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Properties, Ecological Characteristics and Antimicrobial Activities of Ebe Black Pine (*P. nigra ssp. pallasiana var. seneriana*)

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Turkey is one of the richest areas in the middle latitudes in terms of plant diversity. One of Turkey's (Anatolia) endemic plants is Ebe Black Pine (*P. nigra ssp. pallasiana var. seneriana*). This study was carried out to investigate the properties, distribution area, climatic conditions and the antimicrobial activities of this plant. According to de Martonne, this plant lives in passing climates between semi arid and humid climatic conditions and according to Erinç, it grows in semi humid climatic conditions. Additionally, when the results of this study were compared with ampicillin (10 mcg) and nystatin (30 mcg) standards, it was found that extracts of Ebe Black Pine were found to particularly possess stronger antimicrobial activities.

Key Words: *P. nigra ssp. pallasiana var. seneriana*, Endemic plant, Climatic conditions, Antimicrobial activity, Ebe black pine.

INTRODUCTION

The flora of Turkey is rich and diverse with well over 11,000 flowering taxa recorded in a 9-volume set of Prof. Davis' monumental work and its two supplements[1]. In Turkey, the rate of endemism is relatively high when compared with other European countries[2]. One of Turkey's (Anatolia) endemic plants is the Ebe Black Pine (*P. nigra ssp. pallasiana var. seneriana*) which is a variety of the Black Pine family[3].

There are four varieties of *Pinus nigra ssp. pallasiana* in Turkey, which are the Anatolian Black Pine (*Pinus nigra ssp. pallasiana var. pallasiana*), the Ebe Black Pine (*P. nigra ssp. pallasiana var. seneriana*), the Big Fruited

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Black Pine (*P. nigra ssp. pallasiana var. yaltirikiana*) and the Ehrami Black Pine or Pyramidal Black Pine (*P. nigra ssp. pallasiana var. pyramidata*)[3].

Ebe Black Pine (*P. nigra ssp. pallasiana var. seneriana*), is a slow growing tree, the branches of which grow from the bottom. It has a multi-body structure and spherical form. It can grow as long as 17 m. It is a variety of the Anatolian Black Pine spreading in Turkey that has the shortest pine needles and smallest cones[4].

They are found within the borders of the provinces of Bolu, Eskisehir, Afyon, Manisa and Kütahya in Turkey dispersed as individual trees or as infrequent small groups spread over 800-1250 meters. The tallest ones are around Bolu, the South and Manisa-Alasehir; the most decorative ones in terms of appearance are around Kütahya[4,5].

Many of the plants used today were known to the people of ancient cultures throughout the world and they were valued their preservative and medicinal properties[6]. Different parts of Pinus species are used for the same purpose regardless of the species[7]. Due to their tanning contents, the dried barks have been used as a tanning agent and as an infusion to produce constipation. The leaves can also be used as a mucus remover and as an antiseptic[8,9].

Due to a rapid ascending population, developing technology and needs more than enough of humans, natural sources and biological wealth are under considerable threat. Being one of the native tree species of the naturally evertaken in limited area, the Ebe Black Pine (*P. nigra ssp. pallasiana var. seneriana*) was selected as the research material. The aim of the present study is (1) to investigate properties, climatic conditions, distribution area, ecological characteristics of *P. nigra ssp. pallasiana var. seneriana*, (2) and to detect the antimicrobial activities of this plant.

EXPERIMENTAL

The survey area of these black pines is in Alagöz Serial of the Directorate of Domaniç Forest Enterprise which is located in the Kiran area; in the south of Çokköy and Aksu villages of Domaniç belonging to Kütahya Province situated in Central Eastern Anatolia of the Aegean Region. Study area is located at 28°-32° N latitude and 38°-42° E longitude (Fig. 1). Climate type is semi arid to humid and semi humid.

The field study was carried out on the research site in 2002 in order to identify the natural growing conditions of Ebe Black Pine. Particular emphasis was placed on its current situation and ecological facts of Ebe Black Pine.

Meteorological station's report: Records from meteorology stations provided by the Turkish State Meteorological Service placed on the site and earth reports provided by the Ministry of Agriculture and Rural Affairs contributed extensively.

