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Research Article / Araştırma Makalesi

Assessing the Susceptibilty of Some Gut Bacteria to the Extract from Needles of Turkish Pine

Bazı Bağırsak Bakterilerinin Türk Çamı İğnelerinden Elde Edilen Ekstrakta Duyarlılıklarının Değerlendirilmesi

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Abstract: Plant extracts have the potential to be safe alternatives to antibiotics that disrupt the gut flora. The aim of the present study was to assess the susceptibility of some gut bacteria to the extract from needles of Turkish pine (*Pinus brutia* Ten.) using microdilution method in an anaerobic chamber. Turkish pine needle extract promoted the growth of *Bifidobacterium bifidum*, *Bifidobacterium infantis*, and *Lactobacillus acidophilus* from gut commensals at 0.2-6.25 mg/mL, 0.4-6.25 mg/mL, and 0.4-1.6 mg/mL dose ranges, respectively (P<0.05). However, the extract had a potential inhibitory activity on *Bifidobacterium* species starting from 12.5 mg/mL, on *L. acidophilus* starting from 6.25 mg/mL, and on *L. casei* starting from 3.13 mg/mL concentrations (P<0.05). Minimal inhibitory concentration (MIC) was 25 mg/mL for all commensal species (P<0.05). Turkish pine needle extract also showed a potential inhibitory activity against gut pathogens *Escherichia coli* and *Clostridium perfringens* from 0.4 mg/mL dose and against *Staphylococcus aureus* and *Fusobacterium nucleatum* from 0.8 mg/mL dose (P<0.05). The MICs were 6.25, 12.5, 25, and 50 mg/mL for *S. aureus*, *F. nucleatum*, *E. coli*, and *C. perfringens*, respectively (P<0.05). It was concluded that using the Turkish pine needle extract in a dose range of 0.2-6.25 mg/mL, where it protected most of the commensal bacteria and was toxic against some of the pathogens, might produce desirable impacts in the gut.

Keywords: Antibacterial, Gut bacteria, MIC, Plant extracts, Turkish pine needle.

Öz: Bitki ekstraktları, bağırsak florasını bozan antibiyotiklere güvenli alternatifler olma potansiyeline sahiptir. Bu çalışmanın amacı, anaerobik bir kabinde mikrodilüsyon yöntemi kullanılarak bazı bağırsak bakterilerinin Türk çamı (*Pinus brutia* Ten.) iğnelerinden elde edilen ekstrakta duyarlılığını değerlendirmektir. Türk çamı iğnesi ekstraktı, bağırsak yerleşik bakterilerinden *Bifidobacterium bifidum*, *Bifidobacterium infantis* ve *Lactobacillus acidophilus*'un büyümesini sırasıyla 0,2-6,25 mg/mL, 0,4-6,25 mg/mL ve 0,4-1,6 mg/mL doz aralıklarında uyarmıştır (P<0,05). Bununla birlikte, ekstrakt, *Bifidobacterium* türleri üzerine 12,5 mg/mL, *L. acidophilus* üzerine 6,25 mg/mL ve *L. casei* üzerine ise 3,13 mg/mL konsantrasyonlardan başlayarak potansiyel bir inhibitör aktivite göstermiştir (P<0,05). Minimal inhibitör konsantrasyonun (MİK), tüm yerleşik türler için 25 mg/mL olduğu gözlenmiştir (P<0,05). Türk çamı iğnesi ekstraktı ayrıca bağırsak patojenleri olan *Escherichia coli* ile *Clostridium perfringens*'e karşı 0,4 mg/mL dozdan ve *Staphylococcus aureus* ile *Fusobacterium nucleatum*'a karşı ise 0,8 mg/mL dozdan başlayarak potansiyel bir inhibitör aktivite göstermiştir (P<0,05). *Staphylococcus aureus*, *F. nucleatum*, *E. coli* ve *C. perfringens* için MİK değerlerinin sırasıyla 6,25, 12,5, 25 ve 50 mg/mL olduğu gözlenmiştir (P<0,05). Türk çam iğnesi ekstraktının, yerleşik bakterilerin çoğunu koruduğu ve bazı patojenlere karşı toksik olduğu 0,2-6,25 mg/mL doz aralığında kullanılmasının bağırsaklarda arzu edilen etkiler oluşturabileceği sonucuna varılmıştır.

