Anatolian Journal of Botany
7(1): 60-69 (2023)
Research article *doi: 10.30616/ajb.1259084*

The influence of tannins purified from Eastern Mediterranean Region plants (*Pinus brutia* Ten. and *Quercus coccifera* L.) on carbon mineralization: Antimicrobial and antimutagenic evaluation

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Received : 02.03.2023
Accepted : 05.04.2023
Online : 13.04.2023

Doğu Akdeniz Bölgesi bitkilerinden (*Pinus brutia* Ten. ve *Quercus coccifera* L.) saflaştırılan tanenlerin karbon mineralizasyonu üzerindeki etkisi: Antimikrobiyal ve antimutajenik değerlendirme

Abstract: Tannins, which are polyphenols with a wide variety of quality-quantity that control the carbon and nitrogen cycle in forest ecosystems, are very interesting because of their protein binding abilities and forming a complex structure with other compounds. In this study, the purified tannin content of *Pinus brutia* Ten. and *Quercus coccifera* L., the two dominant plant species of the Eastern Mediterranean region, and the effect of these tannins on C dynamics in a forest soil (O and A horizon) were evaluated. In addition, antimicrobial effects of tannin extracts on *Bacillus subtilis*, *Staphylococcus aureus* and *Proteus mirabilis* bacteria by disc diffusion method and antimutagenic effects on *Allium cepa* root tip cells were evaluated. Total phenol (TP) and condense tannins (CT) concentrations of *P. brutia* and *Q. coccifera* leaves ranged from 0.78–1.33 µg/100mg DW and 4.68–1.35 µg/100mg DW, respectively. With the addition of tannin extract to the soils, C mineralization (27th day) was significantly reduced compared to the control group. Both *P. brutia* tannin extract (PTE) and *Q. coccifera* tannin extract (QTE) exhibited antibacterial activity in the range of 8±0.2–35±1.1 mm zone diameter by inhibiting their microbial growth against test microorganisms. In addition, tannin treatments caused a dose-dependent mitotic index decrease in onion root tip cells and a serious inhibition by showing toxic effects on mitotic division stages. As a result, our data showed that C mineralization in soil is affected by different tannin sources and these tannin extracts have significant antimicrobial activity against pathogens and cytotoxic activity in *A. cepa* root tip cells.

Key words: Carbon mineralization, tannin, antimicrobial activity, antimutagenic activity

Özet: Orman ekosistemlerinde karbon ve nitrojen döngüsünü kontrol eden çok çeşitli kalite-niceliğe sahip polifenoller olan tanenler, protein bağlama yetenekleri ve diğer bileşiklerle kompleks bir yapı oluşturmaları nedeniyle oldukça ilgi çekicidir. Bu çalışmada, Doğu Akdeniz bölgesinin iki baskın bitki türü olan *Pinus brutia* Ten. ve *Quercus coccifera* L.'nın saflaştırılmış tanen içerikleri ve bu tanenlerin bir orman toprağında (O ve A horizonu) C dinamiklerine etkisi değerlendirilmiştir. Ayrıca tanen ekstraktlarının disk difüzyon yöntemi ile *Bacillus subtilis, Staphylococcus aureus* ve *Proteus mirabilis* bakterileri üzerindeki antimikrobiyal etkileri ve *Allium cepa* kök ucu hücreleri üzerindeki antimutajenik etkileri değerlendirilmiştir. *P. brutia* ve *Q. coccifera* yapraklarının toplam fenol (TP) ve kondanse tanen (CT) konsantrasyonları sırasıyla 0.78–1.33 µg/100mg DW ve 4.68–1.35 µg/100mg DW arasında değişmiştir. Topraklara tanen ekstraktı ilavesi ile C mineralizasyonu (27. gün) kontrol grubuna göre önemli ölçüde azalmıştır. Hem *P. brutia* tanen ekstresi (PTE) hem de *Q. coccifera* tanen ekstresi (QTE), test edilen mikroorganizmalara karşı 8±0.2–35±1.1 mm zon çapı aralığında antibakteriyel aktivite sergilemiştir. Ayrıca tanen uygulamaları soğan kök ucu hücrelerinde doza bağımlı mitotik indeks azalmasına ve mitotik bölünme evrelerinde toksik etki göstererek ciddi bir inhibisyona neden olmuştur. Sonuç olarak, elde edilen veriler topraktaki C mineralizasyonunun farklı tanen kaynaklarından etkilendiğini ve bu tanen ekstraktlarının patojenlere karşı önemli antimikrobiyal aktiviteye ve *A. cepa* kök ucu hücrelerinde sitotoksik aktiviteye sahip olduğunu göstermiştir.

Anahtar Kelimeler: Karbon mineralizasyonu, tanen, antimikrobial aktivite, antimutajenik aktivite

Citation: Ulusu F, Darıcı C (2023). The influence of tannins purified from Eastern Mediterranean Region plants (*Pinus brutia* Ten. and *Quercus coccifera* L.) on carbon mineralization: Antimicrobial and antimutagenic evaluation. Anatolian Journal of Botany 7(1): 60-69.

