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RESEARCH ARTICLE

In vitro evaluation of the antimicrobial activity of black pine (Pinus nigra) cone syrup traditionally used in the western black sea region

Batı karadeniz bölgesinde geleneksel olarak kullanılan karaçam (Pinus nigra) kozalak şurubunun antimikrobiyal aktivitesinin in vitro değerlendirilmesi

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Abstract

Aim: Pine cone syrups are widely used to treat various diseases or to strengthen the immune system. The aim of this study was to determine the antimicrobial efficacy of black pine (Pinus nigra) cone syrup traditionally used in the western Black Sea region against important pathogenic microorganisms under in vitro conditions, to compare sugared and sugar-free forms, and to investigate its efficacy against standard antibiotics used in routine practise.

Methods: In this study, the antimicrobial activity of sweetened (3%) and sugar-free pinecone syrup against *Staphylococcus aureus* (ATCC 29213), *Enterococcus faecalis* (ATCC 10876), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Candida albicans* (ATCC 10231) strains was investigated. The diameters of the inhibition zones were determined using the agar disc-diffusion method and the minimum inhibitory concentration (MIC) was determined using the microdilution method. The susceptibility profiles of the test microorganisms to standard antibiotics (ampicillin (AM10), erythromycin (E15), gentamicin (CN10), cefixime (CFM5), oxacillin (OX1), penicillin (P10), ceftriaxone (CRO30), amoxicillin/clavulanic acid (AMC30) and itraconazole (ITC10) were analysed comparatively.

Results: Pine cone syrup showed antimicrobial activity against *P. aeruginosa* and *C. albicans* among the microorganisms tested. The diameters of the inhibition zones were determined to be 20 mm and 22 mm, respectively, and the MIC values were 125 mg/ml for both microorganisms. The relationship between pine cone syrup and gentamicin (21 mm) against *P. aeruginosa* and between pine cone syrup and itraconazole (21 mm) against *C. albicans* was found to be statistically significant ($p<0.05$). No significant difference was found between sugared and sugar-free forms in terms of antimicrobial activity ($p>0.05$).

Conclusion: Black pine cone syrup was found to have limited spectrum antimicrobial activity and was particularly effective against *P. aeruginosa* and *C. albicans* which have multidrug resistance problems. In vivo studies and tests against a broader spectrum of microorganisms are required to determine clinical efficacy.

Keywords: *Pinus nigra*, pine cone syrup, antimicrobial activity.

Özet

Amaç: Çam kozalağı şurupları halk arasında çeşitli hastalıklarının tedavisi veya bağışıklık sisteminin güçlendirilmesi amacıyla kullanılmaktadır. Bu çalışmada, Batı Karadeniz bölgesinde geleneksel olarak kullanılan Kara Çam (Pinus nigra) kozalak şurubunun önemli patojen mikroorganizmalara karşı antimikrobiyal etkinliğinin in vitro koşullarda belirlenmesi, şekerli ve şekersiz formların karşılaştırılması ve rutinde kullanılan standart antibiyotiklere karşı etkinliğinin araştırılması amaçlanmıştır.

Yöntemler: Çalışmada şekerli (%3) ve şekersiz çam kozalağı şurubunun *Staphylococcus aureus* (ATCC 29213), *Enterococcus faecalis* (ATCC 10876), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) ve *Candida albicans* (ATCC 10231) suşlarına karşı antimikrobiyal aktivitesi değerlendirilmiştir. Agar disk difüzyon yöntemi ile inhibisyon zon çapları belirlenmiş ve mikrodilüsyon yöntemi ile minimum inhibisyon konsantrasyonu (MİK) değerleri tespit edilmiştir. Test mikroorganizmalarının standart antibiyotiklere (ampicillin (AM10), erythromycin (E15), gentamicin (CN10), cefixime (CFM5), oxacillin (OX1), penicillin (P10), ceftriaxone (CRO30), amoxicillin/clavulanic acid (AMC30) and itraconazole (ITC10)) karşı duyarlılık profilleri karşılaştırmalı olarak analiz edilmiştir.

Bulgular: Çam kozalağı şurubu, test edilen mikroorganizmadan *P. aeruginosa* ve *C. albicans*'a karşı antimikrobiyal aktivite göstermiştir. Her iki mikroorganizma için inhibisyon zon çapları sırasıyla 20 mm ve 22 mm, MİK değerleri ise 125 mg/ml olarak belirlenmiştir. *P. aeruginosa*'ya karşı çam kozalağı şurubu ile Gentamisin (21 mm) arasında, *C. albicans*'a karşı çam kozalağı şurubu ile İtrakonazol (21 mm) arasındaki ilişki istatistiksel olarak anlamlı bulunmuştur ($p<0,05$). Şekerli ve şekersiz formlar arasında antimikrobiyal aktivite açısından anlamlı fark bulunmamıştır ($p>0,05$).

