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Research Article

Influence of *Pinus brutia* bark extract containing phenolic compounds on some commensal and pathogenic bacteria from the intestinal microflora

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ABSTRACT

The microflora of the intestinal tract is vital to many physiological functions, mainly fermentation and processing of dietary components, control of intestinal epithelial cell proliferation, development of the immune system, and protection against pathogens. Plant extracts have potential for treatment options that protect commensal or beneficial microflora in the intestines while eliminating pathogens. The aim of the present study was to investigate the influence of *Pinus brutia* (Turkish red pine) bark extract containing phenolic compounds on some commensal and pathogenic bacteria from the intestinal microflora using a microdilution method. *Pinus brutia* bark extract did not completely inhibit any intestinal bacteria. However, the extract showed a potential inhibitor activity on *Salmonella* Typhimurium and *Staphylococcus aureus* from 75 µg/mL, on *Escherichia coli* and *Fusobacterium nucleatum* from 150 µg/mL, and on *Clostridium perfringens* from 300 µg/mL concentrations (P<0.05). Commensal bacteria were observed to be less sensitive to the extract than those of the pathogenic strains. The extract stimulated moderately the growth of *Bifidobacterium bifidum* from 75 µg/mL dose (P<0.05). The extract did not show any activity on *Lactobacillus acidophilus*. A potential inhibitor activity was observed for *Bifidobacterium infantis* and *Lactobacillus casei* at 600-2400 µg/mL (P<0.05). As a conclusion, *P. brutia* bark extract, at 75-300 µg/mL dose range, had a potential to restrict pathogenic bacteria in the intestines while protect commensal or beneficial ones. Specified effects might be mainly attributed to its polyphenolic content.

Fenolik bileşikler içeren *Pinus brutia* kabuğu ekstraktının bağırsak mikroflorasında bulunan bazı yerleşik ve patojenik bakteriler üzerine etkisi

ÖZ

Bağırsak kanalı mikroflorası, başlıca diyet bileşenlerinin fermentasyonu ve işlenmesi, bağırsak epitel hücre çoğalmasının kontrolü, bağışıklık sisteminin gelişimi ve patojenlere karşı koruma olmak üzere birçok fizyolojik fonksiyon için hayati öneme sahiptir. Bitki ekstraktları bağırsaklardaki patojenleri elimine ederken yerleşik veya iyi huylu mikroflorayı koruyan tedavi seçenekleri için potansiyel taşımaktadır. Bu çalışmanın amacı, fenolik bileşikler içeren *Pinus brutia* (Türk kızılçamı) kabuğu ekstraktının, bağırsak mikroflorasında bulunan bazı yerleşik ve patojenik bakteriler üzerindeki etkisini mikrodilüsyon yöntemi kullanarak araştırmaktır. *Pinus brutia* kabuğu ekstraktı hiçbir bağırsak bakterisini tamamen baskılamamıştır. Bununla birlikte, ekstrakt *Salmonella* Typhimurium ve *Staphylococcus aureus* üzerine 75 µg/mL'den, *Escherichia coli* ve *Fusobacterium nucleatum* üzerine 150 µg/mL'den ve *Clostridium perfringens* üzerine 300 µg/mL'den başlayan konsantrasyonlarda bir potansiyel baskılayıcı aktivite göstermiştir (P<0,05). Yerleşik bakterilerin ise ekstrakta patojenlerden daha az duyarlı olduğu gözlenmiştir. Ekstrakt, *Bifidobacterium bifidum*'un üremesini 75 µg/mL dozdan başlayarak ılımlı bir şekilde uyarmıştır (P<0,05). Ekstrakt, *Lactobacillus acidophilus* üzerinde herhangi bir aktivite göstermemiştir. *Bifidobacterium infantis* ve *Lactobacillus casei* için 600-2400 µg/mL doz aralığında potansiyel bir inhibitör aktivite gözlenmiştir (P<0,05). *Pinus brutia* kabuğu ekstraktının, 75-300 µg/mL doz aralığında bağırsaklardaki yerleşik veya faydalı bakterileri korurken patojenik bakterileri kısıtlama potansiyeline sahip olduğu sonucuna varılmıştır. Belirtilen etkiler başlıca, ekstraktın polifenolik içeriği ile ilişkilendirilebilir.

