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Antioxidant and Antibacterial Activities of Methanol Extracts from Various Plant Parts of Pomegranate and Anatolian Black Pine

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Abstract

Oxidative stress and bacterial infections threaten human and animal health. Different parts of the plants have a great potential to be used as a source of antioxidant and antibacterial agents for human or animal welfare, because of their active metabolites. This study was conducted to assess the antioxidant and antibacterial activities of methanolic extracts from the leaves, flowers, whole fruits, and woods of pomegranate (*Punica granatum* L.), and the leaves, cones, and woods of Anatolian black pine [*Pinus nigra* Arn. subsp. *pallasiana* (Lamb.) Holmboe]. Antioxidant activity was screened by DPPH and CUPRAC assays. Antimicrobial activity was examined by disc diffusion test against fish pathogens, *Aeromonas hydrophila*, *Vibrio anguillarum*, *Yersinia ruckeri* and *Lactococcus garvieae*.

Pomegranate whole fruit extract possessed superior antioxidant activity even higher than ascorbic acid. All parts of pomegranate, except wood, also exhibited significant antibacterial activity against fish pathogens. Black pine cone extract slightly inhibited the growth of fish pathogens while other pine extracts were inactive. This study reveals that the whole fruit of pomegranate is a prominent source of antioxidant and antibacterial metabolites. Cones of Anatolian black pine also seem to be a source of antibacterial compounds against fish pathogens.

Key Words: *Punica granatum*, *Pinus nigra*, *Aeromonas hydrophila*, *Vibrio anguillarum*, *Yersinia ruckeri*, *Lactococcus garvieae*

1. Introduction

Plant kingdom is the most prominent source for bioactive substances, like antioxidant and/or antimicrobial compounds. For ages, even today plant-based traditional remedies are used by various societies in the World. Up to date, a number of ethno-pharmaceuticals, nutraceuticals and cosmeceuticals are approved by the authorities as well (EMA, 2020; Kakade et al., 2021; Kına et al., 2021). Thus, plants seem to be persistent center of interest of pharmaceutical, food/feed and cosmetic industries.

Natural antioxidants attract attention of scientists since reactive oxygen metabolites (ROMs) including free radicals resulted from oxidative stress are involved in many harmful conditions for health, especially during the aging and many diseases (Uysal et al., 2021). Aside from their uses for medicinal purposes, antioxidants have a great potential to be used in various fields, which also directly or indirectly correlate with wellness, as a constituent of animal feed, as a preventive agent against deterioration in some foods or as an ingredient in anti-aging cosmetics.

Bacterial infections lead or contribute to undesirable health problems that threaten human and animal life. Attempts for discovery of new antibiotics become more important today more than ever because of multidrug resistance developed by the microorganisms against known antibiotics (WHO, 2013).

Pomegranate (*Punica granatum* L.) (Punicaeae), a special fruit whose name is commemorated in mythological records and sacred books since ancient times (Bhandari, 2019), is one of the most prominent natural product used in human nutrition concerning its health benefits. In Turkey, Mediterranean, Aegean and Southeast Anatolia are the regions producing the most amount of pomegranate (Kurt and Şahin, 2013). Its fruits are consumed in all over the world, and all parts (fruit, seed, root, leaf, flower and peel) are used as traditional remedies by various cultures since ancient times (Shaygannia et al., 2016; Bhandari, 2019). Therefore, its functional and medicinal activities such as antioxidant, antimicrobial, anti-inflammatory, antidiabetic, anti-aging, antimutagenic, anticarcinogenic, lipid-lowering, antiatherogenic, hepato-, dermo- and nephroprotective effects have been investigated intensely, especially in the last decade (Sevindik et al., 2017; Bhandari, 2019; Mohammed et al., 2020; Akgül et al., 2022). There is a number of publications on the antioxidant activity of plant parts that are consumed as fruit (arils) (Sravanthi and Rao, 2015; Yan et al., 2017; Amir et al., 2019) or juice (Les et al., 2015) as well as other plant parts (peels, barks, rinds, seeds, leaf) (Tozetto et al., 2017; Yan et al., 2017). *In vivo* antioxidant efficacy of pomegranate juice has been exerted on brine shrimp, *Artemia salina* (Les et al., 2015) and on human (Noori et al., 2016). Both antioxidant and antimicrobial activities of peels, rinds or seeds (Wendy et al., 2017; Ali Redha et al., 2018; Demir et al., 2019) and juice or arils (Ali Redha et al., 2018) have been investigated together in some studies.