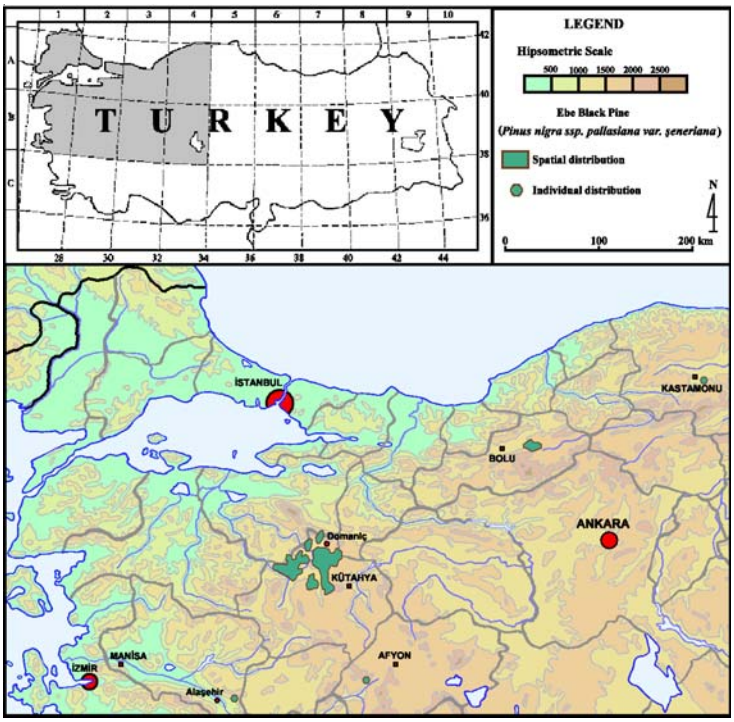

Fig. 1. Distribution of Ebe Black Pine in Turkey

Climatic change: Drought and humid indexes were calculated according to the De Martonne and Erinç method.

$$\text{De Martonne 1942: } \frac{\frac{P}{T+10}+\frac{12p}{t+10}}{2}$$

where P = annual average precipitation amount (mm); p = the precipitation for the driest month (multiplied by 12, number of months in a year); T = annual average temperature (ºC); t = the temperature in the driest month; 10 = constant coefficient used to avoid negative values.

$$\text{Erinç: } I_m = \frac{P}{T_{om}}$$

where I_m = precipitation efficiency indices; P = annual average precipitation amount (mm); T_{om} = annual average maximum temperature (ºC).

The various parts (leaves, cones, barks) of Ebe Black Pine used in this study were collected from the region of Domaniç, Kütahya (Western Anatolia) during winter. The species collected were authenticated according to the conventional method[10]. The collected parts were ground using a blender.

The solvents selected (ethyl alcohol, methanol, ethyl acetate, acetone) were purchased from Merck, Darmstadt and used without further purification[11,12].

In the study, *Escherichia coli* DM, *Micrococcus luteus* LA 2971, *Staphylococcus aureus* Cowan1, *Mycobacterium smegmatis* CCM 2067, *Pseudomonas aeruginosa* ATCC 9027, *Enterococcus faecalis* ATCC 15753, *Bacillus megaterium* NRS, *Streptococcus faecalis* DC 74 bacteria and *Candida albicans* ATCC 10239 fungus were used. The microorganisms tested in this study were provided from the culture collections by the Microbiology Laboratory of the Science and Arts Faculty, University of Kahramanmaras Sütçü Imam, Kahramanmaras, Turkey.

Preparation of model extracts: The collected parts of the species mentioned above were identified and broken into pieces and aliquots (20 g) were separately extracted with ethyl alcohol, methanol, ethyl acetate, acetone solvents (150 mL) over 24 h using a soxhlet apparatus[13]. Most of the solvents of ethyl alcohol, methanol, ethyl acetate or acetone extracts were evaporated *in vacuo* at 30 °C using a rotary evaporator until 1 mL.

Test of antimicrobial activity: Screening plant extracts for their antimicrobial activity was conducted using the disc assay described by Bauer *et al.*[14]. All the extracts thus obtained were injected into empty sterilized antibiotic discs having a diameter of 6 mm (Schleicher & Schüll No: 2668, Germany) in the amount of 50 µL. Discs injected with pure ethyl alcohol, methanol, ethyl acetate, or acetone served as negative controls[15,16]. Standard antibiotic discs such as ampicillin (10 mcg) and nystatin (30 mcg) used for comparison and also used as positive controls were provided by the Microbiology Division of the Medical Faculty, Kahramanmaras Sütçü Imam University, Kahramanmaras, Turkey. Spread plates were then kept at 4 °C temperature for 2 h to allow diffusion of extracts prior to incubation.

