International Journal of Secondary Metabolite
2023, Vol. 10, No. 2, 158–165
https://doi.org/10.21448/ijsm.1231833
Published at https://dergipark.org.tr/en/pub/ijsm
Research Article

Phytochemical profiling of the different organs of *Cupressus sempervirens* L. by LC-HR/MS

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Abstract: *Cupressus sempervirens* L. which is largely used in traditional medicine was collected from the Florya Atatürk Forest (İstanbul, Türkiye) to investigate the phytochemical profiling and antioxidant capacity of the seeds and cones. The antioxidant activities of hexane and methanol extracts of *C. sempervirens* L. were assessed *in vitro* using five complementary methods, including the *β*-carotene-linoleic acid assay for lipid peroxidation activity, the DPPH•, ABTS•+ assays for radical-scavenging activity, the CUPRAC method, and metal chelating methods. In addition, the phenolic profiling of the methanol extracts of the seeds and cones was analyzed using LC-HR/MS, for the first time. According to the findings, the antioxidant activity of the methanol extract obtained from seeds appears to be higher than that of cones in all assays. The methanol extracts of the seeds showed higher activity with an IC_{50}: 24.08±1.06, IC_{50}: 6.08±0.19, and $A_{0.5}$: 18.60±0.63 µg/mL in the DPPH•, ABTS•+, and CUPRAC assays, respectively than the BHA, and *α*-TOC. Also, the methanol extract of the cones showed strong activity with an IC_{50}: 38.87±0.03 and $A_{0.5}$:103.53±4.33 in ABTS•+ scavenging and CUPRAC assays. Moreover, twenty-eight phenolics were determined in the seeds while twenty-one phenolics were determined in the cones of the *C. sempervirens* using LC-HR/MS. The amounts of fumaric acid, vanilic acid, (-)-epicatechin, quercetin, hispidulin 7-glucoside, hyperoside, and quercitrin in the seeds are higher than those in the cones. Therefore, the results suggested that there was a strong relationship between the antioxidant activities of the extracts and their phenolic ingredients.

ARTICLE HISTORY

Received: Jan. 11, 2023
Accepted: Apr. 19, 2023

KEYWORDS

Cupressus sempervirens L.,
Phenolics,
LC-HR/MS,
Antioxidant activity,
Cones and seeds.

1. INTRODUCTION

Cupressus sempervirens L., commonly known as the Mediterranean cypress or Italian cypress, is an evergreen tree species in the Cupressaceae family. It is native to the Mediterranean region and is widely cultivated in many parts of the world for its attractive, narrow, columnar shape and its ability to grow well in warm, dry climates. The other hand, *C. sempervirens* is found throughout Northern America, North Africa, Asia and Europe (Hassan Javed Chaudhary, 2012). *C. sempervirens* is a medicinal tree which is generally called as “servi” in Turkish because the leaves are used to cure hemorrhoids and diabetes, and the fruit of *C. sempervirens* is used to

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e-ISSN: 2148-6905 / **© IJSM 2023**

treat inflammation, toothache, and laryngitis, as well as a contraceptive and astringent. The essential oil obtained by hydrodistillation from the leaves and cones is applied topically to cure headaches, colds, coughs, and bronchitis. Its dried seeds have also been used to cure bruises, ulcers, and wounds. Due to the medical and pharmacological advantages of *C. sempervirens*, particularly its essential oil derived from leaves, it is usually applied as a cosmetic component in the industry (Batiha *et al*., 2022; Selim *et al*., 2014).

As far as we know, many studies that focus on the chemical ingredients of essential oils from *C. sempervirens* grown in the northern Mediterranean basin have been published (Batiha *et al*., 2022; Sacchetti *et al*., 2005; Tumen *et al*., 2010). Some of the research was carried out on the total phenolic potential of the polar extract of the cones of *C. sempervirens* by using spectrophotometric analysis (Selim *et al*., 2014; Semerci *et al*., 2020). However, there have also been no studies on the phenolic profiling of the seeds and cones of *C. sempervirens*, separately. The current study used $DPPH^{\bullet}$, $ABTS^{\bullet+}$ scavenging assays, CUPRAC, β-carotene/linoleic acid, and metal chelating assays to evaluate the *in vitro* antioxidant activity of the nonpolar (hexane) and polar (methanol) extracts of these different parts of *C. sempervirens*. Moreover, the phytochemical profiling to obtain detailed information about the chemical composition of the plant extracts, including the types of compounds present and their relative abundance, was carried out by using LC-HR/MS on the methanol extracts obtained from the seeds and cones of *C. sempervirens*. This information can be used to guide further investigations into the potential biological activities of these compounds, as well as to develop new products or therapies based on the natural products of the plant.