Sonuç: Kara Çam kozalağı şurubunun sınırlı spektrumda antimikrobiyal aktivite gösterdiği ve özellikle çoklu ilaç direnci problemi yaşanan *P. aeruginosa* ve *C. albicans*'a karşı etkili olduğu belirlenmiştir. Klinik etkinliğin belirlenmesi için in vivo çalışmalara ve daha geniş mikroorganizma spektrumuna karşı test edilmesine ihtiyaç duyulmaktadır.

Anahtar Kelimeler: *Pinus nigra*, çam kozalağı şurubu, antimikrobiyal aktivite.

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INTRODUCTION

Throughout human history, plants have been one of the most important sources for treating health problems and meeting nutritional needs (1). Archaeological findings show that herbal treatment methods were practised in Mesopotamia around 5000 BC, with around 250 different herbal medicines being used during this time (2).

Plants that have been used from the past to the present in various areas especially in the health sector are still important today. Medicinal and aromatic plants contain important bioactive compounds that are effective in the treatment of diseases and are used in both traditional and modern medicine (3). Due to the increasing global demand for herbal medicines and aromatic compounds, the cultivation of medicinal and aromatic plants has increased significantly in recent years (4).

Today, many researchers are investigating the chemical composition of plants with medicinal potential, and interest in this area continues to grow. In developing countries, about 80% of the population relies on herbal products for therapeutic purposes (5). In many countries, including Turkey, extracts from plants such as linden, ginger, black cumin, chamomile, fennel and pine are widely used for medicinal purposes. Preparations derived from pine trees including their bark, cones, needles, and other parts are widely used and increasingly recognized by the general public. In East Asian countries, various components of pine trees, such as needles, cones, bark, and pollen, are commonly consumed as food or dietary supplements to support health (6).

Herbal medicines are traditionally prepared by boiling or mechanically crushing plants to extract their active ingredients. This traditional practise has inspired modern scientists to carry out content analyses and identify the active ingredients. The aim is to determine the key substances in plants that act on disease-causing factors and to commercialise these compounds.

The content of bioactive substances in extracts from pine fractions indicates that they could serve as a promising and innovative ingredient for the development of functional foods. However, there are only a limited number of studies on the use of different forms of these fractions such as extracts, powders and capsules as food additives or dietary supplements.

Pine cones, which are particularly rich in antioxidant and antibacterial properties, are now often consumed in the form of pine cone syrup (7). Pine cone syrup is traditionally obtained by collecting the cones of the black pine (Pinus nigra), which grows in the western Black Sea region, in April–May when they are fresh and green, and concentrating them by boiling them for a long time. This product, which is made into syrup either pure or with added sugar, is filled into jars and sold at local markets. Pine cone syrup is often used in the treatment of various diseases or to strengthen the immune system.It has been reported in various studies that secondary metabolites in pine cone extract show antimicrobial activity and have therapeutic potential (8,9). Traditionally, pine cone syrup is widely used in the Western Black Sea region for the treatment of various infections and as an immune system-supportive supplementary food.

In this study, pine cone syrup obtained from the cones of black pine *(Pinus nigra)*, which grows naturally in the forests of the Western Black Sea region, was tested under in vitro conditions against *Staphylococcus aureus* (ATCC 29213), *Enterococcus faecalis* (ATCC 10876), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Candida albicans* (ATCC 10231).

METHODS

In this study, the antimicrobial activity of pine cone syrup obtained from the cones of black pine *(Pinus nigra)*, a species native to the forests of the western Black Sea, was investigated.

Supply of Pine Cone Syrup

Syrup from black pine cones *(Pinus nigra)* collected in the western Black Sea region and sold at local markets has been used. Two types of pine cone syrup- sweetened (3%) and unsweetened (no sugar) were sourced from three different suppliers and a total of six samples were included in the study

Test Microorganisms

Standard strains available in the culture collection of the Medical Microbiology Laboratory of the Faculty

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of Medicine of Karabuk University were used for the antimicrobial activity tests. The microorganisms used in the study were *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 10876, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* ATCC 10231.