Black pine (*Pinus nigra*) (Pinaceae) is a conifer, widely but fragmentally distributed across Europa and Asia (Enescu et al., 2016). *P. nigra* subsp. *pallasiana* grows naturally in Turkey, west side of cross Anatolia (Akkemik, 2018). Kızılarslan and Sevgi (2013) reviewed ethnobotanical uses of the *Pinus* L. genus in Turkey, and stated that *P. nigra* was the most preferred species used by local communities, especially in wood production or folk medicine. Various parts of *P. nigra*, such as branches, roots, tar or resin, are primarily used for medicinal purposes, mainly against respiratory system and skin problems, and usage of leaf and cone is in the second rank followed by other *Pinus* species, namely *P. sylsvestiris* and *P. brutia* (Kızılarslan and Sevgi, 2013). Antimicrobial and antioxidant activities of different parts and/or constituents from various *Pinus* species have

been investigated previously (Dığrak et al., 1999; Kilic et al., 2011; Eryilmaz et al., 2016; Sirakov et al., 2018; Fkiri et al., 2018). However, varieties of the *Pinus* species are undetermined or unspecified in some reports. Anatolian black pine [*P. nigra* Arn. subsp. *pallasiana* (Lamb.) Holmboe var. *pallasiana*] is one of the *Pinus* taxa naturally grown and widely distributed within pine flora of Turkey (Akkemik, 2018). It is mainly used in traditional tar production in central Western Turkey. Recently, potential use of cones of this variety as a natural dye material with antibacterial effect was investigated by Bahtiyari and Yilmaz (2018).

Metabolite content, thus bioactivity of the plant extracts depends on plant species, geographic conditions that the plant grown, plant part used in the extraction and the extraction solvent and method (Altemimi et al., 2017).

The present study aimed to determine the antioxidant and antibacterial activities of the methanolic extracts prepared from different parts of pomegranate and Anatolian black pine grown in the Southeastern Anatolia district of Turkey. Since polyphenolic compounds in plants are the basic constituents of natural antioxidants, and their radical scavenging activity is thought to have an important role in the prevention of numerous chronic diseases, total phenolic content of the extracts was also determined. The results were evaluated by the comparison within the parts of the same plant, as well as between the data reported in the literature.

2. Material and Methods

2.1. Plant Materials

Pomegranate (*Punica granatum* L.) and black pine [*P. nigra* Arn. subsp. *pallasiana* (Lamb.) Holmboe var. *pallasiana*] were collected from Adıyaman district (Turkey) and surroundings, and authenticated by Dr. Ahmet Zafer TEL at Adıyaman University, Faculty of Science, Department of Biology. Voucher specimens were deposited at the same department. After plucking the plant parts, all materials were washed with first tap water, then distilled water, and dried in the shade at room temperature. The dried materials were ground into powder, and stored in opaque containers with lid at refrigerator in desiccator (+4ºC) to avoid deterioration effect of light and high temperatures. Whole fruit of pomegranate included peels and carpelar membranes surrounding the edible parts (Machado et al., 2019). Plant materials were summarized in Table 1.

Table 1. Plant materials used in the study

Common Name	Systematical Name	Plant Parts	Herbarium No.	Collection Region and Habitat
Anatolian Black Pine	*Pinus nigra* Arn. subsp. *pallasiana* (Lamb.) Holmboe	Leaf Cone Wood	1377	Ulubaba Mountain, forest
Pomegranate	*Punica granatum* L.	Leaf Flower Whole fruit Wood	1378	Nemrut Mountain, forest-shrub

2.2. Preparation of Methanolic Extracts

Certain amount of each powdered plant material (5 g of all plant parts of pomegranate; 2 g of pine leaves, 10 g of pine cones and 5 g of pine woods) was weighed and extracted with 80 mL methanol using solvent extractor at 210 ºC for total 1 h 45 min (1 h immersion-30 min washing-15 min recovery). Methanolic extracts were then evaporated to dryness under reduced pressure using rotary evaporator, and the extraction yield was calculated. Each crude extract was then dissolved in a certain volume of methanol and centrifuged at 12.000xg at 4 ºC for 5 minutes, to eliminate insoluble materials. Supernatants were used as test materials, and kept in dark bottles at +4ºC until use.