1. Introduction

Tannins (condensed-CT and hydrolyzable-HT), which are from polyphenolic plant secondary compounds, affect many ecosystem processes such as soil formation, organic matter mineralization, humus formation, carbon and nitrogen cycle, and they are common in plant flora (Norris et al., 2011; Ingold et al., 2021). Tannins, which constitute an essential part of the carbon pools of the forest ecosystem, are found in different proportions (up to 40%) in the bark, wood, fruit and leaves of the plants. This ratio varies depending on the plant species, environmental factors (precipitation, temperature, salt or water stress, etc.), presence of infection, extraction method-time (soxhlet, maceration, etc.) and solvent used (Kraus et al., 2003; Abilleira et al., 2021). In woody species, tannin concentrations in the leaf are usually between 150 and 250 mg/g DW (Kraus et al., 2004a). Especially the O and A horizons of forest soils with plant communities rich in tannins (such as pine, oak, acacia) have a large amount of

plant-derived tannin content and they meet about 90% of the world tannin production (Pizzi, 2003).

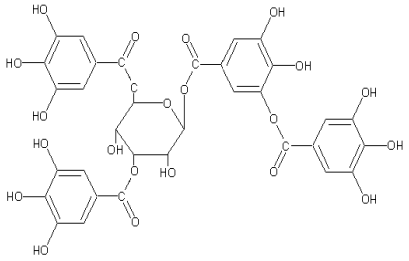

Figure 1. Molecular structure of tannic acid

Soil, which is the main source of organic and inorganic compounds among all ecosystem components, carbon and nitrogen mineralizations occurring in its structure changes over time under the influence of biotic/abiotic factors (Lal, 2002). The microbiological group, which will contribute to the nutrient mobilization required for the life cycle on Earth (Treseder and Lennon, 2015; Bardgett, 2016), has various enzymatic mechanisms for decomposition (Waldrop and Firestone, 2004; Shah et al., 2016). However, soil physical properties (texture, structure, pore size, etc.) and organic materials (plant-animal residues, secondary metabolites) contribute to the relationship between the microbial composition and nutrient cycle in the soil from different perspectives (Cai et al., 2016; Zhang and Marschner, 2017). Tannins, which are a secondary metabolite, can affect the nutrient cycle in terms of edaph by creating toxicity and inhibiting enzyme activities in microorganisms. For example, for tannins, it has been stated that NO_3, which is the source of N that plants can absorb, reduces mycotoxin production in order to reduce the loss in soil, thereby inhibiting nitrification and thus suppressing nitrogen mineralization (Zhang and Laanbroek, 2018; Peng et al., 2018; Elrys et al., 2019). Again, tannins added in the presence of cellulose in the soil showed a negative effect on carbon mineralization by reducing cumulative soil respiration (Madritch et al., 2007). Therefore, tannins help to reduce nutrient loss by slowing down the rapid transition of compounds from organic to inorganic form in the soil. Tannins, which are environmentally friendly secondary metabolites, have an important place in the field of pharmacology due to their many therapeutic properties (anticancer, antioxidant, antimicrobial, etc.) (Gomes de Melo et al., 2010; Ekambaram et al., 2016).

P. brutia (Turkish pine, Pinaceae) and *Q. coccifera* (Kermes oak, Fagaceae) are two important species distributed in the Eastern Mediterranean region of Turkey. In both species, they are used for the production of wood, tannin, seeds and resin, as well as preventing soil erosion (Petins et al., 2021; Abilleira et al., 2021; Jaramillo et al., 2022; Ghazghazi et al., 2022). Valuable secondary metabolites (monoterpenes, tannins, flavonoids, lignans and saponins) in *Pinus* and *Quercus* genera (Ito et al., 2002; Sakar et al., 2005; Sancho-Knapik et al., 2017; Kanchan et al., 2020) provide the use of these plant extracts for phytotherapy purposes (treatment of diabetes, diarrhea, hyperpigmentation and wounds) (Bulut et al., 2017). In addition, the antioxidant (Makhlouf et al., 2018; Zhang et al., 2021), anti-inflammatory (Alizade Naini et al., 2021; Kuo et al.,2021; Yang et al., 2021), antibacterial (Semwal et al., 2018; Mitić et al., 2019; Elkady et al., 2021) and antidiabetic (Zulfqar et al., 2020) effects of *Pinus* and *Quercus* extracts reflect the pharmacological properties of these plants. In recent years, the preference of compounds of natural origin in food additives due to the harm they cause to humans and nature has made medicinal-aromatic plants focus of attention in terms of gastronomy, pharmacology and medicine and has led to an increase in research in this direction.

The majority of tannins, which constitute a significant part of the compounds in the dead plant material that constituent the soil organic matter, originate from the leaves (Qualls and Bridgham, 2005). Therefore, the leaves of forest trees selected as tannin source during the research period were preferred. Antimicrobial activities and antimutagenic effects of tannins have been demonstrated in some previous studies. In this study, it was tried to determine the level of tannins purified from *P. brutia* and *Q. coccifera* grown in the Eastern Mediterranean Region in terms of these effects. Thus, determining the effect of low and high doses of tannins mixed in the soil on carbon mineralization will help to better understand the function of these polyphenols in nature, and also to support the pharmacological importance of these environmentally friendly compounds as an alternative to synthetic inputs.