INTRODUCTION

Commensal microflora in the gastrointestinal tract has various physiological activities almost equal to a virtual organ. Intestinal microflora acts like a metabolic reactor, fermenting non-digestible dietary residue, turning them into short-chain fatty acids which are absorbable energy substrates for the host. Three main short chain fatty acids (acetate, propionate, and butyrate) also stimulate proliferation and differentiation of the intestinal epithelial cells (1). Another important physiological activity of the intestinal bacteria is to form a defensive barrier against to invasion of intestinal epithelium by exogenous microorganisms. Germ-free animals were reported to be very susceptible to infections (2). Gastrointestinal tract also host opportunistic pathogens, but they have restricted growth when there is an equilibrium between species of resident bacteria.

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The indiscriminate use of antibiotics can disrupt the microbial balance in the intestines and cause the overgrowth of pathogenic species which are manifested as different intestinal disorders such as irritable bowel syndrome, pseudomembranous colitis, Crohn's disease, and colon cancer (3, 4). The faecal numbers of *Faecalibacterium prausnitzii* and *Bifidobacterium adolescentis* from intestinal commensals were lower and *Ruminococcus gnavus* higher in patients with Crohn's disease than in healthy relatives (5). Thus, many studies have focused on the new alternative antimicrobial agents that protect commensal or beneficial intestinal bacteria while affecting pathogenic ones (6-8). Plant extracts and secondary plant metabolites have been reported as a possible natural treatment option for diseases caused by bacteria (9).

Pinus brutia Ten. (Turkish red pine) is naturally grown in the Mediterranean, Aegean and Black Sea regions of Turkey (10). The bark of this species is used in order to produce timber in our country. The remnants of the trees after timber production are not much in use, and therefore have a big potential as a waste material (11). Galactoglucomannan oligosaccharides which were extracted from pine wood increased growth performance, villus height and villus surface area, and decreased *Salmonella typhimurium* colonization in the intestines of broiler chicks (12). The bark of *P. brutia* is also rich in antimicrobial phenolic compounds or polyphenolics, i.e. flavonoids and phenolic acids which are particularly monomers of tannins (11, 13). Various studies indicated that plant extracts rich in polyphenolics had a potential to restrict intestinal pathogens (14), and to enhance growth of beneficial cultures (15). There are also reports that extracts from the bark of *P. brutia* had antimicrobial activities on several bacteria and fungi species (16, 17). However, the effects of *P. brutia* bark extract on intestinal bacteria particularly on the commensal ones have not been previously reported. Therefore, the aim of the present study was to investigate the influence of *P. brutia* bark extract on some commensal and pathogenic bacteria from the intestinal microflora.

MATERIAL and METHODS

Pinus brutia bark extract

The extract of *P. brutia* bark was provided by Kale Naturel Herbal Products Company, Ltd., Balıkesir, Turkey. As specified by the manufacturer, air-dried, ground and screened bark samples (powder) were extracted by distilled water with solid/liquid ratio of 1/10 for 6 h at 55°C and filtered to give homogenous liquid. The extract concentrated to a solids concentration of 20% in a rotary vacuum evaporator and dried with a spray-dryer.

Analyses of phenolic compounds of *P. brutia* bark extract

Phenolic compounds (Table 1) of *P. brutia* bark extract were quantified using a high-performance liquid chromatography (HPLC) (Shimadzu) device equipped with a photodiode array detector. An Agilent Eclipse XDB-C18 (250 × 4.60 mm) 5 µm column at 30ºC and 0.8 mL/min flow speed was used.