Anahtar Kelimeler: Antibakteriyel, Bağırsak bakterileri, Bitki ekstraktları, MİK, Türk çamı iğnesi.

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Introduction

The gut flora is a large and dynamic bacterial community that participates in normal physiological functions, but also protects against pathogens by forming a defensive barrier and competing for available substrates (Ahn et al., 1998; Canny and McCormick, 2008). Balance between commensal and pathogenic species has great importance in terms of maintaining the gut health. Conventional antibiotics can prevent the growth of both commensal and pathogenic species and decrease diversity of the gut flora (Bäumler and Sperandio, 2016). In recent years, many studies have been focused on plant extracts as natural alternatives for antibiotics.

Turkish pine or Turkish red pine (*Pinus brutia* Ten.) is the most common pine species in Turkey which has ability to grow on a wide range of Mediterranean- and Black Sea regions (Balaban Ucar et al., 2013). The use of Turkish pine in the forest products industry has been widely accepted because of its suitability for the manufacture of desirable products (Üner et al., 2011). In a previous study, we observed that the extract from barks of Turkish pine, which containing phenolic compounds, had a potential to inhibit pathogenic bacteria in the gut while protect commensal ones (Demirtaş, 2020). Furthermore, *Pinus densiflora* (Japanese pine) leaf derived components, (1R)-(+)-α-pinene and limonene, strongly inhibited the growth of *Staphylococcus aureus*, *Escherichia coli,* and *Clostridium perfringens* without adverse effects on the growth of five commensal bacteria (*Bifidobacterium bifidum, B. longum*, *B. adolescentis*, *Lactobacillus acidophilus*, and *L. casei*) (Hwang and Lee, 2002). The needle of Turkish pine also contains several flavonoids (Kaundun et al., 1997) and essential oil components (Yener et al., 2014) with antioxidant and antibacterial capacity. However, the effects of extract from Turkish pine needle on gut bacteria have not been evaluated previously. Therefore, the aim of the present study was to assess the susceptibility of some gut bacteria to the extract from needles of Turkish pine.

Materials and Methods

Turkish pine needle extract

Turkish pine needle extract was provided by Kale Naturel Herbal Products Company, Ltd., Balikesir, Turkey.

Preparation of bacteria

Commensal bacterial species used in antibacterial tests were *Bifidobacterium bifidum* ATCC 29521, *Bifidobacterium longum* subsp. *infantis* ATCC 15697, *Lactobacillus acidophilus* ATCC 4356, and *Lactobacillus casei* ATCC 393. Pathogenic bacterial species were *Staphylococcus aureus* subsp. *aureus* ATCC 12600, *Escherichia coli* ATCC 11775, *Clostridium perfringens* ATCC 13124, and *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586. The growth medium was Mann Rogosa Sharpe (MRS) broth for *B. infantis*, *L. acidophilus*, and *L. casei*; MRS broth with 0.05% cysteine (MRS-C) for *B. bifidum*; tryptic soy broth (TSB) for *S. aureus*; Luria–Bertani (LB) medium for *E. coli* and; liquid form of medium 2 (Hobson, 1969) for *C. perfringens* and *F. nucleatum*. Medium 2 was prepared under CO_2 as described by Hobson (1969) with only slight modification. Trypticase peptone was used instead of casitone in medium 2 (Table 1). All strains were grown for 24 h at 37°C under an atmosphere of 80% N_2, 10% CO_2, and 10% H_2 in an anaerobic chamber (Don Whitley, Whitley DG250, West Yorkshire, UK).