Preparation of microorganism culture: All the bacteria mentioned above were incubated in Nutrient Broth (NB) (Difco) at 37 ± 0.1 °C for 24 h and the yeast was incubated in Sabouraud Dextrose Broth (SDB) (Difco) at 25 ± 0.1 °C for 24 h. The bacteria and yeasts (prepared as above) were injected into petri dishes (9 cm) in the amount of 0.01 mL (10^5-10^6/mL for the bacteria and 10^5/mL for the fungi)[17], 15 mL of Mueller Hinton Agar (MHA, Oxoid) and Sabouraud Dextrose Agar (SDA) (sterilized in a flask and cooled to 45-50 °C) were homogenously distributed onto the sterilized petri dishes[15]. Sterilized blank paper discs 6 mm in diameter were saturated with extracts by micropipette per disc, then placed onto the agar plates which had previously been inoculated with the above organisms. The petri dishes were left at 4 °C for 2 h and then the injected plates with bacteria were incubated at 37 ± 0.1 °C for 24 h, plates inoculated with fungi were incubated at 25 ± 0.1 °C for 48 h[7,15,16]. At the end of the period, inhibition zones were measured with a transparent ruler in millimeters (mm). These studies were performed in triplicate.

RESULTS AND DISCUSSION

Ebe Black Pine is one of the native plants of Turkey, was initially discovered by Saatçioglu around Çaydurt (Bolu) between 1000 and 1100 meters. It was named as "*Pinus nigra var. seneriana Saatçioglu*" by Saatçioglu and then was added into "*subsp. pallasiana*" sub species by Yaltirik[5].

It can be seen as single trees dispersed over land or in infrequent small groups spread between 800 and 1250 meters within the borders of Bolu, Eskisehir, Afyonkarahisar, Manisa and Kütahya provinces in Turkey. One of the places of its natural occurrence is Domaniç (Kütahya), the western part of Anatolia. In this research, the spread of the Ebe Black Pine over Domaniç is introduced according to the results of the study area made in 2002. The pine clusters are found to be between nearly 750-850 meters in the South of Çokköy and Aksu villages in the area.

Limeless brown forest soil is common in the research area. This soil generally has a loam or sandy loam structure and has a neutral and slight acid reaction. According to Saatçioglu[18], this pine species grows into forests in brown forest soil which is rich in terms of organic material and lime; in limeless brown forest soil with slight acid and in red Mediterranean clay soil (terra rossa). It grows best in soil types which range between wet, deep fine sand loam and coarse sand loam and heavy loam. The findings of the present research are in line with the findings of Saatçioglu[18].

Ebe Black Pines within the scope of survey area were generally 610 cm. Three different forms draw attention in the area according to the shape of the crown: (1) **Full spherical head form:** in this form, the body starts to have branches from the bottom and dense branches lead to a spherical head structure. (2) **Semi spherical form:** There are branches on multi-body pines up to 3-5 m. The body can be seen from a distance. (3) **Umbrella form:** In this type, an umbrella shaped head is above the 10-15 tall bodies. The body can be seen from a distance.

In present studies, Ebe Black Pine does not compose a single forest on its own but shares its place with Anatolian Black Pine (*Pinus nigra ssp. pallasiana*) separately. The area is composed of 70 % Anatolian Black Pine and 20 % Ebe Black Pine. Additionally, 10 % Turkey Oak (*Quercus cerris*) is included in this mixture. The dominant plants on the surface of the forest are Prickly Juniper (*Juniperus oxycedrus*), Rhus (*Rhus* sp.) and Cistus (*Cistus* sp.).

The research area is in "Mediterranean-Central Anatolia Continental Transition Type" in terms of thermal regime. The temperature is approximately 0 ºC in January and increases up to 20 ºC in July. The total annual precipitation is nearly 650 mm; 41 % in winter, 26 % in spring, 21 % in autumn and 12.1 % in summer. According to this data, the research area is in Marmara Transition Type.

The area which is in "Semi-humid Marmara Climate" in terms of humidity and drought is in a transition climate between semi-arid areas and humid regions (indis 19.1) according to de Martonne. On the other hand, it is classed in semi-humid climate type (Indis 39.8) according to Erinç. The area is suitable for Ebe Black Pines in terms of its general climate features. However, in other Ebe Black Pine areas, annual average temperatures are between 10.2-16.9 ºC, the annual average relative humidity is 57-73 % and the annual precipitation ranges between 487.1-702.7 mm[5].