2. Materials and Method

2.1. Site, plant and soil descriptions

The research area was chosen from Cukurova University Balcalı campus (18.024 da) located in the Eastern Mediterranean Region (Adana, Turkey). The local climate is a subtropical humid Mediterranean climate type with an annual average temperature of 18.7 °C, annual average precipitation 668.7 mm, and annual average humidity of 66% (Adana meteorological station, Adana Meteorology Bureau, 2021) (Fig. 2). The vegetation in the natural habitats of the campus is concentrated on the Pliocene clay deposits and conglomeratic series. The physicochemical properties of the soil (0-10 cm deep) are shown in Table 1. The samples were obtained from areas that best represent the plant communities (*Q. coccifera* and *P. brutia*) and are protected as much as possible from natural and human destruction (Fig. 3). The dominant and characteristic plant species of this region is *Q. coccifera*.

2.2. Physicochemical analysis of plant and soil samples

Soil samples were taken from the rhizosphere of the plant samples (0-10 cm deep). Soil samples were air-dried and sieved (<2mm) for physical and chemical analysis.

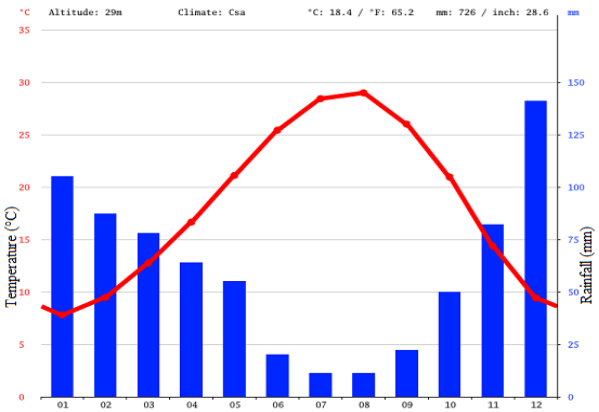

Figure 2. Climate diagram of Adana, Turkey (https://en.climate-data.org/)

Figure 3. *Pinus brutia* (Station 1) (A) and *Quercus coccifera* (Station 2) (B) populations

Leaf samples were cut from parts of the plants up to 1.5 m high to include young shoots and perennial branches, and the dried leaves were ground into powder with a blender. All samples were stored at +4 °C until analysis.

Soil texture was determined by Bouyoucos hydrometer (Bouyoucos, 1951), soil pH was measured at a soil-to-water ratio of 1:1 (w/v) with a pH meter, $CaCO_3$ content (%) by a Scheibler calcimeter, field capacity (%) was determined by vacuum pump. Total nitrogen (%N) and organic carbon (%C) contents of dried and ground plant and sieved soil samples were determined by Kjeldahl method and Anne method (Walkley and Black, 1934).

2.3. Quantitative phytochemical analysis

2.3.1. Tannin extraction

Tannin extract was performed with minor modifications to the procedure described by Makkar (2003). This tannin extract was used in all quantitative assay. For the tannin extract, the ground leaf samples (100 mg) were extracted with cold acetone (70%) in a magnetic stirrer (12 h). After, the extract was twice kept in an ultrasonic homogenizer mixer for 5 min. The extracted samples were centrifuged (3000 rpm, 15 min, 4 °C) and supernatant was stored at +4 ˚C for analysis.

2.3.2. Determination of total phenolic content

Total soluble phenols (TP) in the extract were determined by Folin-Ciocalteu method (Makkar, 2003). In this method, different concentrations of tannic acid (20–80 µg/mL) were used as standard phenolic compound. 500 µL of Folin-Ciocalteu reagent is added to 50 µl of extract in a test tube. After 3 min, 2500 µL of 2% (w/v) Na_2CO_3 were added and mixed well. The final volume was made up to 4 mL with dH_2O. The solution was incubated for 1 h under dark room conditions and measured at 725 nm (T60 UV Visible Spectrophotometer). TP was determined using the calibration curve of tannic acid standard ($y = 0{,}0819x+0{,}1112$ $R^2 = 0{,}9911$).

2.3.3. Determination of total tannins

Polyvinylpolypyrrolidone (PVPP) was used to separate tannin phenols from non-tannin phenols, condensed tannins (CT) were determined by the butanol-HCl-iron reagents (Makkar, 2003). 2 mL tannin extract and 2 mL dH_2O were added to 200 mg of PVPP (tannins precipitate with PVPP) into a test tube. The mixture was vortexed, then kept in cold water for 15 min, again vortexed and centrifuged (3000 rpm, 15 min, 4 °C). Supernatant was stored at +4 ˚C for analysis. The extracts were prepared as in the total phenol content determination and measured at 725 nm. Total tannins (TT) were calculated as the difference between TP and non-tannin phenols (NTP). TP and TT were expressed as tannic acid equivalent (TAE).

2.3.4. Determination of condensed tannins

250 µl of tannin extract, 1.5 mL of Butanol-HCl reagent (95:5 v/v) and 50 µl of Fe ($FeCl_3$ solution) were added to a test tube and vortexed. The test tube was kept in a water bath at 97-100 °C for 1 h. Then the absorbance of solution was measured spectrophotometrically at 550 nm (Bate-Smith, 1975).

