the woods and chunky barks are not as edible as needles (Erdemoğlu 1999). In parallel with this statement, rather than methods damaging the forests, the present project utilized needle sampling for the production of taxol and derivatives from needles.

As a result of the research, it was determined that many compounds in *Taxus* species such as 10-DAB (diacetyl baccatin) III, 10-DAT, baccatin III, and cephalomannine can be used in taxol production *via* semi-synthesis (Fang *et al.* 1993; Lauren *et al.* 1995; Mroczek *et al.* 2000; Zu *et al.* 2006). For this reason, it would be useful to carry out a countrywide study in Türkiye by adding other compounds. Given the results achieved here, it was determined that certain individuals in certain populations of *Taxus baccata* L. in Marmara and Western Black Sea regions were suitable for cultivation aiming to produce these

materials. Moreover, because climatic, physiographic, and edaphic conditions of these regions were examined, opinions were acquired about individuals grown under which conditions would more efficiently produce these compounds. Aksoy (1985) drew attention to the importance of utilization of the economic potential of yew, which grows in Türkiye as a natural source. Reducing restrictions on drugs is important for Türkiye. *Taxus* resources can be used to produce pharmaceutical raw materials with high economic value in Türkiye. This is because *Taxus* contains substances with high economic value that are preferred by the pharmaceutical industry. Ensuring and finding the continuity of the provision of these resources is also important in terms of public health. In combination with the results achieved in the present study, the materials obtained from yew populations with sufficient taxol content in Türkiye will contribute to the tissue culture and synthetic compound production studies. In recent studies (Kutlutürk 2019), taxol compound was examined in hazelnut (*Corylus avellana* L.) tissues and fruits and it was stated that hazelnut might be an alternative source of raw material.

Stating that "maintaining, protecting, and even artificially cultivating yew in its current locations should be considered an important silvicultural responsibility for protecting the nature," Saatçioğlu (1976) emphasized that the characteristics of this species should be studied. Through a comprehensive action plan for yew, as stated by Aksoy (1985), this species should be protected as a gene source. It could be improved by the General Directorate of Forestry (OGM) and General Directorate of Nature Conservation, and National Parks (DKMP). It is thought that, following the principle of protection-usage balance, researchers, and practitioners should conduct more studies on "knowing the silviculture" (pro-yew interventions, plantation under shelter or in small gaps) of this species, which is in danger of extinction in natural forests, and guaranteeing its future.

CONCLUSIONS

1. As a result of the statistical analysis, statistically significant relationships were found between slope and humus content and compound concentration. Correlation analyses showed that there were positive and significant relationships between total nitrogen content (%) and taxol concentration and between potassium (K) and taxol concentration. Given these findings, it can be stated that, for the sapling cultivation for pharmaceutical purposes, combination of less sloping lands with hummus or fertilizer (N-P-K) addition at an optimal level could yield qualitatively and quantitatively qualified compound sources. New multidisciplinary studies on this subject are needed.
2. In field observations, needles and trunks with different colors and appearances were observed in ecological regions. Thus, in addition to the academic studies on biology and varieties of the yew tree, it is thought that examining the taxoid concentrations of different forms and sub-species would be useful.
3. The most poisonous and lethal part of the *Taxus baccata* L. plant is the seeds (except for soft and red arillus). Birds eat the cone without being poisoned, but the seeds do not disintegrate in birds' stomachs but rather are defecated whole. Hence, the seeds are transferred to different locations and seeds reaching the mineral soils create the next generations of plants. Thus, it is thought today that sapling production from seeds might be possible for yew using methods similar to those used for juniper.
4. In the vegetation analyses in the present study, it was found that hazelnut (*Corylus avellana* L.) was cultivated/grew in natural distribution areas of yew trees. Hendek-Çamdağı (hazelnut farming), Alaplı-Gümeli-Fındıklı neighborhood, Dirgine Aksu-

Pisfındık zone, and Bartın-Gölderesi (hazelnut cleaning was performed here) are such types of areas. This intersection should be scientifically analyzed and new studies should be conducted.

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