2. MATERIAL and METHODS

2.1. Plant Material

The cones of *Cupressus sempervirens* L. were collected from Florya Atatürk Forest in İstanbul-Türkiye in November 2022. The taxonomic identification of the plant was confirmed by Dr. Cagla Kizilarslan Hancer in the Department of Pharmaceutical Botanic at Bezmialem Vakif University.

2.2. Extraction Procedures

The seeds were hand-picked and isolated directly from the cones of *Cupressus sempervirens* L. The separated and air-dried plant organs were ground to a fine powder using a laboratory-type grinder. The Soxhlet extractor was used to extract 80 g of the powdered seeds using 800 mL of hexane (CSHS). The solution was filtered and concentrated to give an extract by using a rotary evaporator under reduced pressure at 38–40 °C. The remaining portions of the seeds were exposed to the same extraction technique to obtain a methanol extract after the hexane extraction (CSMS). Also, 300 g of the powdered remaining part of the cones was extracted by employing maceration techniques with 1500 mL of hexane (CSHM) and methanol (CSMM), respectively. Then, a freeze dryer was used to completely dry the extract after the removal of the metanol by using the rotary evaporator. Following the removal of the solvents under vacuum and lyophilizer, all crude extracts were stored at 4 °C in the dark until further analysis.

2.3. Antioxidant Activities

2.3.1. *Free-Radical scavenging activity (DPPH assay)*

The DPPH test was used to assess the free radical-scavenging capacity of the extracts (Blois M.S., 1958). The hexane and methanol extracts were resolved in DMSO and methanol, respectively, to prepare solutions in eight different concentrations: 6.25, 12.5, 25, 50, 100, 200, 400, and 800 µg/mL. Following the addition of the DPPH solution, the absorbance was determined at 517 nm after 30 minutes of room temperature incubation in the dark. BHA and α-

TOC were used as the standard chemicals, while methanol and DMSO were employed as the control solvents. The reaction mixture's lower absorbance showed a stronger capacity for scavenging free radicals. The data are presented as a 50% inhibition concentration in µg/mL (IC_{50}) (Ferhat *et al*., 2017).

2.3.2. *ABTS-Cation radical scavenging activity ($ABTS^{+\cdot}$ assay)*

According to the literature (Re R., 1999) the ABTS-cation radical scavenging activity of the extracts was assessed. The 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) and $K_2S_2O_3$ were used to create a 7 mM $ABTS^{\bullet+}$ radical solution. Then, the same concentrations of the extracts used in the DPPH assay were tested to affect ABTS-cation radical scavenging at 734 nm after 10 min of incubation in the dark. The data are presented as a 50% inhibition concentration in µg/mL (IC_{50}) (Chemsa *et al*., 2016).

2.3.3. *Cupric reducing antioxidant capacity (CUPRAC assay)*

With a few minor adjustments, the procedure described by Apak was utilized to assess the antioxidant activity of the extracts in reducing cupric (Apak *et al*., 2004). The 50 µL of 10 mM Cu (II), 50 µL of 7.5 mM neocuproine, and 60 µL of NH_4Ac buffer (1 M, pH 7.0) solutions were added to each well in a 96-well plate. After that the same concentration, range of the extracts was used to measure the absorbance at 450 nm after 1-hour incubation at room temperature. For the purpose of comparing the activity, BHA and *α*-TOC were employed as standard antioxidants.

2.3.4. *β-Carotene/Linoleic acid assay*

The antioxidant activity of the obtained extracts was evaluated using a slightly modified version of the *β*-carotene-linoleic acid assay (Miller, 1971). For this purpose, 25 µL of linoleic acid and 200 µL of a Tween-40 emulsifier combination were added to 0.5 mg of *β*-carotene in 1 mL of chloroform. After vacuum-assisted chloroform evaporation, 100 mL of distilled water saturated with oxygen was added. Using a 96-well plate reader, the zero-time absorbance at 470 nm was determined as soon as the emulsion was applied to each tube. The emulsion system was incubated at 50 °C for two hours. Also, BHA and *α*-TOC were used as standard antioxidants.

