Indian Journal of Biochemistry & Biophysics
Vol. 57, June 2020, pp. 298-303

Phenolic constituents, antioxidant and antimicrobial activities of methanolic extracts of some female cones of gymnosperm plant

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Received 05 September 2019; *revised 12 March 2020*

In the present study, the total antioxidant activity, the total phenolic content, and the antimicrobial activity of methanol extracts obtained from the female cones of *Pinus brutia*, *Pinus nigra*, *Cupressus sempervirens* L., *Thuja orientalis* L., and *Cedrus libani* were evaluated. The *in vitro* total phenolic content, the antioxidant, and the antimicrobial activities were determined using the Folin-Ciocalteu reaction, the DPPH radical scavenging assay and the disc diffusion method, respectively. The results of the present study showed that the extracts of the female cones prepared in methanol had the antimicrobial activity against the test microorganisms and the highest antimicrobial effect was observed against *Staphylococcus epidermidis*, *Staphylococcus aureus*, and *Bacillus subtilis.* The total phenolic constituents of the extracts were determined to be in the ranges from 69 to 220 mg GA/100 g. All extracts exhibited antioxidant activity with concentrations necessary to inhibit the activity of DPPH radical by 50% (IC_{50}) ranging from 0.35 to 17.21 µg/mL. The results indicate that the extracts of the cones exhibit acceptable antioxidant and antimicrobial activities and suggest that these cones' extracts may serve as a potential source of natural antioxidants and antimicrobials for food or medical purposes.

Keywords: Antioxidant, Antimicrobial effect, Cupressaceae, Pinaceae

Plant extracts obtained from aromatic and medicinal plants have been known to possess biological activity, most notably antibacterial, antifungal and antioxidant properties for long times. With growing interest in the use of essential oils in both the food and the pharmaceutical industries, the systematic and the potential usefulness of plant extracts studies have become increasingly important[1]. Therefore, the antimicrobial and antioxidant activities of medicinal plants can be explored to maintain alternative drugs in the pharmaceutical industry or to protect food quality in the food industry[2-4].

On the other hand, increasing the incidence of multi-resistant microbial strains represents the major [illegible] for new, efficient, and safe antimicrobial agents. Therefore, there are many studies focused on finding new drugs with antimicrobial properties, which are, in many cases, inspired by the traditional utilization of medicinal plants[5,6].

Also, gymnosperm species have antioxidant activities and thus they have great potential as sources for natural health products[7].

The buds, leaves, young needles, and female cones of the families of Cupressaceae and Pinaceae and their essential oils and resins are used in various diseases in the traditional treatments[8,9]. Also, these species have been used for anti-inflammatory, antioxidant, antiseptic, antipyretic, anthelminthic, astringent, anti-rheumatic, anti-hemorrhoidal, anti-diarrheal and vasoconstrictive properties[10].

The aim of this study is to determine the total phenolic constituents, antioxidant and antimicrobial activities of the methanolic extracts of the female cones of *Pinus brutia*, *Pinus nigra*, *Cupressus sempervirens* L., *Thuja orientalis* L., and *Cedrus libani*.

Materials and Methods

Chemicals and reagents

[illegible]gents (Folin-Ciocalteu, [illegible] Acid, 2,2 diphenyl-1-picrylhydrazyl (DPPH), Methanol, Mueller Hinton Agar(MH agar), Tryptic Soy Broth, Saboraud Dextrose Agar, Sodium Carbonate, Ascorbic Acid) used in this study were of analytical grade and obtained from Merck Company, Germany.

Plant materials

The female cones of *Pinus brutia*, *Cupressus sempervirens* L., *Thuja orientalis* L., and *Cedrus libani* were randomly collected from natural populations in Esentepe campus of Sakarya University, Sakarya, Turkey; *Pinus nigra* were randomly collected in National Park of Camlık, Yozgat, Turkey in June and July 2018.