2.3. Free Radical Scavenging Activity (DPPH Test)

DPPH (2,2-Diphenyl-1-picrylhydrazyl) free radical scavenging activity was measured by the determination of absorbance of the sample mixtures containing 40 µL of each extract (in a concentration of 0.065 - 1 mg/mL) and 160 µL of DPPH solution in methanol (0.2 mM) at 520 nm (Erol-Dayi et al., 2011). Blank sample was prepared mixing of DPPH solution with methanol instead of extract. All mixtures were kept in dark at RT for 10 min prior to measurement. Percentage inhibition of DPPH for each concentration was estimated by using following formula.

$$\% \text{ Inhibition of DPPH} = 1-(A_{sample} / A_{blank}) \times 100$$

where A_{sample} = absorbance of the sample mixture (extract + DPPH) and A_{blank} = absorbance of DPPH solution containing methanol instead of extract.

Ascorbic acid solutions in a concentration range of 0.005–0.1 mg/mL were used as reference antioxidants. The results were expressed as IC_{50}, which is defined as the concentration of sample required to inhibit DPPH by 50%.

2.4. Cupric Reducing Antioxidant Capacity (CUPRAC Test)

Cupric ion reducing powers of the extracts were determined using spectrophometric method described by Apak et al. (2006), with a slight modification. In this method, 250 µL of each, 10^{-2} M $CuCl_2$, 7.5×10^{-2} M ethanolic Neocuproine solution and 10^{-3} mM ammonium acetate buffer, pH 7.0, were added in each well of a 24-microwell flat bottom plate, respectively, and followed by 25 µL of each extract. The final volume of the reaction mixture was accomplished by adding 250 µL distilled water to each well. The mixtures were then kept in dark at RT for 1 h, and the absorbance was measured at 450 nm. Trolox was used as reference antioxidant, and results were expressed as one mmole trolox equivalent antioxidant capacity per gram dry weight of extract (mmole TEAC/g DW).

2.5. Total Phenolic Content

Total phenolic contents of the extracts were determined using Folin-Ciocalteu method (Singh et al., 2016), with slight modification. In this method, a stock solution of gallic acid as reference was prepared in a concentration of 125 µg/mL and serially diluted to 62.5 µg/mL, 31.25 µg/mL, 15.62 µg/mL, 7.8 µg/mL and 3.9 µg /mL. Into one milliliter of each crude extract or gallic acid solution, 0.5 mL of Folin's reagent, followed by 3 mL of Na_2CO_3 (Merck) solution (200 g/L) and 5.5 mL of distilled water were added. The mixtures were centrifuged at 1250×*g* at RT for 5 min. Two hundred microliter from each supernatant was transferred into the wells of a 96 microwell plate and the absorbances were recorded at 725 nm against blank containing of methanol instead of sample. The results were expressed as milligram gallic acid equivalent per gram of dry weight of extract (mg GAE/g DW), using standard gallic acid calibration curve.

Spectrophotometric data represented the mean ± SD of at least three independent experiments.

2.6. Antibacterial Activity Test against Fish Pathogens

Isolated strains of fish pathogens, *Aeromonas hydrophila*, *Vibrio anguillarum*, *Yersinia ruckeri* and *Lactococcus garvieae*, kept in the culture collection of the Department of Aquaculture and Fish Diseases, Faculty of Aquatic Sciences, Istanbul University (Table 2) were used in antibacterial activity tests. In addition, as a human, fish and aquatic bird pathogen (Miniero Davies et al., 2018), commercial strain of *Edwardsiella tarda* Ewing and McWhorter (ATCC® 15947) from human feces was employed.