2.3.5. *Ferrous ions chelating activity*

The chelating activity of the extracts on Fe^{2+} was determined using Ferene (Decker & Welch, 1990) with a few minor adjustments. 40 µL of 0.20 mM $FeCl_2$ was added to the extract solution, which was resolved in DMSO and methanol, respectively, to prepare solutions in eight different concentrations: 6.25, 12.5, 25, 50, 100, 200, 400, and 800 µg/mL. 80 µL of 0.5 mM ferene was added to start the reaction. After a 10-minutes incubation at room temperature, the mixture was measured at 562 nm. Also, EDTA was used as standard.

2.4. Quantification of Phenolic Compounds by LC-HR/MS

A reverse phase C18 column (150 x 3 mm x 5 µm particle size, Troyasil) and a Thermo Orbitrap Q-Exactive mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) were used for the LC-HR/MS investigations (İstanbul, Türkiye). LC-HR/MS analysis of the 100 phenolic standards was conducted with a Thermo Orbitrap Q-Exactive. The mobile phases A and B contained 1% formic acid in both the water and the methanol, respectively. The gradient programs were 50% A and 50% B for 0–1 min, 100% B for 1–3 min, 100% B for 3–6 min, 50% B for 6–7 min and 100% B for 7–15 min. The column temperature was set at 35 °C, and the mobile phase flow rate was 0.35 mL/min. The temperature and relative humidity were set at 22.0±5.0 °C and 50±15%, respectively. We selected the electrospray ionization (ESI) source for the applicable approach since it offers one of the best ionizations for small polar molecules. In the instrument's high-resolution mode, the ions with *m/z* values between 100 and 900 were scanned. By comparing retention times and HR-MS data with those of reference compounds

substances were identified. In order to lessen the repeatability issue brought on by external factors, such as ionization repeatability, in mass spectrometry measurements, dihydrocapsaicin was utilized as an internal standard for LC-HR/MS measurements. Quantitative analysis involves determining the amount of a phenolics in the extracts (µg/g extracts) using a calibration curve. All the phenolic compounds were bought and used as standards.

2.5. Statistical Analysis

All the assays were carried out in triplicate. The data were recorded as mean ± standard error meaning (SEM). Significant differences between means were determined using the student's *t*-test, while *p* values <0.05 were regarded as significant.

3. RESULTS

3.1. Strong Antioxidant Activity Profile

The hexane and methanol extracts of the seeds and cones of *C. sempervirens* were determined by five complementary methods, namely the *β*-carotene-linoleic acid assay for lipid peroxidation activity, DPPH•, ABTS•+ assays for radical-scavenging activity, and CUPRAC method and metal chelating methods as in Table 1. The antioxidant activity of the extracts studied was compared to standards such as BHA, *α*-TOC, and EDTA. According to the current findings, the activity of methanol extract obtained from seeds appears to be higher than that of cones in all antioxidant assays.

Table 1. Antioxidant activity of the extracts of *C. sempervirens* L.[a,b]

		DPPH assay	ABTS assay	CUPRAC assay	*β*-carotene/linoleic acid assay	Metal Chelating assay
		IC_{50}	IC_{50}	$A_{0,5}$	IC_{50}	IC_{50}
Seeds	CSHS	>200	411.55±4.13	149.97±1.45	>200	>200
	CSMS	24.08±1.06	6.08±0.19	18.60±0.63	30.90±1.11	>200
Cones	CSHM	>200	353.50±2.38	130.07±2.75	>200	>200
	CSMM	95.50±1.30	38.87±0.03	103.53±4.33	92.32±1.39	>200
Standards	BHA	28.59±0.06	7.23±0.01	24.49±0.19	1.34±0.04	nt
	α-TOC	36.35±0.24	27.70±0.28	134.53±0.19	2.10±0.08	nt
	EDTA	nt	nt	nt	nt	26.85±1.50

[a]IC_{50} values expressed are means ± SEM of three parallel measurements (p<0.05).
[b]IC_{50} and $A_{0.5}$ values are given as µg/mL.
[c]Reference compounds, BHA: Butylated hydroxy anisole; *α*-TOC: *α*-tocopherol; EDTA: Ethylenediaminetetraacetic acid.; nt: not tested.

In DPPH•, ABTS•+ and CUPRAC assays, both methanol extracts exhibited excellent activity, where the CSMS (IC_{50}: 24.08 ± 1.06, IC_{50}: 6.08 ± 0.19 and $A_{0.5}$: 18.60 ± 0.63 µg/mL, respectively) showed a higher activity than the standard BHA and *α*-TOC. In addition, methanol extract of the cones (CSMM) also exhibited good activity with an IC_{50}: 38.87±0.03 and $A_{0.5}$:103.53±4.33 in ABTS•+ scavenging and CUPRAC assays. The hexane extracts of the seeds and cones were less active in all assays when compared to the methanol extracts. In comparison to the other assays, all extracts demonstrated metal chelating with IC_{50}> 200 µg/mL by EDTA ferrous ions, which was used as a standard.