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Preparation of methanolic extracts

Ten grams of powdered cones were placed in the soxhlet device, with 200 mL of solvent (methanol), and subjected to 12 h of extraction. The solvents in the extracts were evaporated by using a rotary evaporator (Heidolph) under vacuum at 45°C for 15 min and the dried extracts were used for all investigations. The extract concentrations were adjusted by adding own solvent (methanol) to each extract at the doses of 6400 µg/mL for the antimicrobial activity tests and 1000 µg/mL for the antioxidant activity and the total phenolics analyses.

Disc diffusion method

All strains used throughout this study have been obtained from the Microbiology Research Laboratory of Sakarya University. For the determination of the antimicrobial activity, the disc diffusion method was used according to the National Committee for Clinical Laboratory Standards[11]. Briefly, microbial suspension with adjusted density (0.5 McFarland) was spread on the MH solid media plate by using sterile swabs. Filter paper discs (6 mm in diameter, Himedia) were impregnated with 10 µL of the methanol extracts. After keeping at room temperature for 2 h, the discs impregnated with extracts were slightly pressed on the inoculated plates under aseptic conditions. Then, they were incubated 37°C for 24 h. Methanol-impregnated discs were used as negative controls and the commercial antibiotic discs (Gentamicin and Amphotericin B) were used as positive ones. At the end of the incubation, the diameters of the inhibition zone were measured by using an electronic digital caliper.

Antioxidant activity (DPPH assay)

The modified Blois method was used for antioxidant activity determination[12]. Briefly, 1 mL of 0.004% solution of DPPH radical in methanol was mixed with 1 mL of extract solution in methanol (containing different concentrations of dried extract). These solutions were kept in dark for 30 min and the optical density was measured at 517 nm using spectrophotometer and methanol was used for the blank. The following equation was employed to determine the % DPPH radical scavenging activity.

$$\%\ \text{DPPH radical scavenging} = \frac{(\text{control absorbance} - \text{extract absorbance})}{(\text{control absorbance})} \times 100$$

Total phenolic content (TPC)

The total phenolic content was determined according to Singleton and Rossi method with a slight modification[13]. For the total phenolic content determination, the plant extracts were prepared at 100 µg/mL concentrations.100 µL of the sample was mixed with 200 µL of 50% Folin and Ciocalteu's phenol reagent (Sigma). After 3 min, 1 mL of 2% Na_2CO_3 (~35%) (Riedel-de Haën) was added to the mixture. The reaction was kept in dark for 60 min, after which its absorbance was read at 760 nm. A calibration curve was constructed with different concentrations of gallic acid (Sigma) (0.01-0.1 mM) as a standard.

Statistical Analysis

All measurements were performed in triplicate and the results were represented as mean ± SD. Statistical analyses were realized with the SPSS 20.0 statistics program. Data statistical analyses were achieved by using One-way ANOVA and Duncan-test. The level of significance was set at $P < 0.05$.

Results

Antimicrobial activity

In the present study, the antimicrobial activity of the female cones of *Pinus brutia*, *Pinus nigra*, *Cupressus sempervirens* L., *Thuja orientalis* L., and *Cedrus libani* against *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 8739, *Enterecoccus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, *Salmonella typhimurium* ATCC 14028, *Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 1029 were determined.

The results of the antimicrobial screening of five conifer species against the seven bacteria and one yeast have been summarized in (Table 1) (the diameters of the inhibition zone against the test microorganisms).

All the tested extracts revealed the antimicrobial activity showing different selectivity for each microorganism. The methanolic extracts of cones showed that the greatest inhibitory effects were determined to be 12-15 mm, 11.5-15.2 mm, 10.5-13 mm against *B. subtilis*, *S. epidermidis*, and *S. aureus*, respectively. Additionally, no inhibitory effect against *C. albicans*, and *S. typhimurium* was observed for all extracts. Activities of the cones towards the test microorganisms were found to be lower than those of the known antibiotics. Only the methanolic extract of *Cedrus libani* showed antibacterial activity against *E. coli* and *E. faecalis*. The results of the present study demonstrated that *Cedrus libani* cones had a broad spectrum of activity on various microbial infections.