Table 1. Composition of medium 2 (for 100 mL)

Component	
Trypticase peptone (BD 211921 Bacto™)	1.0 g
Yeast extract (Sigma Y1625)	0.25 g
Mineral solution 1	15 mL
Mineral solution 2	15 mL
Clarified rumen fluid	20 mL
Resazurin (Sigma R7017)	0.0001 g
Sodium lactate (70% w/v)	1.0 g
Glucose	0.2 g
Maltose	0.2 g
Cellobiose (Sigma 22150)	0.2 g
Cysteine HCl (Sigma C7880)	0.05 g
$NaHCO_3$ (Sigma S5761)	0.4 g
Deionized water	to 100 mL

Mineral solution 1 – 3 g/L K_2HPO_4 (Sigma P3786); Mineral solution 2 – 3 g/L KH_2PO_4 (Sigma P9791), 6 g/L $(NH_4)_2SO_4$ (Sigma A4915), 6 g/L NaCl (Sigma S7653), 0.6 g/L $MgSO_4 \bullet 7H_2O$ (Sigma 230391), and 0.6 g/L $CaCl_2$ (Sigma C1016). Clarified rumen fluid – ruminal fluid brought from the slaughterhouse was mixed and filtered through three layers of cheesecloth to partition into liquid and solid (digesta) fractions. The liquid fraction was centrifuged at 15000 rpm, and the clear supernatant was used as a component of the anaerobic medium.

Antibacterial screening

The effect of Turkish pine needle extract on the growth of gut bacteria was tested by a broth dilution method in the anaerobic chamber (CLSI, 2016). A stock solution was prepared by dissolving pine needle extract in 50% ethanol. Ten serial dilutions of the extract starting at a concentration of 50 mg/mL were prepared from the stock solution in the bacterial strain specific growth media. Two hundred microliters of each dilution were added to wells of a 96-well plate. Next, 20 µL of the test bacteria suspension was inoculated into each well. Each bacterium was tested in triplicate wells. Plates were incubated for 24 h at 37°C in the anaerobic chamber. Bacterial growth was detected with a microplate reader at 600 nm (Epoch, BioTek, USA). The minimal inhibitory concentration (MIC) is defined as the lowest concentration of added extract at which no significant bacterial cell growth was observed. A significantly lower OD_{600} value compared to control dose (0 mg/mL) was accepted as potential inhibitory activity (Ko et al., 2018) while significantly higher value was accepted as stimulatory effect (Das et al., 2015).

Statistical analyses

Statistical analysis was carried out by the use of one-way ANOVA followed by Dunnett's test. Each well of a 96-well plate was an experimental unit. A probability value at $P<0.05$ was considered statistically significant.

Results

Effects of Turkish pine needle extract on gut bacteria are showed in Figure 1 and Figure 2. Turkish pine needle extract promoted the growth of *B. bifidum*, *B. infantis*, and *L. acidophilus* from gut commensals at 0.2-6.25 mg/mL, 0.4-6.25 mg/mL, and 0.4-1.6 mg/mL dose ranges, respectively ($P<0.05$). That effect was more obvious for *B. infantis*. However, the extract had a potential inhibitory activity on *Bifidobacterium* species starting from 12.5 mg/mL, on *L. acidophilus* starting from 6.25 mg/mL, and on *L. casei* starting from 3.13 mg/mL concentrations ($P<0.05$). The MIC was 25 mg/mL for all commensal species ($P<0.05$) (Table 2). Turkish pine needle extract also showed a potential inhibitory activity against gut pathogens *E. coli* and *C. perfringens* from 0.4 mg/mL dose and against *S. aureus* and *F. nucleatum* from 0.8 mg/mL dose ($P<0.05$). The MICs were 6.25, 12.5, 25, and 50 mg/mL for *S. aureus*, *F. nucleatum*, *E. coli*, and *C. perfringens*, respectively ($P<0.05$) (Table 2).