Cultures were maintained in Nutrient Broth (NB) medium, and disc diffusion assay was employed for antimicrobial activity tests (Jorgensen and Turnidge, 2015). Two hundred microliter of stock cultures adjusted to 0.5 McFarland standard were added to 9800 µL of NB medium and incubated at 21ºC for 24 h. One hundred microliter from each fresh culture was transferred onto the solid medium of Mueller Hinton Agar (MHA) using "Drigalski" spatula, and spread with sterile cotton. Methanol extracts were sterilized by passing through the filters with 0.22 µm pore size. Certain volumes of the extracts in known concentrations were applied onto the aseptic filter paper discs with the diameter of 6 mm. Methanol in a volume equivalent to one in applied sample was used as blank. Following the methanol evaporated from the discs under the aseptic conditions, the discs were embedded onto the cultures in MHA media and incubated at 21ºC for 24 h. Diameters of inhibition zones as millimeter were measured and used as antimicrobial activity criteria. Ampicillin, oxytetracycline, kanamycin, trimethoprim sulfamethoxazole, flumequine, enrofloxacine and ciprofloxacin were used as reference antibiotics. Values were given as the mean ± SD of at least three independent experiments.

2.7. Statistical Analyses

The quantitative data were presented as mean ± standard deviation (SD) based on at least three independent experiments. Statistical analysis and graph generation were performed using the GraphPad Prism Software version 7.01. The statistical evaluation was performed with one-way analysis of variance (ANOVA) followed Tukey's multiple comparisons. The *P* value of<0.05 was taken as the criterion of statistically significance.

3. Results and Discussion

3.1. Antioxidant Activity

Antioxidant potential of the samples varied depending on the plant species, and different plant parts for the same plant (Table 3). DPPH scavenging and cupric ion reducing activities and total phenolic contents of the samples were compared in Figure 1.

Pomegranate extracts were the most active ones, as expected. Among the pomegranate samples, the whole fruit extract exhibited superior DPPH scavenging activity with the IC_{50} value of 0.030±0.007 mg/mL, even better than the reference antioxidant, ascorbic acid (0.048 mg/mL) (Table 3). Similar superiority of pomegranate extracts was also detected in CUPRAC assay. Cupric reducing activity of these extracts ranged between 1.409-2.323 mmole TEAC/g DW, with the priority of whole fruit extract. Surprisingly, the antioxidant activity of pomegranate wood extract was also prominent. There was no statistically significant difference between the antioxidant activities of wood and whole fruit extracts ($P>0.05$) in both DPPH and CUPRAC tests, although total phenolic content of wood extract was very low ($P<0.001$). This result indicated that wood extract might have bioactive constituents other than phenolic compounds.