3.2. Qualitative and Quantitative Analysis of Phenolic Compounds by LC–HR/MS

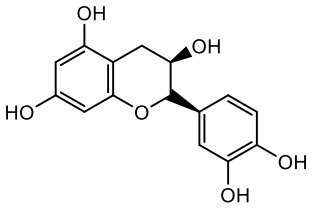
The LC-HR/MS, that stands for liquid chromatography-high resolution mass spectrometry, was used to analyze the phenolic compounds of the methanol extracts of the seeds and cones of *C. sempervirens* applying both positive and negative ionization modes. In this study, twenty-eight phenolic compounds were identified in the seeds while twenty-one phenolic compounds were identified in the cones of *C. sempervirens* (Table 2). The phenolic compounds (+)-catechin (6.735 µg/g extract), chlorogenic acid (0.018 µg/g extract), nepetin-7-glucoside (0.220 µg/g extract), scutellarein (1.743 µg/g extract), genistein (1.308 µg/g extract), kaempferol (.0383 µg/g extract), homogentisic acid (1.710 µg/g extract), and pyrogallol (0.343 µg/g extract) were identified in the seeds while they were not determined in the cones of *C. sempervirens*. And also, salicylic acid (0.115 µg/g extract) was determined only in the cones. The amounts of fumaric acid (93.518 µg/g extract), vanilic acid (37.008 µg/g extract), (-)-epicatechin (9.108 µg/g extract), quercetin (5.345 µg/g extract), hispidulin 7-glucoside (3.515 µg/g extract), hyperoside (2.783 µg/g extract), and quercitrin (2.530 µg/g extract) in the seeds are higher than those in the cones of *C. sempervirens* (Figure 1). It is well known that the identified phenolic compounds have potent antioxidant properties. For this reason, the LC-HR/MS results and the antioxidant activities were found to be correlated, indicating that the identified phenolic compounds in the sample were responsible for its antioxidant activity.

Table 2. Qualitative and quantitative analysis of the phenolic compounds in different organs of the *C. sempervirens* L. by LC-HR/MS.[a]

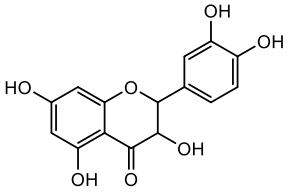
Phenolic Compounds	Molecular Formula	CSMS (seeds)	CSMM (cones)	U%
Ascorbic acid	$C_6H_8O_6$	8.948	27.623	3.94
(+)-Catechin	$C_{15}H_{14}O_6$	6.735	-	3.31
Chlorogenic acid	$C_{16}H_{18}O_9$	0.018	-	3.58
Fumaric acid	$C_4H_4O_4$	93.518	61.363	2.88
(-)-Epicatechin	$C_{15}H_{14}O_6$	9.108	1.458	3.17
(-)-Epicatechin gallate	$C_{22}H_{18}O_{10}$	0.065	0.073	3.05
(+)-*trans* taxifolin	$C_{15}H_{12}O_7$	0.438	0.070	3.35
Vanilic acid	$C_8H_8O_4$	37.008	32.515	3.49
Hyperoside	$C_{21}H_{20}O_{12}$	2.783	0.148	3.46
Aromadendrin	$C_{15}H_{12}O_6$	0.353	0.783	2.86
Apigenin 7-glucoside	$C_{21}H_{20}O_{10}$	1.250	0.053	3.59
Ellagic acid	$C_{14}H_6O_8$	0.188	0.185	4.20
Quercitrin	$C_{21}H_{20}O_{11}$	2.530	0.500	3.78
Myricetin	$C_{15}H_{10}O_8$	0.125	0.125	4.18
Nepetin-7-glucoside	$C_{22}H_{22}O_{12}$	0.220	-	3.07
Scutellarein	$C_{15}H_{10}O_6$	1.743	-	2.84
Quercetin	$C_{15}H_{10}O_7$	5.345	0.440	2.95
Salicylic acid	$C_7H_6O_3$	-	0.115	1.89
Naringenin	$C_{15}H_{12}O_5$	1.110	2.580	4.20
Luteolin	$C_{15}H_{10}O_6$	0.460	0.030	3.42
Genistein	$C_{15}H_{10}O_5$	1.308	-	3.28
Kaempferol	$C_{15}H_{10}O_6$	0.383	-	3.56
Apigenin	$C_{15}H_{10}O_5$	0.615	0.045	2.87
Chrysin	$C_{15}H_{10}O_4$	0.035	0.015	3.24
Homogentisic acid	$C_8H_8O_4$	1.710	-	4.35
Pyrogallol	$C_6H_6O_3$	0.343	-	4.50
Hispidulin 7-glucoside	$C_{22}H_{22}O_{11}$	3.515	0.633	4.57
Dihydrocaffeic acid	$C_9H_{10}O_4$	0.535	0.630	0.86
Chrysoeriol	$C_{16}H_{12}O_6$	0.560	0.063	2.08

[a] Values in µg/g extract.