Media and Chemicals

Nutrient Agar (NA), Nutrient Broth (NB), Potato Dextrose Agar (PDA), Potato Dextrose Broth (PDB), Mueller-Hinton Agar (MHA), Mueller-Hinton Broth (MHB), Malt Extract Agar (MEA), Malt Extract Broth (MEB) and Sabouraud Dextrose Broth (SDB) were used for antimicrobial activity assays. All media were commercially available in powder form (Merck, Germany) and were prepared in the laboratory according to the manufacturer's instructions.

Agar Disc Diffusion Method

The antimicrobial activity of pine cone syrup was determined using the agar disc diffusion method described by Hudzicki (2016) (10). Standard microorganism strains were passaged on appropriate media according to their species; then the resulting colonies were activated twice in Mueller-Hinton Broth (MHB) medium at 37°C. One millilitre was inoculated onto Petri dishes containing Mueller-Hinton agar (MHA) using a sterile swab.

Discs impregnated with 20 µl of 1/1, 1/2 and 1/10 dilutions of pine cone syrup, negative control discs impregnated with 20% dimetil sülfoksit (DMSO) (Merck, Germany) and antibiotic discs for the positive control were placed on the agar surface using sterile forceps. The media were incubated for 24 hours at 35°C and the diameters of the inhibition zones were measured in millimetres.

Determination of the Minimum Inhibitory Concentration (MIC)

The MIC test was performed using 96-well microplates and the microdilution method described by Zgoda and Porter (2001) (11). Bacteria were incubated in Mueller-Hinton broth and yeast in Sabouraud dextrose broth at 35°C for 24 hours. The optical density (OD) of the microorganisms was measured at 600 nm with a spectrophotometer and set to 0.1 (corresponds to the McFarland 0.5 standard). The bacteria were diluted 1/10 and the yeast was used directly.

A concentration of 250 µg/ml was achieved in the first well with 1 mg/ml pine cone syrup. Serial 1:2 dilutions were made and the concentrations were adjusted to 125, 62.5, 31.25, 15.625, 7.8125, 3.906, 1.953 and 0.976 µg/ml, respectively. The final volume in each well was set to 100 µl. Well 10 served as a microorganism growth control (medium+microorganism), well 11 as a contamination control (medium+extract solution) and well 12 as a solvent control (medium+20% DMSO+microorganism). The microplates were incubated for 24 hours at 35°C and the lowest concentration at which growth was inhibited was reported as the MIC value. The evaluation was performed according to the guidelines of the European Committee for Antimicrobial Susceptibility Testing (EUCAST v.10.0).

Antibiotic Susceptibility Test

One hundred µl of bacterial samples activated in Mueller Hinton Broth (MHB) were transferred to Petri dishes containing Nutrient Agar. The antibiotic platelets were placed on Mueller Hinton agar (MHA) and incubated at 37°C for 24 hours. The antibiotics used in the study were ampicillin (AM10), erythromycin (E15), gentamicin (CN10), cefixime (CFM5), oxacillin (OX1), penicillin (P10), ceftriaxone (CRO30), amoxicillin/clavulanic acid (AMC30) and itraconazole (ITC10).

Statistical Analysis

All analysis results were analysed using SPSS statistical software. The antimicrobial activity of the different dilutions of pine cone syrup and their relationships with the microorganism species were statistically analysed, with $p<0.05$ considered statistically significant.

RESULTS

Results of the Antimicrobial Activity Test

The diameters of the inhibition zones determined by the agar disc diffusion method and the minimum inhibitory concentration (MIC) values determined by the microdilution method for pine cone syrup with and without sugar (3%) against the tested microorganisms were evaluated comparatively. The zones of inhibition

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(mm) and MIC values (mg/ml) of the microorganisms tested against pine cone syrup are listed in Table 1.

Pine cone syrup showed antimicrobial activity against two of the five microorganisms tested: *Pseudomonas aeruginosa* (ATCC 27853) and *Candida albicans* (ATCC 10231). Against *P. aeruginosa* ATCC 27853, both the unsweetened and the sweetened pine cone syrup formed an inhibition zone with a diameter of 20 mm and an MIC value of 125 mg/ml. Both forms were also effective against C. albicans ATCC 10231, with an inhibition zone of 22 mm and an MIC of 125 mg/ml.

Neither form of pinecone syrup showed antimicrobial activity against Gramme-positive strains of *Staphylococcus aureus* (ATCC 29213) and *Enterococcus faecalis* (ATCC 10876) or the Gramme-negative strain *Escherichia coli* (ATCC 25922).