Scavenging effects on DPPH radical

Antioxidant activity is most commonly evaluated by the DPPH scavenging activity test[14]. The IC_{50} of a

Table 1 — Inhibition zone diameters of extracts

Test Microorganisms	Inhibition Zones (mm) (±SD) Methanolic Extracts (6400 µg/mL)					Antibiotics	
	P. brutia	*P. nigra*	*C. sempervirens*	*T. orientalis*	*C. libani*	GC	AMB
B. subtilis	15.0±0.5	13.0±0.02	12.0±0.0	12.5±0.0	13.0±0.05	26.0	-
C. albicans	0	0	0	0	0	-	17.0
E. coli	0	0	0	0	11.0±0.0	28.0	-
E. faecalis	0	0	0	0	12.0±0.02	23.0	-
P. aeruginosa	0	15.0±0.0	0	0	13.0±0.05	22.0	-
S. aureus	13.0±0.5	12.0±0.0	13.0±0.5	12.0±0.5	10.5±0.1	28.0	-
S. epidermidis	15.2±0.1	14.0±0.5	13.0±0.5	14.0±0.0	11.5±0.1	21.0	-
S. typhimurium	0	0	0	0	0	25.0	-

GC: Gentamicin, AMB: Amphotericin B

compound is related to its antioxidant capacity inversely, as it expresses the amount of antioxidant required to decrease the DPPH concentration by 50%, which is obtained by interpolation from a linear regression analysis. A lower IC_{50} indicates a higher antioxidant activity of a compound[15].

Table 2 shows the IC_{50} values in the DPPH radical scavenging activity assay of the extracts. The DPPH radical-scavenging activities of plant extracts display a concentration-dependent manner. The IC_{50} values were found to range from 0.35 to 17.20 µg/mL. The highest antioxidant activity was determined in the *Cedrus libani* cone and the lowest antioxidant activity was determined in the *Thuja orientalis* L. cone. The methanolic extracts, obtained from the cones of *Cedrus libani*, *Pinus brutia*, and *Pinus nigra*, showed higher antioxidant activity than ascorbic acid.

The antioxidant activities of the cones belonging to the conifer species used in the present study exhibited higher activity than the fruits and roots belonging to the same species in the literature[16-18].

Total phenolic contents (TPCs)

Antioxidant compounds are usually in the phenolic form. Phenols, which are the most abundant structures in plants, are compounds that have the ability to destroy radicals because they contain hydroxyl groups; hence, they play an important role in the antioxidant activity[19]. Therefore, determination of the quantity of the phenolic compounds is of importance in order to regulate the antioxidant capacity of plant extract[20].

The total content of phenolics in the extracts has been evaluated according to the Folin-Ciocalteu method using gallic acid as the standard (Table 2).

TPCs of the extracts range from 69 to 220 mgGA/100 g, with *C. libani* showing the highest value of 220 mgGA/100 g, followed by *P. nigra*, *P. brutia*, *T. orientalis*, and *C. sempervirens* with TPC values of 135, 91, 80, and 69 mgGA/100 g, respectively (Table 2). Total phenolic compounds and IC_{50}values show an inverse relation.