2.4. Measurement of the carbon mineralization

Moist soil samples (80 g–80% field capacity) and 40 mL saturated $Ba(OH)_2$ (as an alkaline trap) were placed in 0.75 L incubation vessels for aerobic incubation. Incubation vessels were closed and incubated at 28 °C for 30 d. Empty vessels were used as blanks. The trap was titrated with $C_2H_2O_4$ every 3 d after the precipitation of the carbonates and the CO_2 resulting from microbial respiration was measured. In order to determine the effect of grain on C mineralization, after *Pinus* and *Quercus* soils were prepared as described above, the first day *P. brutia* tannin extract (PTE) (0.8 µg) and *Q. coccifera* tannin extract (QTE) (1.27 µg) were separately added to the soils and incubated (the amount of tannin was determined in accordance with their content). On the 11th and 23rd d of incubation, 2.5 times the first dose of tannin was added to both soil groups and incubation was continued. The effect of tannin on carbon mineralization was calculated separately for a total of 39 days and compared with the control. Cumulative CO_2 production throughout the incubation (39 d) was determined according to a nonlinear equation. First order kinetic model was used for organic carbon mineralization (Bernal et al., 1998).

$C_m=C_o(1-e^{-Kt})$

$C_{m:}$ The mineralised carbon (%C) at time *t* (days)

C_o: The potentially mineralisable C (%C)

K: The C mineralization rate constant (days^{-1})

2.5. Antibacterial activity of tannin extracts

Tannin extracts were tested against the Gram-positive bacteria strains *Bacillus subtilis* (ATCC® 6633), *Staphylococcus aureus* (ATCC® 29213), and the Gram-negative *Proteus mirabilis* (ATCC® 25933) bacteria strain by disc diffusion assay. Fresh bacterial cultures grown overnight were seeded onto Muller-Hinton Agar (MHA) (20 mL) plate medium using a sterile cotton swab. Sterile discs (6 mm in diameter) loaded separately with different concentrations of tannin extract (10-75 µl) were placed on MHA plates inoculated with bacteria strains from fresh culture (1.5 x 10^8 CFU/mL). Acetone (70%) was used as negative control. The inoculated plates were incubated at 37 °C for 24 h and the diameters of the inhibition zones (mm) were measured after incubation (Bauer et al., 1966).

2.6. Onion root sprouting, tannin extract treatment and preparation of mitotic phases from root apical meristem cells

Onion (*A. cepa*) root apical meristems (48 h aged and 2-3 cm length) were used to determine the cyto-genotoxic potential of tannin extracts. Mitotic abnormalities and chromosome morphology were analyzed to evaluate the cyto-genotoxic effects induced by tannin extracts on onion

root tip cells. For this purpose, onion bulbs of similar size were first rooted in distilled water (25–27°C) and after the roots reached a length of approximately 1.5-2 cm, the onion roots were treated with *Pinus* and *Quercus* leaf tannin extracts at different concentrations (1, 2, 4%) for 20 h. Then the roots were fixed in carnoy's fixative (ethyl alcohol:glacial acetic acid (3:1 v/v). The control group was kept in distilled water simultaneously with the treatment groups and their bulbs (1.5-2 cm) were fixed directly in carnoy's fixative. All groups were removed from carnoy's fixative after treatment and maintained in ethyl alcohol (80%). Preparations were prepared according to Feulgen's squash technique (Rencüzogulları et al., 2001). The prepared preparations were examined under a bright-field light microscope to observe and score cellular abnormality.

2.7. Statistical analysis

All experimental results were done in triplicate (n=3). The significant differences between groups were performed via the one-way analysis of variance (ANOVA) and Tukey HSD test. Statistical analyzes were performed with the IBM SPSS Statistics Version 24 package program. $p < 0.05$ was considered as statistically significant.

A clear and complete information should be provided about the materials, and the procedures followed.

3. Results and Discussion

3.1. Plant leaves and soil physiochemical properties and tannin contents

Physicochemical properties of plant leaves and soil samples shows Table 1. Both soil samples are of the same color (brown-red) and have a sandy loam texture. The pH of soils is slightly basic and lime-free and there is no significant difference between them ($p < 0.05$). Field capacity of *P. brutia* and *Q. coccifera* soils were 26.23% and 28.34%, respectively. Although the highest C/N ratio in both leaf and soil was determined in *P. brutia* soil at 52.37 and 15.67, respectively, the highest nitrogen content was observed in *Q. coccifera* soil at 1.34% and 0.26%, respectively. Also, although the highest TT content (40 µg/100mg) was determined in *Q. coccifera* leaves, CT content was higher in *P. brutia* leaves (4.68 µg TAE/100mg) (Table 1). Crop production inputs are the main source of carbon and nitrogen in soils (Updegraff et al., 1995; Bridgham et al., 1998). The C/N ratio is significantly affected by the diversity of tree species, plant litter, roots and microbial communities in the research areas (Landesman and Dighton, 2010). Differences in quality and quantity of aboveground and underground inputs transferred from tree species to soil have indirect effects on soil pH, C/N ratio, organic matter and tannin content (Lovieno et al., 2010). In a study, changing C/N ratio, organic matter and pH were observed in the soil with different tree species (*Mytilaria laosensis* and *Cunninghamia lanceolata*) (Wan et al., 2015). Leaf nutrient content, which is a very important component in the nutrient cycle, is affected by various edaphic factors (physicochemical structure and nutrient content of the soil, moisture, temperature, microorganisms, etc.) (Liu et al., 2017; Wu et al., 2023). In our analyzes, the effectiveness of edaphic factors on plants was once again demonstrated by the consistency of C and N ratios obtained in soil and leaves on a species basis.