Table 1. Phenolic compounds of *P. brutia* bark extract

Phenolic compounds	µg/g
Gallic acid	2.2
Protocatechuic acid	1.4
Catechin	6.4
P-hydroxy benzoic acid	0.9
Caffeic acid	1.2
Epicatechin	5.8
Vanilin	0.4
P-coumaric acid	0.2
Ferulic acid	0.2
Quercetin	17.7
Luteolin	0.2
Kaempferol	0.2
Apigenin	0.3

Bacterial strains and culture conditions

Bifidobacterium bifidum ATCC 29521, *Bifidobacterium longum* subsp. *infantis* ATCC 15697, *Lactobacillus acidophilus* ATCC 4356, and *Lactobacillus casei* ATCC 393 were used as commensal bacterial species in the tests. Pathogenic bacterial species tested were *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586,

Table 2. 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 $MgSO_4 \cdot 7H_2O$ (Sigma 230391), and 0.6 g/L $CaCl_2$ (Sigma C1016).

Clostridium perfringens ATCC 13124, *Staphylococcus aureus* subsp. *aureus* ATCC 12600, *Escherichia coli* ATCC 11775, and *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 29630.

The media used to culture different intestinal strains were as follows: *B. bifidum*, Mann Rogosa Sharpe (MRS) broth with 0.05% cysteine (MRS-C); *L. acidophilus* and *L. casei,* MRS broth; *E. coli* and *S*. Typhimurium, Luria–Bertani (LB) medium; *S. aureus,* tryptic soy broth (TSB); and *B. infantis, C. perfringens*, and *F. nucleatum,* liquid form of medium 2 (18). Medium 2 was prepared under CO_2, as previously described (18) with only slight modification. Trypticase peptone was used instead of casitone in medium 2 (Table 2). Ruminal fluid which was used as a component of the anaerobic media brought from the slaughterhouse, 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 media (Table 2). *Escherichia coli, S*. Typhimurium, and *S. aureus* were grown aerobically at 37°C for 24 h. All others were grown at 37°C for 24 h under an atmosphere of 80% N_2, 10% CO_2, and 10% H_2 in an anaerobic cabinet (Whitley DG250, Don Whitley, West Yorkshire, UK).

(100 mg/mL) was prepared dissolving extract in 50% (v/v) ethanol. Dilutions of extract (2400, 1200, 600, 300, 150, 75, 37.5, 18.8, 9.4 and 4.7 µg/mL) were made from the stock solution in the bacterial strain specific growth media. For broth microdilution, 200 µl of each dilution was distributed over a 96-well plate (Flat bottom, Corning 3599). A 20 µl of inoculum which compromising 4×10^{10} cell/mL overnight bacterial culture were transferred into each well. Each strain was tested in triplicate wells. At the same time, negative control wells without extract and media control wells without bacteria were maintained for each set. Plates were incubated for 24 h at 37°C in the anaerobic cabinet and in an incubator for *E. coli, S*. Typhimurium, and *S. aureus*. Bacterial growth was detected with a microplate reader at 600 nm (Epoch, BioTek, USA). A significantly lower OD600 value compared to control dose (0 µg/mL) was accepted as potential antibacterial activity (20) while significantly higher value was accepted as stimulatory effect (21).

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 value of $P<0.05$ was taken to indicate a significant difference.