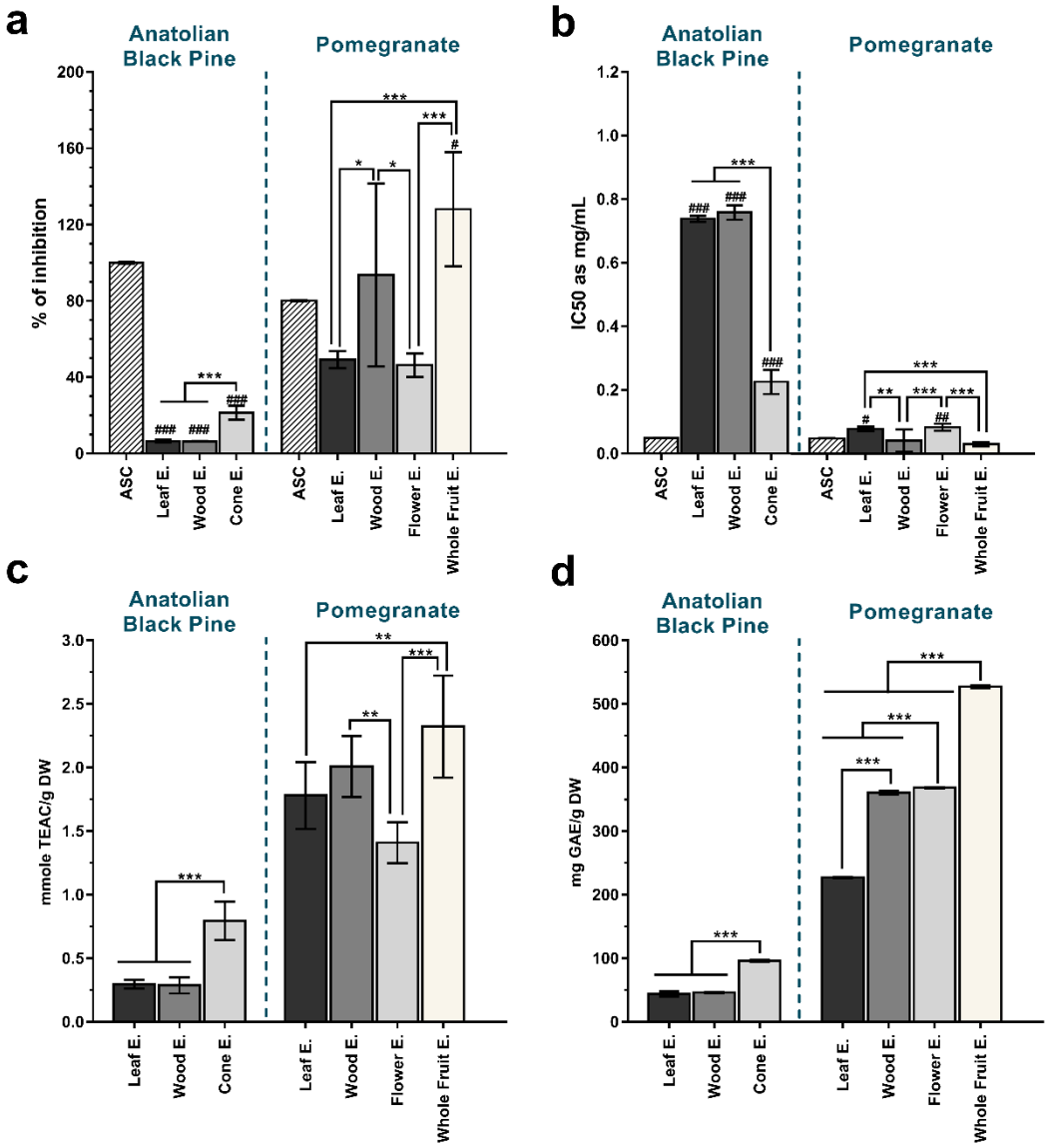

Fig 1. Comparison of **a)** percentage inhibition of DPPH, **b)** the half maximal DPPH inhibitory concentration (IC_{50}), **c)** CUPRAC value, and **d)** total phenolic content of the samples. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus ascorbic acid. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, which show multiple comparisons between different groups. *P* values were determined by one-way ANOVA using Tukey's multiple comparison test. (ASC: ascorbic acid, E.: extract).

Table 2. Fish pathogens and their origins

Isolate Name	Culture Collection Code	Collected Region of Fish	Isolated Fish	Isolated Organ	Isolation Media
Aeromonas hydrophila	PŞF	Sapanca, Marmara region, Turkey	Rainbow trout	Liver	TSA*
Vibrio anguillarum	HVA	Bodrum, Aegean district, Turkey	Sea bass	Liver	MA*
Yersinia ruckeri	YR38	Fethiye, Mediterranean district, Turkey	Rainbow trout	Liver	TSA
Lactococcus garvieae	EYJK-B1	Fethiye, Mediterranean district, Turkey	Rainbow trout	Spleen	TSA

*TSA: Tryptone Soy Agar; MA: Marine Agar

Table 3. Antioxidant activity and total phenolic content of the extracts prepared from different parts of the plants

Plant/parts	DPPH Scavenging Activity (IC_{50} as mg/mL)	Cupric Reducing Activity (mmole TEAC/g DW)	Total Phenolic Content (mg GAE/g DW)
Pomegranate/leaves	0.078±0.007	1.781±0.263	227.08± 0.960
Pomegranate/flowers	0.083±0.011	1.409±0.161	368.24±0.868
Pomegranate/ whole fruits*	0.030±0.007	2.323±0.401	526.85±2.128
Pomegranate/woods	0.041±0.035	2.008±0.240	360.30±3.290
Anatolian Black Pine/leaves	0.738±0.009	0.297±0.034	44.02± 4.032
Anatolian Black Pine/cones	0.225±0.038	0.793±0.151	96.14± 1.701
Anatolian Black Pine/woods	0.758±0.023	0.288±0.064	46.26± 0.790
Ascorbic acid	0.048±0.0002	-	-

*Whole fruit: Entire fruit with the rind [calyx, mesocarp, seeds (arils)] and a small portion of the stem peels and carpelar membranes surrounding the edible parts.