There was no significant difference in antimicrobial activity between sweetened and unsweetened pine cone syrup ($p>0.05$). Both syrups showed similar inhibition zone diameters and MIC values against the same microorganisms.

Results of the Antibiotic Susceptibility Test

To evaluate the antimicrobial activity of pine cone syrup, the susceptibility profiles of the test microorganisms to standard antibiotics were also determined. The comparative analysis showed that the pine cone syrup exhibited comparable activity to certain conventional antibiotics (Table 2).

Against P. aeruginosa (ATCC 27853), pinecone syrup produced an inhibition zone diameter of 20 mm, while gentamicin (CN10) produced a zone of 21 mm. The similarity of the zones of inhibition was statistically significant ($p<0.05$).

Pinecone syrup showed an inhibition zone of 22 mm for *C. albicans* (ATCC 10231), while itraconazole (ITC10)

Table 1. Inhibition zone diameters (r) (mm) and MIC values (mg/ml) of the microorganisms tested against pine cone syrup

Microorganisms	Pine Cone Syrups			
	Unsweetened		Sweetened (%3)	
	r (mm)	MIC(mg/ml)	r (mm)	MIC(mg/ml)
Staphylococcus aureus (ATCC 29213)	-	-	-	-
Pseudomonas aeruginosa (ATCC 27853)	20 mm	125 (mg/ml)	20 mm	125 (mg/ml)
Enterococcus faecalis (ATCC 10876)	-	-	-	-
Escherichia coli (ATCC 25922)	-	-	-	-
Candida albicans (ATCC 10231)	22 mm	125 (mg/ml)	22 mm	125 (mg/ml)

Table 2. Inhibition zones of the tested microorganisms against antibiotics (mm)

Antibiotics	Microorganisms				
	Staphylococcus aureus	Enterococcus faecalis	Escherichia coli	Pseudomonas aeruginosa	Candida albicans
Ampicillin (AM10)	-	36 mm	-	-	-
Erythromcin (E15)	36 mm	-	-	-	-
Gentamicin (CN10)	-	38 mm	-	21 mm	-
Cefixime (CFM5)	-	-	-	-	-
Oxacillin (OX1)	-	-	-	-	-
Penicillin (P10)	28 mm	-	-	-	-
Ceftriaxon (CRO30)	-	-	36 mm	-	-
Amoxicillin (AMC30)	-	-	40 mm	-	-
Itraconazole (ITC10)	-	-	-	-	21 mm

"- : No activity detected"

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produced a zone of 21 mm. This similarity was also statistically significant ($p<0.05$).

For the other test microorganisms: *S. aureus* (ATCC 29213) showed sensitivity to erythromycin (36 mm) and penicillin (28 mm); *E. faecalis* (ATCC 10876) was sensitive to ampicillin (36 mm) and gentamicin (38 mm); and *E. coli* (ATCC 25922) to ceftriaxone (40 mm) and amoxicillin (36 mm). However, no inhibition zones with pinecone syrup were observed in any of these strains, indicating resistance.

DISCUSSION

Products obtained from the bark, cones and leaves of the pine tree are widely used in the treatment of various diseases and as dietary supplements. In our study, pine cone syrup extracted from the cones of black pine (Pinus nigra) was tested against *S. aureus* (ATCC 29213), *E. faecalis* (ATCC 10876), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853) and *C. albicans* (ATCC 102312) and proved to be effective against the latter two.

In national and international literature, various studies have reported that the secondary metabolites in pine cone extract show antimicrobial activity and have therapeutic potential (8-12). In a study conducted in Nevşehir province, the antibacterial effects of the extract obtained from the cones of some conifers were evaluated and it was reported that antimicrobial activities against P. aeruginosa and E. coli were observed (8). However, no antimicrobial activity against E. coli was observed in our study. This difference could be due to differences in the pine species used, extraction method and concentration.

Various studies in the literature have reported that the secondary metabolites in the extract of pine cones show antimicrobial activity and have therapeutic potential (8-12). In a study conducted in Nevşehir province, it is suggested that the differences in the antimicrobial activity of pine cones are due to the differences in phenolic compounds, flavonoids, terpenes and essential oil components (13,14). In particular, monoterpenes such as α-pinene, β-pinene, limonene and phenolic compounds such as caffeic acid and quercetin are thought to be responsible for the antimicrobial activity (15). These compounds are thought to exert antimicrobial effects by increasing the permeability of cell membranes, inhibiting protein synthesis and preventing DNA replication (16,17). The selective antimicrobial activity observed in our study indicates that these bioactive compounds may have different mechanisms of action depending on the microorganism species. The fact that the outer membrane structure of *P. aeruginosa* and the fungal cell wall of *C. albicans* are more sensitive to these compounds could explain the observed selective effect.