Table 2 — Antioxidant activities and total phenolic contents of the extracts

Species	Antioxidant Activity		Total Phenolics	
	IC_{50} (µg/mL)±SD	R^2	mg GA/ 100 g±SD	R^2
Cedrus libani	0.58±0.09[a]	0.88	220.0±0.08[d]	0.98
Cupressus sempervirens L.	9.83±0.72[b]	0.82	69.0±0.05[a]	0.98
Pinus brutia	2.13±0.13[a]	0.94	91.0±0.04[b]	0.98
Pinus nigra	0.35±0.01[a]	0.98	135.0±0.22[c]	0.98
Thuja orientalis L.	17.20±0.39[b]	0.93	80.0±0.04[a,b]	0.98
Ascorbic acid	3.20±0.01[a]	0.96	-	-

Data were represented as mean ± SD of three measurements. Different letters symbolized significant differences (P <0.05) by mean of the ANOVA Duncan-test

Xie *et al*. determined that the TPCs of the essential oil extracts obtained from needles of six *Pinus* species ranged from 86.60 to 138.34 mg GA/100 g[21]. In the present study, it was determined that the total phenolic values of *Pinus* cones were within this range. In the literature, it has been reported that the total phenolics content was significantly correlated with the antioxidant activities as in the present study[15,22].

Discussion

Different parts of *Thuja* plants have been found to contain secondary metabolites such as flavonoids[23,24], phenolics, and terpenes[25-27]. It is known that the essential oils of *Thuja orientalis* L. have antimicrobial properties[28]. Akers *et al.* reported that *Thuja orientalis* L. leaf extract exhibited antimicrobial activity for

Salmonella typhimurium c and d strains and concluded that the antimicrobial activity arise from α, β, γ thujaplicin in the extract[29]. Bissa *et al.* and Jasuja *et al*. reported that the leaves of *Thuja orientalis* L. had more antimicrobial activity than the stem against selective bacterial species[30,31]. In the research of Jasuja *et al*., 70% methanol extract of the leaves of *Thuja orientalis* L. plant contained more phenolic compounds than the methanol extract of female cones with respect to our study[31]. This may be the reason why the female cone has less antimicrobial activity.

Xu *et. al*. specified the compounds obtained from the fruit extracts such as catechin, quercitrin, hypolaetin 7-O-β-xylopyranoside, isoquercitrin, myricitrin shown antioxidant activity due to a catechol group or a pyrogallol group in ring Dubey and Batra (found that the IC_{50} values of the extract obtained from dried branches were 202.45 μg/mL[32,33]. The IC_{50} value in our study was lower than Dubey and Batra's study[33]. Due to this fact that this could be related to the female cones containing more metabolites than branches.

Cupressus sempervirens L. is a plant that is used for medical purposes. Cypresses have antioxidant properties exhibiting phenolic and flavonoid compounds in different parameters[34]. In various studies, the extracts and essential oils of *Cupressus sempervirens* L. have been found to have high antioxidant activity and it was reported that these extracts had α-pinene and 3-carene in abundance[35-37]. However, in some studies, the extracts of this plant have a moderate antioxidant activity[38]. Zouaghi *et al*. compared the antioxidant acitvity of *C. sempervirens* stem, leaf and cone extract and concluded that the inhibition features of essential oils obtained from stem were similar to those of BHA and BHT activities but the extract of cones has shown less DPPH radical scanvenging activity and they suggested that this could be caused by diterpenoids[39]. In the present study, DPPH scavenging activity of *C. sempervirens* cone extracts has lower positive control value as in the study of Zouaghi *et al*.[39].

In various studies, *E. faecalis* and *P. aeruginosa* have been shown to have less sensitivity than *S. aureus* against the essential oils of leaves and the aerial parts of *Cupressus sempervirens* L.[2,40]. Selim *et al.* determined that the *Cupressus sempervirens* L. essential oil had no antimicrobial activity against *E. coli* and *C. albicans*[40]. The results of these studies are similar to the results of *Cupressus sempervirens* L. female cones in the present study.