Pomegranate fruits are known to have several phenolic compounds, such as gallic acid, protocatechuic acid, p-hydroxybenzoic acid, vanillic acid, caffeic acid, p-coumaric acid and ferulic acid (Singh et al., 2018) that contributes the antioxidant activity, and many other active metabolites, including flavonoids, anthocyanins, alkaloids, fatty acids and vitamins (Shaygannia et al., 2016).

In fact, promising antioxidant and antimicrobial activities of pomegranate have been manifested by a number of studies, previously. Although plant parts seem to be ambiguous in some of the publications, peels (barks, rinds) are the most studied parts in antioxidant activity tests, probably due to the attempts for utilization of large quantities of waste materials produced by food industry (Wendy et al., 2017; Yan et al., 2017; Tozetto et al., 2017; Ali Redha et al., 2018; Demir et al., 2019). Arils, seeds and fruits are in the second rank in research, since they are the plant parts consumed by humans or used for juice production (Sravanthi and Rao, 2015; Les et al., 2015; Yan et al., 2017; Ali Redha et al., 2018; Amir et al., 2019). Some researchers have investigated antioxidant activity of commercial or hand-made juices and some by-products (bagasse) (Yan et al., 2017). However, no study on the antioxidant activity of woods appeared in the literature. Our results demonstrate that not only the solvent (MeOH) but also the method (continuous Soxhlet extraction) used here seems to be highly effective for extracting total phenolics from pomegranate, since TPC of the extracts ranged from 227.08 to 526.85 mg GAE/g DW. Especially, TPC of whole fruit extract (526.85) was at least 40-fold higher than that of fruits from 5 varieties (4-11.8 mg GAE/g DW) grown in India (Sravanthi and Rao, 2015).

In this study, IC_{50} values of MeOH extracts from different parts of Anatolian black pine in DPPH test were ranged between 0.225 mg/mL and 0.758 mg/mL (Table 3). Cone extract having at least two fold phenolic compounds than the other parts (leaf and wood) showed the highest radical scavenging activity. Phenolic content of cones from *P. nigra* grown in Northwest Turkey (Bartın) have been previously investigated by Kilic et al. (2011), and catechin, a known flavonoid was detected as major phenolic, in addition to much lower of vanillin and 3,4-dihydroxybenzoic acid. Very recently, Topal (2020) reported

antioxidant activity and secondary metabolites of ethanol extracts of cones from Scots pine (*P. sylvestris*) trees grown in three different provinces in Eastern Anatolia (Gumushane, Erzurum and Sarikamis). Ethanolic extract of *P. sylvestris* grown in Gumushane seems to contain more total phenolic compounds (131.82 µg GAE/mg) than Sarikamis sample (99.09 µg GAE/mg) as well as than the Anatolian black pine cone extract studied here (96.14 µg GAE/mg DW). Although phenolic contents of the cone extracts from Scots pine grown in Sarikamis and Anatolian black pine grown in Adiyaman were very close, Scots pine cone extract with IC_{50} value of 30.13 µg/mL seems to be more effective scavenger than Anatolian black pine cone extract with IC_{50} value of 225±0.038 µg/mL obtained in this study. More than seven-fold difference between these samples may be dependent on the lack of some bioactive compounds because of species-specific genetic factors and environmental/climatic conditions, and/or partially loss of them during the extraction.