Akkemik and Aras[19] specified that Black pine trees in lower altitudes in the Turkish Mediterranean region have a significantly positive response to precipitation. Saatcioglu[18], Mayer and Aksoy[20] stated that *Pinus nigra* is resistant to climatic extremes in the areas from humid sites in high mountainous regions to semi arid sites in central Anatolia. The general distribution of *P. nigra* is especially high mountainous zones of the Mediterranean basin[21]. Its widest distribution in the world is in Turkey with *ca.* 2,527,675 ha. Most parts of the natural distribution of this species in Turkey have water deficiency during summer[22].

Since Ebe Black Pine is not suitable for timber utilization, it is not destroyed. However, due to the fact that areas are open to grazing and the area is used freely, this harms especially young trees and the growth of plants. In addition, natural reproduction becomes difficult since the seeds' development is weakened and productivity is low, cone yield is observed only once every 4-5 years and their numbers are very low[5].

Ebe Black Pine, in terms of timber, is not valuable or economic. However, these pines can be utilized for decorative purposes in parks and as garden trees. When the fact that biodiversity is considerably decreasing in the world and in Turkey is taken into account, it is obligatory to protect this species since it has a limited area for expansion.

Although the Ebe Black Pine does not have any economic value, it is used by local people against diseases. The bark, cones and resin of black pines are used for diarrhea, internal diseases, erysipelas, ulcers, common colds, coughing, burns, abscess, preventing distention, snake and scorpio bites and fractures[23,24].

Furthermore, in this study, the microbial effects of Ebe Black Pine were investigated. Table-1 depicts the antibacterial and antifungal activities of the ethyl alcohol, methanol, ethyl acetate and acetone extracts of the leaves, cones and bark of Ebe Black Pine on various bacteria and fungi.

As shown in this Table-1, all the extracts of the tree had antifungal effects against *Candida albicans*. All the extracts of all the parts of the tree inhibited the growth of all the bacteria tested with zones of inhibition between 7-20 mm. Methanol extract of bark showed the highest effect against *Escherichia coli* with zones of inhibition 20 mm. Yet, the ethyl alcohol

TABLE-1
ANTIMICROBIAL ACTIVITIES OF THE EXTRACTS OF EBE BLACK PINE

Microorganisms	Diameter of inhibition zone (mm)														
	Leaf				Cone				Bark				Std.		Con.
	a	b	c	d	a	b	c	d	a	b	c	d	Amp	Nst	a,b, c,d
Escherichia coli	7	15	15	14	7	14	15	7	8	20	15	7	10	NT	0
Micrococcus luteus	10	9	9	10	0	12	11	9	9	15	11	12	10	NT	0
Staphylococcus aureus	8	12	11	11	0	12	9	8	9	12	9	9	12	NT	0
Mycobacterium smegmatis	9	13	12	11	0	11	13	7	10	15	13	7	19	NT	0
Pseudomonas aeruginosa	10	16	16	10	0	12	11	7	11	14	13	7	10	NT	0
Enterococcus faecalis	14	11	12	10	0	11	12	7	8	12	11	7	16	NT	0
Bacillus megaterium	11	15	12	11	0	12	12	0	11	15	14	8	20	NT	0
Streptococcus faecalis	7	8	12	9	0	13	8	7	7	17	7	7	20	NT	0
Candida albicans	9	12	10	9	7	8	8	7	7	7	7	7	NT	18	0

a = ethyl alcohol, **b** = methanol, **c** = ethyl acetate, **d** : acetone, **Con**. = Control, **Amp** = Ampicillin 10 mcg, **Nst** = Nystatin 30 mcg, **Std.** = Standard antibiotics, **NT** = Not tested.

extract of the cone of this species did not inhibit the growth of all tested bacteria except for *Escherichia coli*, likewise the acetone extract of the cone of this species did not inhibit the growth *Bacillus megaterium*. These results are in agreement with those found in other studies[7,25]. Some differences determined in the results are thought to result from different chemical components in the Ebe Black Pine and different microorganisms studied.

The inhibition of *B. megaterium* growth by the ethyl alcohol, methanol, ethyl acetate and acetone extracts of the leaf.

(1) This study agrees with the reports of Tan[26], that transfer of these plant genetic resources to future generations, can only be possible by preserving them. In order to maintain their existence, it is essential to use them consciously and would be cultivated medicinal and aromatic plants, (2) *P. nigra* has rich biological resources such as antimicrobial agents. Recently, the utilization of plants for drugs, spice and the drink industry have shown a remarkable increase[7]. Then, it has either the work of agronomists to use them as sources of genetic material for the development of new crops or chemists to synthesize it.

Therefore, it is essential that the Pyramidal Black Pine, which is one of the most prominent biological species of Turkey, should be protected and necessary precautions taken for the survival of the species.

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