Tannins, which contribute to the nutrient cycle by affecting the organic matter degradation, mineralization, carbon and nitrogen ratios in the soil (Kraus et al., 2003), constitute an important part of terrestrial biomass carbon (Hernes and Hedges, 2000). The highly variable tannin concentrations in plants are associated with a large amount of genetic and environmental variation (Northup et al., 1995; Siemens et al., 2002). Even in different parts of a plant such as leave, root, stem, fruit, there are different tannin concentrations (Kraus et al., 2004b). In this study, tannin production in plants also differed according to species. In previous studies, *P. muricata* (198 mg CE/g), *P. radiata* (93.7 mg CE/g), *P. rijida* (70.4 mg CE/g), *P. densiflora* (19.6 mg CE/g) (Ku et al., 2007), *P. pinaster* (5.15 mg CE/g) (Chupin et al., 2013), *Q. incana* (38.96-46.85 mg CE/g) (Makkar and Singh, 1991) condensed tannin and *Q. robur* (44-57 mg TAE/g) (Gonzalez-Hernandez et al., 2003) total tannin contents were determined in different pine and oak species, which is consistent with our results.

3.2. Carbon mineralization in soils of tannin extract treatment

A significant decrease in C mineralization was observed from the first day in both soils with added tannin compared to the control group and in both treatment groups, this microbial activity is very close and parallel to each other ($p<0.01$). It can be said that C mineralization in *Q. coccifera* soil was slightly higher than *P. brutia* soil among the control groups. This can be explained by the *Q. coccifera* organic matter being more suitable for soil microorganisms. Although the acceleration in the carbon mineralization of the control and treatment groups continued periodically until the 23rd day, a serious slowdown was observed in the carbon mineralization rate after the last tannin addition to the treatment soils and even after the 27th day, the mineralization activity tended to stop (Figs. 4, 5). The significant difference in carbon mineralization between control and application soils clearly

Table 1. Physicochemical properties of plant leaves and soil samples (0–10 cm deep)

Plant material	Physicochemical properties												Tannin contents of leaves (µg TAE/100mg)		
	Soil									Leave					
	Texture (%)														
	Sand	Silt	Clay	Field capacity (%)	pH	$CaCO_3$ (%)	C%	N%	C/N	C%	N%	C/N	Total tannin	TP	CT
Pinus brutia	57.93 ±0.09	27.13 ±0.13	14.93 ±0.04	26.23 ±0.77	7.80 ±0.05^{a}	1.45 ±0.75^{a}	22.56 ±0.43^{a}	0.14 ±0.00^{b}	15.67 ±0.59^{a}	52.86 ±0.31^{a}	1.01 ±0.02^{b}	52.37 ±0.74^{a}	29 ±0.72^{b}	0.78 ±0.0^{b}	4.68 ±0.12^{a}
Quercus coccifera	50.35 ±0.27	13.20 ±0.50	36.43 ±0.28	28.34 ±1.36	7.26 ±0.02^{b}	0.26 ±0.18^{b}	23.55 ±0.77^{a}	0.26 ±0.00^{a}	8.97 ±0.34^{b}	50.94 ±0.41^{b}	1.34 ±0.02^{a}	38.04 ±0.86^{b}	40 ±0.81^{a}	1.33 ±0.04^{a}	1.35 ±0.03^{b}

Note: The experiments were conducted in triplicate independently (n= 3), and the data are expressed as the means ± standard error (SE) with p<0.05

shows the antimicrobial effect of tannin on soil microorganisms (Halvorson and Gonzalez, 2008; Adamczyk et al., 2013). In addition, the decrease in C mineralization in the control groups in the last days of the experiment may be associated with the presence of more difficult to decompose or resistant organic materials in the environment. Fallen leaves to the soil are one of the main sources of primary (carbohydrate, protein, oil), secondary metabolites (polyphenols, alkaloids, etc.) and inorganic compounds. Since carbohydrates and proteins are generally susceptible to microbial degradation, they can quickly become involved in the nutrient cycle (Weiss and Simon, 1999). But, tannins, a class of polyphenols, can affect the biogeochemical cycle in ecosystems by limiting microbial activity (Kuiters, 1990; Kraus et al., 2003). Again, tannins added to the soil may be involved in ionization and oxidation reactions that generate some reactive substances such as phenolate ions and quinones (Rimmer, 2006). However, tannins can polymerize (with reversible ionic or irreversible covalent bonds) the C and N in the soil solution, turning them into more difficult to break down, stubborn substances or heavier molecules (Zibilske and Bradford, 2007). Thus, tannins can help prevent the loss of available C and N and extend their residence time in soil. This interaction appears as an important natural ecological cycle