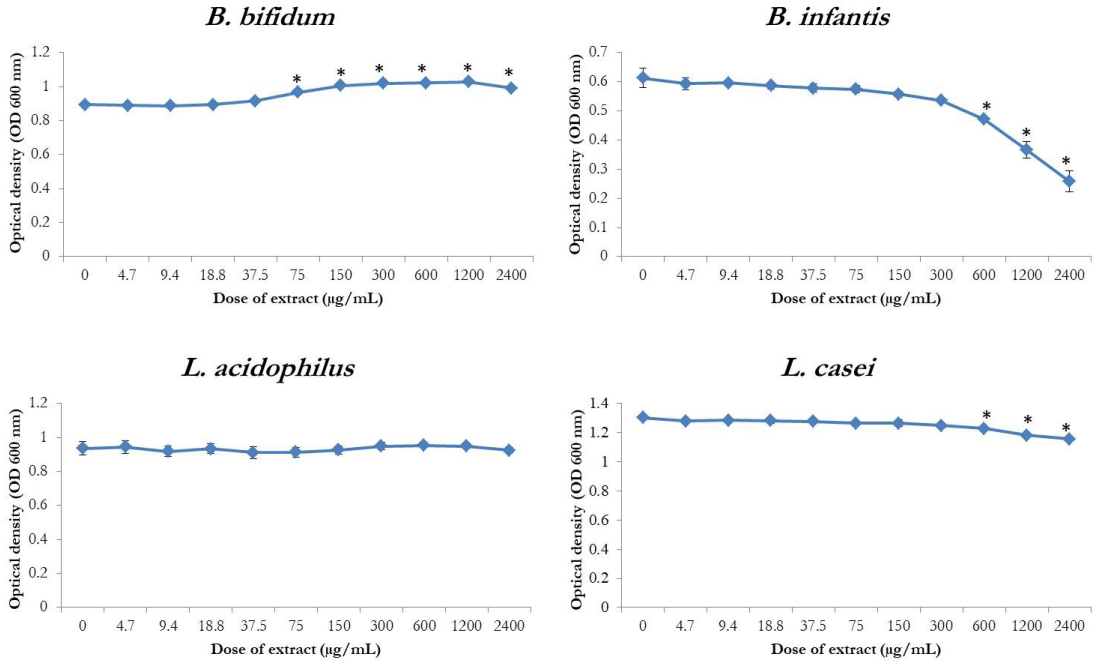

Figure 1. Effects of *P. brutia* bark extract on commensal intestinal bacteria. The results represent the mean ± standard error. * indicates the difference of the treatments compared with the control (0 µg/mL) ($P < 0.05$).

Determination of the influence of *P. brutia* bark extract on bacterial growth

The influence of *P. brutia* bark extract on the growth of intestinal bacterial strains was tested by a broth dilution method on 96-well plates in the anaerobic cabinet (19). Tests for *E. coli, S*. Typhimurium, and *S. aureus* were performed in a laminar flow box. Stock solution of *P. brutia* bark extract

RESULTS

Effects of *P. brutia* bark extract on intestinal bacteria are presented in Figure 1 and Figure 2. *Pinus brutia* bark extract did not completely inhibit any intestinal bacteria. However, the extract showed a potential inhibitor activity on *S*. Typhimurium and *S. aureus* from 75 µg/mL, on *E. coli* and *F. nucleatum* from 150 µg/mL, and on *C. perfringens* from 300

µg/mL concentrations (P<0.05). Commensal bacteria, on the other hand, were observed to be less sensitive to the extract than those of the pathogenic strains. The extract stimulated moderately the growth of *B. bifidum* from 75 µg/mL dose (P<0.05). The extract did not show any activity on *L. acidophilus*. A potential inhibitor activity was observed for *B. infantis* and *L. casei* at 600-2400 µg/mL dose range (P<0.05).

DISCUSSION

The gut is a natural habitat composed of several bacterial communities that are in a dynamic relationship with each other and the host. Commensal bacteria compete with pathogenic species for substrates and binding sites on the intestinal epithelium. Antibiotic treatment decreases the diversity of commensal microflora and leads to expansion of the intestinal pathogens like *C. difficile* and *S.* Typhimurium which cause colitis and gastro enteritis (22). Hence, not to inhibit or even stimulate beneficial bacteria in the intestines during antibacterial treatments is of great importance in terms of health and physiology of both humans and animals.