3.2. Antibacterial Activity

Although there are numerous reports on *in vitro* or *in vivo* antibacterial activity of various extracts of pomegranate and/or its constituents on various bacteria (Jalali et al., 2021), including *Aeromonas hyrophila* (Belal et al., 2009; Wendy et al., 2017), *Yersinia ruckerii* (Acar et al., 2018), *Lactobacillus gravieae* (Goudarzi et al., 2011) and *Edwardsiella tarda* (Wendy et al., 2017), there is no publication appeared in the literature concerning its activity on *Vibrio anguillarum* up to date. Methanolic extracts from all parts of pomegranate, except wood, inhibited most of the bacteria studied here (Table 4). Antibacterial activity in pomegranate extracts against *V. anguillarum* was reported for the first time. Whole fruit extract was the most active one, and *Y. ruckeri* and *L. garvieae* were the most sensitive fish pathogens to this extract. Pomegranate leaf and flower extracts also inhibited growth of 4 out of 5 bacteria species, except *A. hydrophila*, while wood extract was inactive against the test bacteria. No activity was detected in leaf extract against *A. hydrophila*. Within a number of reports indicating antibacterial activity of pomegranate, those on fish pathogens appeared in the literature in the last decade. Plant materials were mostly commercial products or waste materials, such as peels, seeds, fruits, rinds, flowers (Goudarzi et al., 2011, Wendy et al., 2017) in the studies conducted with fish pathogens, and *A. hydrophila* was the most studied organism. Besides, *in vivo* activity of seed oil (Acar et al., 2018) and fruits (Belal et al., 2009) were shown against *Y. ruckeri* in rainbow trout and *A. hydrophila* in mice, respectively.

The data obtained in this study are substantially in agreement with the results of previous reports. It is well known that phenolic compounds act as effective antimicrobials by leading to irreversible changes in the structure and properties of the bacterial membrane (Borges et al., 2013). Hence, methanolic extract of whole fruit consisting of peels and carpelar membranes surrounding the arils, which contain the highest total phenolic content, is expected to have antibacterial activity.

Although antioxidant and antimicrobial activities of needles and/or their essential oils from Anatolian black pine have been

investigated previously (Fkiri et al., 2018), no study was reported on the antibacterial activity of this variety against bacteria tested here. We demonstrated that only the methanolic cone extract of Anatolian black pine was able to inhibit the growth of all bacteria tested here moderately, with the inhibition zones ranging between 8.7±0.6 mm to 11.0±1.2 mm. Pine leaf and wood extracts were inactive against the bacteria tested here, probably due to their insufficient amount on the disc (Table 4).

Table 4. Inhibition zone diameters caused by methanolic extracts and reference antibiotics in disc diffusion assay where all discs used were 6 mm (Conc.: Concentration)

		Inhibition Zone Diameter (mm±SD)				
Plant/parts	**~Amount (mg)**	**Ah***	**Va***	**Yr***	**Lg***	**Et***
Pomegranate/leaves	10	–	11.0±2.0	12.0±0.6	11.0± 0.6	11.0±1.2
Pomegranate/ flowers	10	–	12.0±0.6	13.0±0.6	13.0±1.0	12.0±0.6
Pomegranate/whole fruits**	7	13.0±1.0	12.0±0.6	15.0±0.6	16.0±1.0	14.0±1.5
Pomegranate/ woods	2	–	–	–	–	–
Anatolian Black Pine/leaves	2	–	–	–	–	–
Anatolian Black Pine/cones	10	8.7±0.6	9.3±1.5	9.0±1.0	11.0±2.1	11.0±1.2
Anatolian Black Pine/woods	5	–	–	–	–	–
Reference antibiotics	~Amount (µg)	Ah*	Va*	Yr*	Lg*	Et*
Amp**	10	–	–	–	–	–
Oxytet**	30	18.0±2.5	11.0±2.0	24.0±1.0	13.0±0.5	17.0±1.0
Kan**	30	16.0±0.8	0	23.0±2.0	0	–
Trimet/ Sulfamet**	25	–	–	–	–	–
Flum**	30	13.0±1.0	23.0±2.0	12.0±0.5	14.0±1.5	–
Enrof**	5	15.0±1.0	30.0±2.5	21.0±1.5	16.0±1.0	15.0±1.0
Cipro**	1	15.0±1.0	12.0±2.0	19.0±1.0	14.0±1.5	11.0±1.0

*Ah, Va, Yr, Lg and Et are *Aeromonas hydrophila*, *Vibrio anguillarum*, *Yersinia ruckeri*, *Lactococcus garvieae* and *Edwardsiella tarda*, respectively.
**Amp, Oxytet, Kan, Trimet/ Sulfamet, Flum, Enrof and Cipro are abbreviations for Ampicillin, Oxytetracycline, Kanamycin, Trimethoprim/ Sulfamethoxazole, Flumequine, Enroflaxacine and Ciprofloxacin, respectively.
– means no inhibition zone was observed.