Vainio-Kaila et al. (18) reported that the extract of Pinus sylvestris showed antimicrobial activity against *S. aureus*, *E. faecalis*, *E. coli* and *S. pneumoniae*. Similarly, Karapandzova et al. (19) reported that the essential oil of Pinus mugo needle was particularly effective against Gramme-positive bacteria. Multani et al. (20) compared the antimicrobial activities of essential oils from cones and needles of different Pinus species (P. mugo, P. nigra, P. sylvestris, P. halepensis) and reported that the cone oil of *P. sylvestris* had the highest antimicrobial activity. The different antimicrobial effects of pine cones are thought to be due to differences in the composition of phenolic compounds, flavonoids, terpenes and essential oil components (13,14). In particular, monoterpenes such as α-pinene, β-pinene and limonene as well as phenolic compounds such as caffeic acid and quercetin are thought to be responsible for the antimicrobial activity (15). These compounds are suggested to exert antimicrobial effects by increasing the permeability of cell membranes, inhibiting protein synthesis and preventing DNA replication (16,17). The selective antimicrobial activity observed in our study indicates that these bioactive compounds may have different mechanisms of action depending on the microorganism species. The greater sensitivity of the outer membrane structure of *P. aeruginosa* and the fungal cell wall of *C. albicans* to these compounds could explain the observed selective effect.

In this study, black pine *(Pinus nigra)* cone syrup from the Western Black Sea region was obtained in two forms: sweetened (3%) and unsweetened (without sugar). There was no significant difference in antimicrobial activity between sweetened and unsweetened forms ($p>0.05$). This indicates that the addition of sugar in low concentrations does not affect the stability and activity of the bioactive compounds. The literature reports that the addition of low concentrations of carbohydrates to plant extracts does not significantly alter antimicrobial

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activity and in some cases even provides stability. It is hypothesised that low concentrations of carbohydrates such as sugars may have a long-term protective effect by preventing the oxidation of bioactive compounds and thus contributing to the maintenance of antimicrobial activity (21).

Akar et al. (8) compared the antibacterial effects of the extract obtained from pine cones with the effects of chemical antibiotics (Ampicillin AM10, Erythromycin E15, Gentamicin CN10, Cefixime CFM5, Oxacillin OX1, Penicillin P10, Cefuroksim CXM30, Ceftriaxone CRO30, Amoxicillin AMC30, Cefoksitin FOX30) used commercially today on pathogens. "The researchers found that the antibiotic Cefuroxime CXM30 showed the most potent antimicrobial activity against pathogens; nevertheless, cone extracts showed better efficacy compared to Cefuroxime. They reported that this enhanced antimicrobial activity can be attributed to the high concentration of bioactive compounds in the cone extracts (8). In our study, the susceptibility profiles of the test microorganisms to standard antibiotics were determined in order to evaluate the antimicrobial activity of pine cone syrup. Pine cone syrup and gentamicin (CN10) formed similar zones of inhibition against the strain of *Pseudomonas aeruginosa* (ATCC 27853) ($p<0.05$). Pinecone syrup and itraconazole (ITC10) showed similar activity against the strain of Candida albicans (ATCC 10231) ($p<0.05$). These results suggest that pine cone syrup has comparable activity to conventional antimicrobials against certain pathogens. The activity of pine syrup against *P. aeruginosa* is particularly important as this microorganism is a major cause of nosocomial infections and can develop resistance to most antibiotics (17). Similarly, given the increasing problem of fungal resistance in C. albicans infections, it is crucial to develop alternative treatments of natural origin (21).

CONCLUSION

Our study has some limitations. Only five types of microorganisms were tested, so the broad-spectrum antimicrobial activity could not be evaluated. Secondly, the profile of bioactive substances in pine cone syrup was not analysed in detail. Future studies should consider profiling bioactive compounds using advanced analytical methods, comparing syrup from different pine species and testing with a wider range of microorganisms.

These results suggest that pine cone syrup is not a broad-spectrum antimicrobial, but has comparable efficacy to conventional antimicrobials against certain microorganisms. It could be considered as an alternative or adjuvant treatment, particularly for *P. aeruginosa* infections with multidrug resistance and *C. albicans* infections with increasing fungal resistance.

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