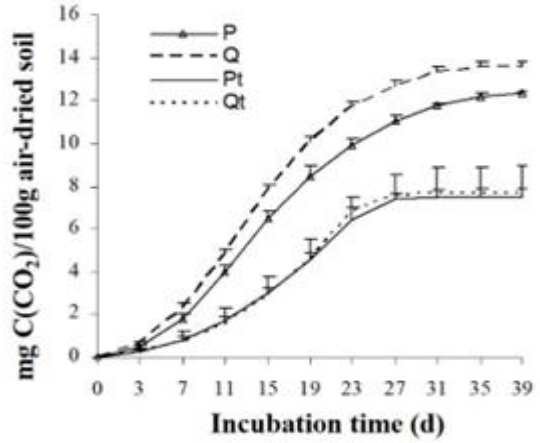

Figure 4. Cumulative C mineralized of soils during 39 days at 28ºC (mean ± standard error, mg $C(CO_2)$/100g oven-dried soil, n=3), P: *P. brutia* soil without treatment, Q: *Q. coccifera* soil without treatment, Pt: *P. brutia* soil with tannin added, Qt: *Q. coccifera* soil with tannin added

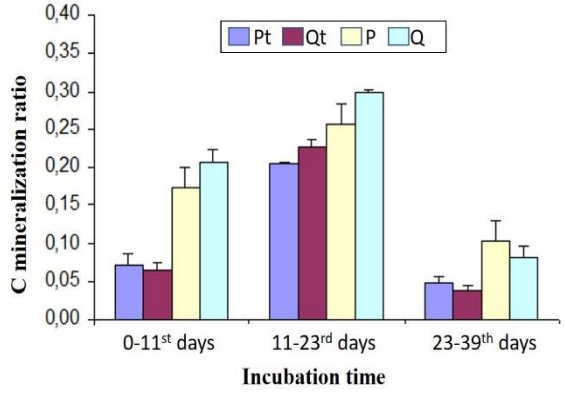

Figure 5. Soil carbon mineralization rates (mean ± standard error, n=3), P: *P. brutia* soil without treatment, Q: *Q. coccifera* soil without treatment, Pt: *P. brutia* soil with tannin added, Qt: *Q. coccifera* soil with tannin added

to preserve soil identity in the longer term by preventing rapid organic matter loss in the soil.

3.3. Antibacterial effect of tannin extracts

The antimicrobial potentials of PTE and QTE were evaluated against Gram-positive (*B. subtilis*, *S. aureus*) and Gram-negative bacteria (*P. mirabilis*) by disc diffusion method. Zones of inhibition (ZOI) exhibited by tannin extracts at different concentrations (10, 30, 45, 60 75 µl) and negative control (70% acetone) against each bacterial strain are shown in the Table 2 and Figure 6. ZOI (mm) indicates that microorganisms could not proliferate around the sample-loaded disc. The ZOIs of PTE and QTE were larger and clearer compared to the control. Antibacterial potentials of tannin extracts were dose dependent against *B. subtilis*, *S. aureus* and *P. mirabilis*. PTE and QTE applied to pathogenic microorganisms at different concentrations had the potential to inhibit their microbial growth at varying rates. The microorganism most affected by both tannin extracts was *S. aureus* and the most resistant microorganism was *B. subtilis*.

It is a frequently encountered and sometimes inevitable fact that different secondary metabolites are included in the extract along with the target molecule during the extraction stage (Jones and Kinghorn, 2012). The fact that PTE is the most effective inhibitory extract against the tested microorganisms can be explained by the species-specific composition of the *Pinus* tannin, as well as the inclusion of other phytochemicals in the extract along with tannin during the extraction. The variety and concentration of phytochemicals of different plant species cause variable inhibitory effects on each microorganism (Nouri et al., 2014; AlSheikh et al., 2020). The difference in ZOIs obtained in the study is due to both the extract concentration and the varying degrees of antibacterial properties of the species-specific phytochemicals. Overall, in the present study, PTE and QTE showed significant antimicrobial potential against the tested microorganisms. Similarly, antimicrobial properties of some *Pinus* and *Quercus* extracts have been shown against *B. subtilis*, *S. aureus* and *P. mirabilis* bacteria, supporting our results. Zone diameters of 24 mm and 20 mm were obtained by applying *Q. infectoria* ethanol extract against *B. subtilis* and *S. aureus* bacteria, respectively (Satirapathkul and Leela, 2011). In another study, *Quercus variabilis* ethanol extract exhibited 10.89mm inhibition for *S. aureus* (Zhou et al., 2019). In the literature, in terms of *Pinus* extracts, on *B. subtilis* and *S. aureus* bacteria the aqueous extract of *Pinus massoniana* formed 21.8 mm and 13.7 mm (Feng et al., 2010) zone diameters and the aqueous extract of *Pinus roxburghii* formed 22.6 mm and 21.6 mm (Kaushik et al., 2013) zone diameters, respectively. Kim et al. (2013) observed that essential oils obtained from 3 *Pinus* species (*Pinus densiflora*, *Pinus thunbergii*, *Pinus rigida*) had mild antimicrobial activity against *B. subtilis*, and the most effective essential oil for *S. aureus* belonged to *P. thunbergii*. In another study, it was stated that the essential oil of *P. roxburghaii* had moderate inhibitory activity against *S. aureus* (Hassan and Amjid, 2009). The antimicrobial properties in plants are attributed to the presence of some secondary metabolites such as tannins,