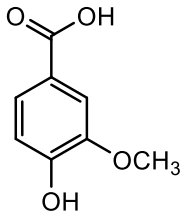
Figure 1. The major phenolic compounds of the seeds and cones of *C. sempervirens*.



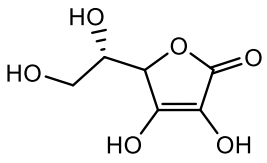
(-)-epicatechin



quercetin



vanilic acid



ascorbic acid

4. DISCUSSION and CONCLUSION

The phytochemical profiling of the seeds and cones of *C. sempervirens* growing in the Atatürk Forest involves the identification and quantification of phenolic compounds. There is much research on biological activity and chemical components of leaves of the cypress and its essential oils, in the literature (Batiha *et al*., 2022; Selim *et al*., 2014). However, there is lack of information and research on the cones and seeds of the *C. sempervirens,* especially on the phenolic compounds of the polar extracts. Phenolic compounds have been shown to have a wide range of health benefits, such as reducing the risk of chronic diseases such as cancer, cardiovascular disease, and neurodegenerative disorders. By analyzing the phenolic compounds present in a sample, researchers can gain insights into the potential antioxidant properties of the sample. One of the previous research on the ethanol extract obtained by maceration of seeds of *C. sempervirens* revealed that the only seven phenolic compounds together with the protocatechuic acid, (+)-catechin, and (−)-epicatechin which are major constituents by using RP-HPLC (Zengin *et al*., 2017). In the current study, the twenty-eight phenolic compounds which are the most potent antioxidant molecules were determined by using LC-HR/MS in the methanol extract of seeds (CSMS) of *C. sempervirens* which is obtained by using Soxhlet extractor. While vanilic acid and ascorbic acid were identified as major compounds in both seed and cones, (+)-catechin (6.735 µg/g extract) was analyzed in seed and naringenin (2.580 µg/g extract) was analyzed in higher amounts in cones. This can be seen as a positive result, as it helps to build upon the existing body of knowledge and reinforces the findings of the previous study.

The seeds and cones of the *C. sempervirens* were conducted *in vitro* experiments using five complementary methods to assess the antioxidant activity such as $DPPH^{\bullet}$, $ABTS^{\bullet+}$ scavenging assays, CUPRAC, *β*-carotene/linoleic acid, and metal chelating assays. The use of multiple complementary methods to assess antioxidant activity can provide a more comprehensive understanding of the antioxidant potential of seeds and cones. It can also help identify the types and quantities of antioxidants present in these plant parts and determine their potential health benefits. Especially, the seeds of the *C. sempervirens* exhibited strong antiradical potential in $DPPH^{\bullet}$ (IC_{50}: 24.08 µg/mL) and $ABTS^{\bullet+}$ (IC_{50}: 6.08 µg/mL) while showing the total antioxidant capacity with $A_{0.5}$: 18.60 µg/mL in CUPRAC assay. Moreover, an IC50 of 30.90 µg/mL obtained using the *β*-carotene/linoleic acid assay by analyzing the change in color of the

solution over time by oxidation is a measure of how methanol extract of the seeds protects β-carotene and linoleic acid. Also, Semerci investigated the DPPH free radical scavenging activity of the cones of *C. sempervirens* (Semerci *et al*., 2020).

Overall, most of the research showed the benefits of the essential oil of *C. sempervirens* seeds. Herein, investigation of the phytochemical composition and biological activity of the seeds and cones of *C. sempervirens* is an important step in understanding the potential health benefits of this plant. The study suggests that the methanol extract obtained from the seeds of *C. sempervirens* using Soxhlet extraction may contain bioactive secondary metabolites. These compounds could have potential applications in traditional medicine, as well as in the development of new drugs and nutraceuticals.

Acknowledgments

The author would like to thank Dr. Cagla Kizilarslan Hancer for plant identification.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

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