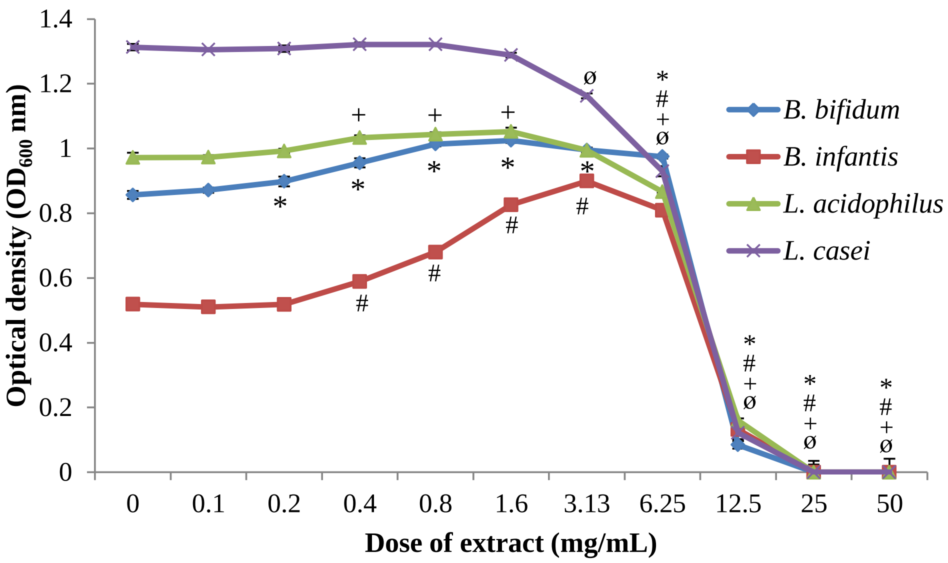

Figure 1. Effects of Turkish pine needle extract against commensal bacteria from the gut. The results represent the mean ± standard error. *$P<0.05$, extract treated culture vs *B. bifidum* control; #$P<0.05$, extract treated culture vs *B. infantis* control; +$P<0.05$, extract treated culture vs *L. acidophilus* control; and ø$P<0.05$, extract treated culture vs *L. casei* control. Control level was 0 mg/mL of the extract.

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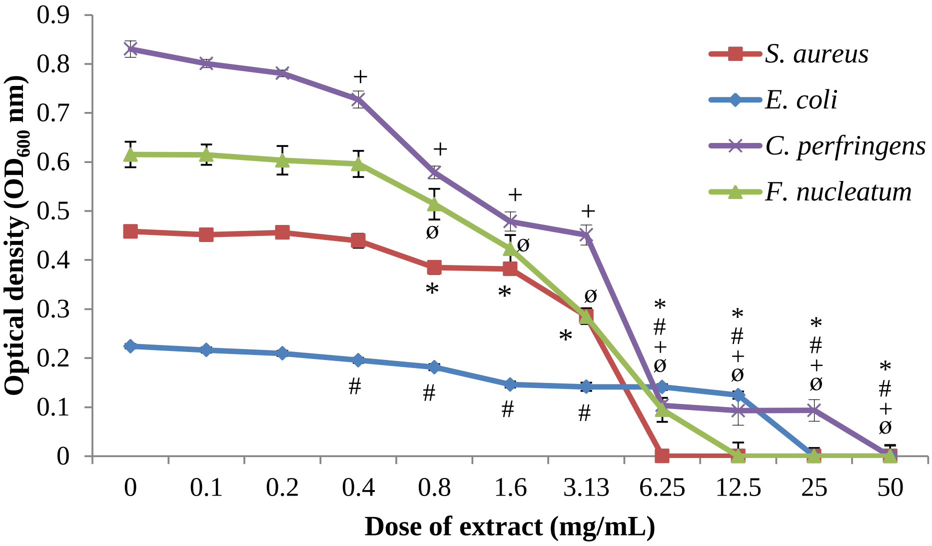

Figure 2. Effects of Turkish pine needle extract against pathogenic bacteria from the gut. The results represent the mean ± standard error. $^{*}P$<0.05, extract treated culture vs *S. aureus* control; $^{\#}P$<0.05, extract treated culture vs *E. coli* control; ^{+}P<0.05, extract treated culture vs *C. perfringens* control; and ^{ø}P<0.05, extract treated culture vs *F. nucleatum* control. Control level was 0 mg/mL of the extract.

Table 2. Minimum inhibitory concentration (MIC) values of Turkish pine needle extract on gut bacteria.