Antimicrobial activity of various part and/or constituents from *Pinus* species has been studied intensively (Sharma et al., 2015, Ramos et al., 2022). In a previous study having a similar concept with this study, antimicrobial activities of chloroform, acetone and methanol extracts from different parts of *P. nigra* in the same habitat have been compared by disc diffusion test (Dığrak et al., 1999). Methanolic extracts from leaves, cones and bark inhibited the growth of most of the bacteria, including *Bacillus megaterium*, *B. subtilis*, *B. cereus*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Staphylococcus aureus*, *Mycobacterium smagmatis*, *Proteus vulgaris* and *Pseudomonas aeruginosa* at different levels. Methanolic cone extract was detected as the most active one against these bacteria, producing the inhibition zone of 17 mm on *P. vulgaris*, even higher than reference antibiotic ampicillin (10 µg). Ethereal extracts of cones from *P. nigra*, *P. brutia* and *P. halepensis* collected from various provinces in Turkey (*P. nigra* from Bartın at Western Black Sea Region, two others from Izmir and Mugla, respectively, at Aegean Region) have been investigated for their antimicrobial activities (Eryilmaz et al., 2016). Only *P. nigra* cone extract was found to be moderately active against *S. aureus* (MRSA), and *P. halepensis* cone

extract against *P. aeruginosa* (Eryilmaz et al., 2016).

Yersinia ruckeri, *Vibrio anguillarum*, *Lactococcus garvieae* and *Aeromonas hydrophila* lead to fish diseases namely yersiniosis, vibriosis, lactococcosis, motile Aeromonas septicemia, respectively, and they are of the bacteria cause great losses in aquaculture economics (Candan et al., 2007; Öztürk and Altınok, 2014). Importance of development and use of herbal drugs against harmful pathogens in aquaculture was discussed by Ramudu and Dash (2013). Our results demonstrated that the methanolic extracts of the pomegranate whole fruits and pine cones could be useful for the development of new natural and economical antibiotics, against the fish pathogens used in this study.

4. Conclusion

Aerial parts of pomegranate, mainly the whole fruits and woods are the most promising plant materials for obtaining antioxidant and antibacterial metabolites. For example, they might be used as a feed additive, especially for fish, or take part in packaging materials to prolong the shelf life in the future.

Moderate antioxidant and antibacterial activities detected in the cone extract of Anatolian black pine in this study make the pine cones an attractive plant source, although they have been regarded as an insignificant source for commercial production of antioxidants (Kilic et al., 2011). However, pine cones, which are produced in large quantities annually around the world (Bahtiyari and Yilmaz, 2018), can be used as a source for both antioxidant and antibacterial compounds and recycled. Plant habitats, especially pine forests have a great potential with not only their known plant sources but also undiscovered or by/waste products and residues, as stated by Ferreira-Santos et al. (2020).

Understanding the biological activities of different parts of these plants may arise their use as a source for active metabolites against oxidative stress and fish pathogens, directly or indirectly.

Further studies on these plant extracts, such as optimization of the extraction method, detection of active constituents and measurement of *in vivo* activities are in progress, in order to evaluate their uses in feed/food, cosmetic and pharmaceutical industries and to obtain standardized products.

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Author Contribution

NA, SK and HY designed the research and responsible for the conceptualization. OHS conducted the experiments and contributed to outline. AZT collected and identified voucher specimens of the plant materials. HY and AZT prepared the plant materials. OHS and NA prepared the extracts. YA and NA helped in collecting the data on antioxidant activity. SK, REY and HY helped in collecting the data on antibacterial activity. NA drafted and HY revised the paper. All authors read and approved the final manuscript.

Conflicts of Interest

The authors have no conflicts of interest to declare and disclose any financial field.

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