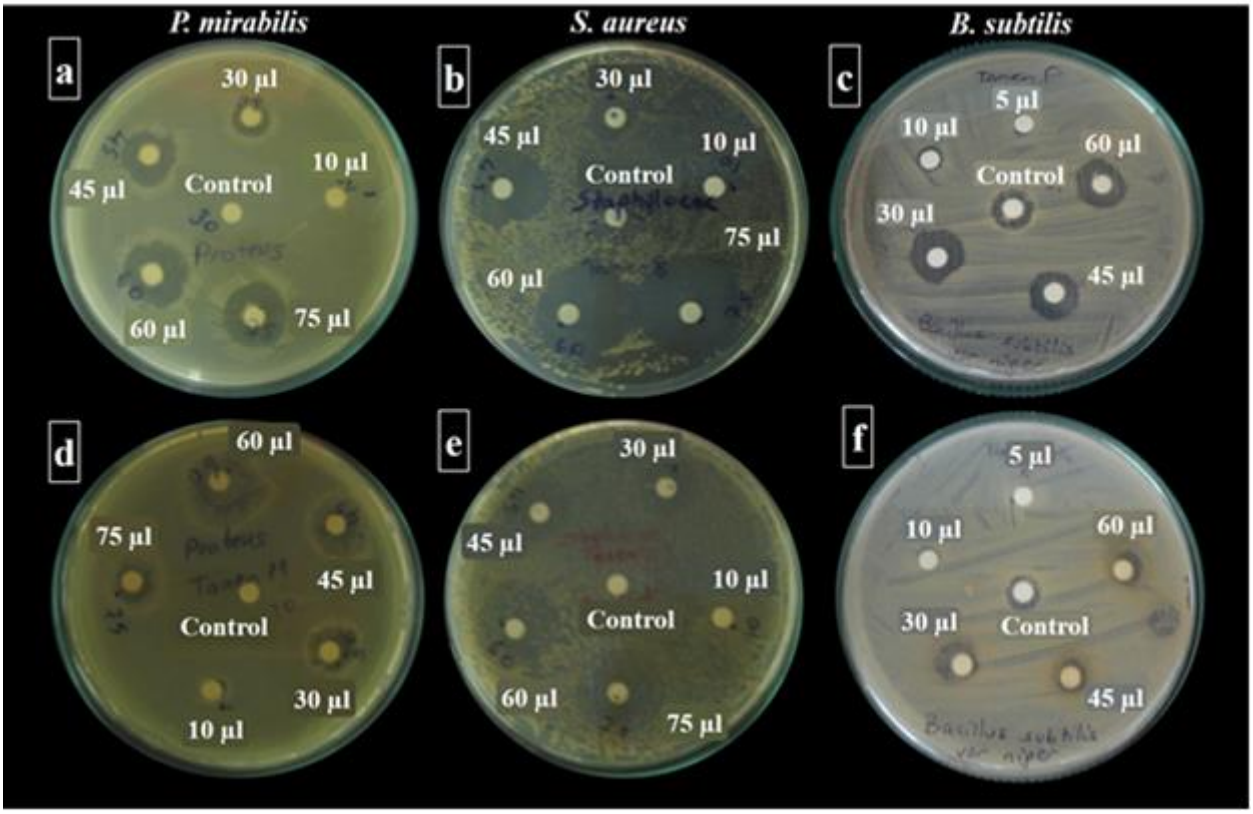

Figure 6. Antibacterial activities of (a-c) *P. brutia* tannin extract, (d-f) *C. coccifera* tannin extract, Acetone (70%) as negative control (all the experiments were performed in triplicate)

Table 2. Antibacterial activity of tannin extracts against pathogenic strains

Pathogenic bacteria	Extract	ZOI (mm)					
		Control	Tannin extract concentrations (µl)				
		Acetone (70%)	10	30	45	60	75
B. subtilis	*P. brutia*	10±0.2	NA	17±0.3	18±0.4	18±0.3	-
	Q. coccifera	7±0.4	NA	12±0.2	8±0.1	9±0.4	-
S. aureus	*P. brutia*	NA	16±0.8	17±0.5	27±0.3	30±0.4	35±1.1
	Q. coccifera	NA	8±0.2	20±0.2	24±0.5	30±0.7	31±0.9
P. mirabilis	*P. brutia*	NA	NA	14±0.4	18±0.7	21±0.6	23±0.5
	Q. coccifera	NA	NA	14±0.2	16±0.3	20±0.3	21±1.4

Note: Data are means of triplicate (n=3) ± standard error, $p<0.05$. NA (no activity)

quinones, alkaloids, phenols, flavonoids, terpenoids, glucosinolates. Tannins bind to the cell wall of bacteria and inhibit protease, thereby preventing the growth of bacteria. Thus, the ability of tannins to disrupt the metabolism and protein synthesis of microorganisms imparts important antibacterial properties to these compounds (Wafa et al., 2016; Chandra et al., 2017).