Bacteria	MIC values (mg/mL)
Commensals	
B. bifidum	25
B. infantis	25
L. acidophilus	25
L. casei	25
Pathogens	
S. aureus	6.25
E. coli	25
C. perfringens	50
F. nucleatum	12.5

Discussion

The presence of a diverse and balanced bacterial community in the gut is of great importance for host physiology. Disruption of commensal flora in the gut is one of the major complications encountered in the treatment of infections with antibiotics (Bäumler and Sperandio, 2016). Accordingly, to determine the safe dose range of therapeutic agents that protects commensal bacteria while suppressing pathogens has great importance in terms of gut health. Turkish pine needle extract at low concentrations stimulated the growth of *B. bifidum*, *B. infantis*, and *L. acidophilus* from commensals, more prominently for *B. bifidum*. However, this stimulatory effect turned into a potential inhibitory effect on *Bifidobacterium* species starting from 12.5 mg/mL and on *L. acidophilus* starting from 6.25 mg/mL concentrations. The extract completely inhibited all commensal species at 25 mg/mL. Although there is no literature on the effects of Turkish pine needle extract on commensal gut bacteria, it was reported that low doses of several plant metabolites could stimulate bacterial growth in the gastrointestinal tract while high doses induced inhibition (Patra et al., 2012; Demirtas et al., 2019; Goker and Demirtas, 2020). Aldehydes, one of the plant secondary metabolites from the green leaf volatiles family, moderately promote the growth of *L. acidophilus* and *B. bifidum* at lower concentrations while had inhibitory effects at higher concentrations in a previous study (Goker and Demirtas, 2020). *Trans*-2-decenal, also an aldehyde from green leaf volatiles, stimulated the growth of *Fibrobacter succinogenes*, which is a fibrolytic bacterium from the rumen, at low doses

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(Demirtas et al., 2019). Similarly, saponins, another group of phytochemicals, encouraged *in vitro* bacterial growth and feed utilization in the rumen at low doses while they exhibited inhibition at high doses (Patra et al., 2012).

Escherichia coli and *S. aureus* are common foodborne pathogens that can cause severe gastro-intestinal illness (Ørskov and Ørskov, 1992; Rajkovic, 2014). The MIC value of Turkish pine needle extract for *E. coli* was 25 mg/mL in the present study. There is no literature on the effects of Turkish pine needle extract on pathogenic gut bacteria. However, Hmamouch et al. (2001) reported that the MIC value of the essential oil extracted from the needles of *P. brutia* grown in Morocco was higher than 10 mg/mL for *E. coli* (ATCC 25922). This result is consistent with the result of this study. On the other hand, it was observed that *S. aureus* was the most sensitive bacterium to Turkish pine needle extract in the present study. The extract exhibited inhibitory activity against *S. aureus* at 6.25 mg/mL dose. Extract from pine needles of *Cedrus deodara* (Himalayan cedar), with the main antibacterial component of shikimic acid, inhibited the growth of *S. aureus* (ATCC 25923) at 0.78 mg/mL (Zeng et al., 2012). The difference in MIC values is probably due to the difference in bacterial strains and also due to active ingredients in needles of the pine trees from the different origins. The dominant flavonoids found in the needles of *P. brutia* were reported as quercetin (41%), kaempferol (29%), and isorhamnetin (%23) (Kaundun et al., 1997) while the main essential oil component was reported as β-pinene (Yener et al., 2014). In this study, one or more of these active ingredients, that were likely to be contained in the extract, might be responsible for the antibacterial effects.

Fusobacterium nucleatum, which is obviously associated with colorectal cancer (Shang and Liu, 2018), was more sensitive to Turkish pine needle extract than *C. perfringens* in the present study. Extract from the barks of *P. brutia* also had an inhibitory potential on this bacterium from 150 µg/mL concentration in a previous study (Demirtas, 2020). On the other hand, *C. perfringens*, which is generally linked to gastrointestinal symptoms such as vomiting and diarrhea (Keeratirathawat et al., 2013), was the most resistant species to the used extract in this study. Turkish pine needle extract inhibited the growth of this bacterium at 50 mg/mL. Keeratirathawat et al. (2013) also reported that oils from the needles of four different *Pinus* species (*Pinus radiata*, *P. pinaster*, *P. sylvestris,* and *P. nigra*) did not exhibit any antibacterial activity against *C. perfringens.*

It was concluded that using the Turkish pine needle extract in a dose range of 0.2-6.25 mg/mL, where it protected most of the commensal bacteria and was toxic against some of the pathogens, might produce desirable impacts in the gut. Further *in vitro* and *in vivo* studies required to clarify its beneficial effects on the gut health.

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