Pinus brutia contains lipophilic compounds such as fatty acids, resin acids, resin alcohols, sterols α-pinene and β-pinene are the main compounds in the essential oil of *P. brutia*[18,41]. The cones include terpene compounds such as monoterpene hydrocarbons, monoterpene alcohol, sesquiterpene hydrocarbons, sesquiterpene alcohols[42]. Ulukanlı *et al*. found that the total phenolic compound content of *P. brutia* was 17.02 mg gallic acid equivalent/g essential oil[18]. This result is higher than the value we found, due to the different extraction methods. The results of the total phenolic compound content contribute significantly to the antioxidant activity of *P. brutia*. Ustun *et al.* reported that the extract of Pinus species exhibits moderate or less DPPH scavenging activity[43]. Yesil-Celiktas *et al.* observed that the bark extracts of *P. brutia* showed high radical scavenging effect[44]. Guri *et al.* reported that the dry leaf and bark extracts of *P. brutia* had antioxidant properties. Guri *et al.* and Yesil-Celiktas *et al*. supported that the antioxidant scavenging effect of *P. brutia* was higher than the antioxidant effect of ascorbic acid in our study[44,45].

In the study of Ulukanlı *et al.*, the antimicrobial activity of essential oils obtained from resin of *Pinus brutia* was determined against *B. subtilis*, *E. coli*, *E. faecalis*, *S. aureus*, and *C. albicans* (inhibition zone values in the range of 8-16 mm); no effect against *P. aeruginosa*[18].

The major constituents in essential oils of *C. libani* leaves were Germacrene D and β-caryophyene[46,47]. *C. libani* cones and the leaves have antimicrobial activity[48]. There is no study in the literature about the antioxidant characteristics of *Cedrus libani*. In our study, the DPPH radical scavenger effect of *Cedrus libani* was higher than ascorbic acid.

Kızıl *et al*. reported that the ethanol extract of resins obtained from the stems and roots of *Cedrus libani* have shown antimicrobial activity (inhibition zone values in the range of 10-14 mm) against *E. coli*, *S. aureus*, *P. aeruginosa*, and *C. albicans*[49].

The cone extracts of *Pinus nigra* contain lipophilics such as fatty acids, resin acids, resin alcohols, resin hydrocarbons, sterols, sterols, and triglycerides[41]. The main compound found in essential oils of *P. nigra* is α-Pinene[50]. It contains also isopimaric acid[41]. Ustun *et al.* observed that *Pinus nigra* extracts displayed a weak scavenging effect compared to gallic acid[43]. Yesil-Celiktas *et al.* reported that the extracts of *Pinus nigra* showed a high scavenging

effect and they found that the antioxidant activity of *P. nigra* was bigger than *P. brutia*[44]. The study of Yesil-Celiktas *et al.* is coherent with our study. In the present study, *Pinus nigra* has a more scavenging effect than the ascorbic acid and its antioxidant activity is due to its constituents[44]. The DPPH radical scavenging activity of *Pinus nigra* is the highest of all the extracts.

Šarac *et al.* reported that *S. aureus* showed sensitivity against essential oils of *Pinus nigra* needle[51]. The result of that study is consistent with the current one. The results of the present study have shown that the cones used in the study have antimicrobial activity and that the cones of forest trees, which are one of the major sources of nature, can be evaluated.

Conclusion

It was determined that all the coniferous extracts obtained in the study had antimicrobial effects on *B. subtilis, S. aureus* and *S. epidermidis* bacteria, while there was no antimicrobial effect on *S. typhimurium* and *C. albicans*. All extracts showed high antioxidant activity compared to ascorbic acid used as standard.

Antioxidant evaluation of the extracts has shown that the antioxidant potency correlated well with the total phenolic content (TPC) and revealed that the cone's extracts exhibit acceptable antioxidant activities. Therefore, it is possible that the cones may take an active role in the prevention of many diseases that are likely to occur as a result of the radical-mediated damage. The results of the antimicrobial and antioxidant activity of the cone's components obtained in pure form will be even more informative.

Conflict of interest

All authors declare no conflict of interest.

Acknowledgment

The authors would like to thank Dr. Mehmet Sağıroğlu and Dr. İbrahim Okur for their invaluable support.

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