Salmonella Typhimurium, *S. aureus*, *C. perfringens,* and *E. coli* are among the most prevalent causes of foodborne infections and gastroenteritis (23-25). In the present study, *P. brutia* bark extract exhibited a potential antibacterial action on these species at various doses starting from the smallest dose of 75 µg/mL, although the extract did not completely inhibit the bacterial growth. There is no report about the effects of this extract on intestinal bacterial species, however Dığrak et al. (16) reported that *E. coli* DM and *S. aureus* Cowan 1 were resistant to acetone and methanol extracts of *P. brutia* bark. Similarly, trunk bark extract of *P. brutia* had no inhibition on *S. aureus* and very low inhibition on *E. coli* (17). On the other hand, potential antibacterial action of *P. brutia* bark extract on intestinal pathogens in the present study might be caused by the polyphenolic compounds it contains. *Pinus brutia* bark extract used in this study contained several polyphenolic compounds mainly flavonoids and phenolic acids, some of which were monomeric units of tannins (Table 1). The main phenolic compound contained in the *P. brutia* bark extract used in this study was quercetin. It was reported that quercetin exhibited antibacterial effect on food-borne pathogens, *S. aureus* (MTCC-3160), *E. coli,* and *S.* Typhimurium (MTCC 3224) starting from the dose of 28.12 ppm (26). Catechin, epicatechin, and gallic acid which were the other dominant phenolic compounds belong to *P. brutia* bark extract in the present study were reported to inhibit strongly the growth of the same strain of *C. perfringens* (27). In that study (27), the minimal inhibitor concentrations of purified polyphenols were lower than that of plant extracts that contain them. Despite the potential antibacterial effect of the extract used in this study, the reason for its lack of inhibition may be the relatively low amount of phenolic components it contains.

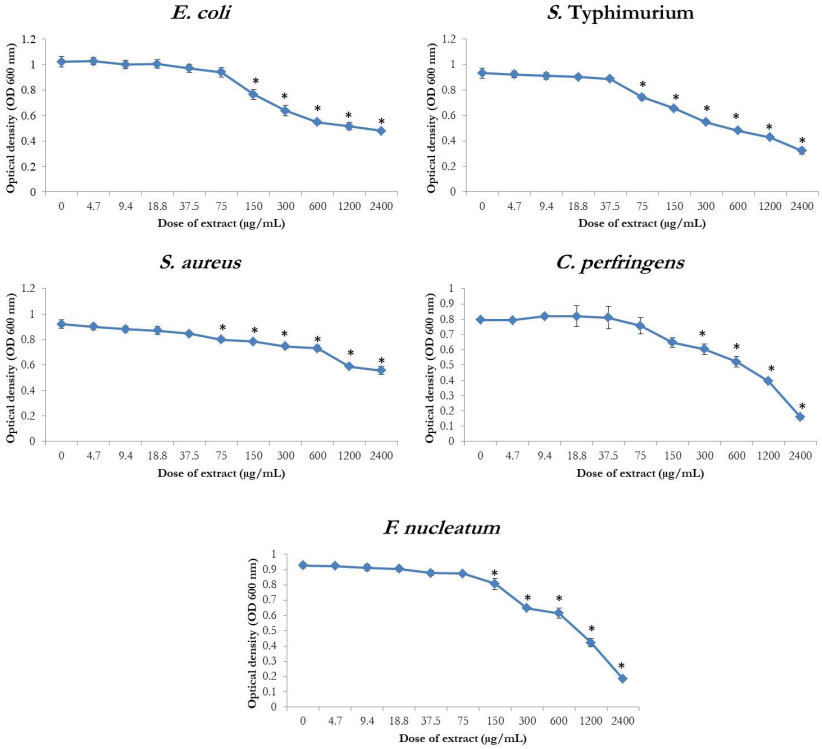

Figure 2. Effects of *P. brutia* bark extract on pathogenic intestinal bacteria. The results represent the mean ± standard error. * indicates the difference of the treatments compared with the control (0 µg/mL) (P < 0.05).