3.4. Effect of tannin extracts on mitotic index, mitotic phase frequency and abnormality of *A. cepa* root meristem cells

The mitotic index (MI), which is considered an indicator of cell cycle progression, is an important test to detect biotic or abiotic factors that pose a risk to DNA during cell divisions (Ray et al., 2013). PTE and QTE (1, 2, 4% (w/v)) were evaluated effects on mitotic phase frequency and mitotic abnormalities in onion root tip cells. MI caused by tannin extract treatments at all stages of mitosis is given in Table 3 in comparison to the control group. The obtained data show that MI in root apical meristem cells varies according to the growth period. MI (48 h) in untreated meristem cells (control) was 5.05±0.21. All doses of PTE and QTE significantly reduced MI in onion root tip cells compared to control. Dose-related decrease in MI (2.26±0.09% - 0.13±0.04%) is quite evident, especially in PTE (1-4%) treated samples. Also, both PTE and QTE significantly decreased the % rates of mitosis stages in onion root tip cells ($p<0.001$). In general, tannin extracts of both plants inhibited metaphase, anaphase and telophase at the highest dose due to toxicity. Although MI below 50% is considered a non-lethal effect (Kundu and Ray, 2017), it has been observed that tannin extract has an arresting effect on the cell cycle progression mechanism in meristematic cells and this explains the anti-proliferative activity of tannin extract in overdose. 1% solutions of PTE and QTE increased the percentage of abnormal cells by 4.35±1.32 and 6.25±1.17, respectively, compared to the control, while at the 2% concentration, this ratio regressed to 1.23±0.64 and 3.56±1.09, respectively. In addition, QTE treatment caused more abnormal cells (%) in onion root meristem cells. At the highest concentration (4%) of both tannin extracts, no abnormal cells could be detected; This may be

Table 3. The MI, the ratio of mitosis stages and abnormal cells in the root tip cells of *A. cepa* treated with tannin extracts

Treatments	Concentration (%)	Prophase (%)	Metaphase (%)	Anaphase (%)	Telophase (%)	MI (%)	abnormal cells (%)
Control		15.75±1.10	10.00±0.81	6.00±1.08	18.75±0.85	5.05±0.21	ND
PTE	1	11.00±0.44**	2.83±0.48**	1.83±0.36**	7.00±0.62***	2.26±0.09***	4.35±1.34*
	2	7.91±0.28***	1.33±0.44***	0.75±0.37**	6.75±0.39***	1.66±0.11***	1.23±0.64*
	4	1.33±0.44***	ND	ND	ND	0.13±0.04***	ND
QTE	1	11.16±0.40**	5.16±0.50*	3.50±0.55*	9.25±0.68**	2.90±0.16**	6.25±1.17*
	2	9.08±0.35***	2.66±0.51**	2.66±0.68**	6.41±0.58***	2.08±0.14***	3.56±1.09*
	4	1.83±0.66***	ND	ND	ND	0.17±0.06***	ND

*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$. ND: Not determined

associated with the inhibition caused by toxicity in the cells and the proportionally low number of cells.

In this study, it is clear that tannin extracts have a cytotoxic effect by inhibiting mitosis in onion root tip cells. Unlike other polyphenols, tannins stand out with their ability to bind to proteins, pigments, basic and large molecule compounds, and exhibit antioxidant, anticancer and antimicrobial activities (Gomes de Melo et al., 2010). It is stated that tannins provide proliferation in healthy cells and rapid closure of the wound (Antunes-Ricardo et al., 2015), as well as have a toxic effect especially against cancer cells. For example, Cuphiin D1 (CD1), the hydrolyzable tannin isolated from *Cuphea hyssopifolia* showed antiproliferative activity against HL-60 cancer cell line (Wang et al., 2000), while ellitannin isolated from *Cistus ladanifer* showed antiproliferative activity against M220, MCF-7/HER2 and JIMT-1 cancer cell lines (Barrajon-Catalan et al., 2010). The fact that bioactive molecules of plant origin, such as tannin, act in animal cells, similar to plant cells (inhibit or encourage) (Ray et al., 2013; Barman and Ray, 2022), gives an idea about the working mechanisms of these important molecules.

4. Conclusion

Tannins extracted from 2 different plant sources (*P. brutia and Q. coccifera*) caused significant differences on C mineralization in soils. This result may be due to the different resistance of the substance components used in the study against microbial decomposition. The presence of these substances resistant to decomposition in the soil prevents the rapid decomposition of organic materials of vegetable or animal origin added to the soil and provides a support in terms of soil fertility. In addition, the present study reveals the significant antimicrobial activity of PTE and QTE against gram positive and gram negative pathogens. In this respect, such extracts are among the natural agents against antibiotic-resistant microorganisms. Again, although tannin has known therapeutic potential, cyto-genotoxic effects (increased mitotic abnormality and mitotic index) induced by tannin extract on onion root tip cells were demonstrated with this study. For this reason, the indiscriminate use of medicinal and aromatic plants containing tannins should be avoided and carefully recommended for drug treatment by paying attention to the dosage. In order to determine and understand the biotic and abiotic reaction interactions between tannin and tannin-like phenolics and soil organic matter composition (humic acid, fulvic acid, glomalin, etc.), its place and effect in the nutrient cycle it should also be evaluated in terms of different plant species and thus more work is needed in this scope. *In vitro* and *in vivo* comparative studies with other pharmacological models of the Eastern Mediterranean region, which has an extremely rich biodiversity, and the purification and identification of possible molecule(s) responsible for pharmacological activity will further increase the importance of the flora of this region.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Contributions

The authors contributed equally.

Acknowledgements

Supported by Çukurova University Research Projects Unit under Project no FEF2006YL59.

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