Fusobacterium nucleatum, the other enteropathogen, is associated with the stages of colorectal neoplasia development (28). *Pinus brutia* bark extract had an inhibitory potential on this bacterium from 150 µg/mL concentration**.** *P*-hydroxy benzoic acid, gallic acid, ferulic acid, caffeic acid, quercetin, catechin, epicatechin, and apigenin presented in the extract were reported to show antibacterial activity against to *F. nucleatum* (ATCC 10953) at a dose range of 62.5-2500 µg/mL (29). Polyphenolic compounds exert antibacterial activity via increasing the permeability of bacterial membranes and

decreasing cell surface charges, causing rupture or forming pores, with consequent leakage of intracellular components (30). Catechin also could chelate metals essentials as enzymatic cofactors involved in bacterial growth (31).

Bifidobacterium spp. and *Lactobacillus* spp. species are part of normal microbiota of the gastrointestinal tract and considered as beneficial bacteria with various physiological functions (32). These bacteria are also the most used species as probiotics in the manufacturing of food products (33). *Pinus brutia* bark extract did not have any adverse effect on *B. infantis* and *L. casei* up to 600 µg/mL dose in the present study. Grape seed extract which is rich in phenolic compounds such as (+)-catechin, (−)-epicatechin, and gallic acid inhibited strains of *L. casei* at some degree at the highest dose (1 mg/mL) but not at the lower doses (0.25 and 0.50 mg/L) similar to the results in this study (34). *Pinus brutia* bark extract did not affect *L. acidophilus*, the other commensal, at any doses. Gallic acid, one of the phenolic components of the extract, did not inhibit the *L. acidophilus* ATCC 4356 that was the same strain used in this study at a dose of 500 µg/mL (35). *Lactobacillus acidophilus* CECT 362 was also resistant to tea phenolic extracts, the composition of which was similar to the extract used in this study, containing caffeine; (–)-epicatechin, (–)-epicatechin gallate, (–)- epigallocatechin, (–)-epigallocatechin gallate, and gallic acid (36).

Pinus brutia bark extract, furthermore, increased the growth of *B. bifidum* which is among to commensals, up to about 15% starting from 75 µg/mL dose in the present study. Quercetin, catechin, and epicatechin were the main polyphenolic compounds found in the extract. Gwiazdowska et al. (37) reported that quercetin increased growth of *B. bifidum* NCFB 2235 up to 20% at 2, 20, and 100 µg/mL concentrations while no significant stimulation effect was observed for *Bifidobacterium adolescentis* except for the concentration of 2 µg/mL. The effect of quercetin seems to depend on both the bacterial strain and the concentration administered. Additionally, Tzounis et al. (38) stated that the polyphenols (+)-catechin and (–)epicatechin found in the diet can be utilized by faecal microflora even in the presence of preferential bacterial energy sources such as fructo-oligosaccharides and sucrose. Authors also reported that (+)-catechin supplementation (150 mg/L) enhanced significantly the growth of *Bifidobacterium* spp. Thus, the stimulating effect of the *P. brutia* bark extract on *B. bifidum* might be due to the polyphenolic compounds it contains. Many other studies also suggest that polyphenols may promote the proliferation, growth or survival of beneficial microorganisms in the gut microflora (39). Lactic acid bacteria such as *Bifidobacterium* spp. and *Lactobacillus* spp. are able to use polyphenols as a substrate (40). *Lactobacillus plantarum* strains can degrade tannic acid to gallic acid and glucose, and then use the end products to obtain energy (40). The fact that phenolic compounds enhance the consumption of nutrients such as sugars by bacteria may be the other possible mechanism for stimulatory effects of phenolic compounds (41).

CONCLUSION

Pinus brutia bark extract, at 75-300 µg/mL dose range, had a potential to restrict pathogenic bacteria in the intestines while protect commensal or beneficial ones. Specified effects might be mainly attributed to its polyphenolic content. Further *in vitro* and *in vivo* studies required on the effects of this extract on mixed cultures of intestinal bacteria to clarify its beneficial effects on the gut health.

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