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Bioguided Isolation of Secondary Metabolites from *Salvia cerino-pruinosa* Rech. f. var. *cerino-pruinosa*

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Abstract: In the current study, the ethanol extracts prepared from the aerial parts and roots of an endemic species, *Salvia cerino-pruinosa* Rech. f. var. *cerino-pruinosa* were fractionated on silica gel columns and tested for determination of their antioxidant activity using DPPH free radical and ABTS cation radical scavenging, and cupric reducing antioxidant capacity (CUPRAC) test assays. Twenty known secondary metabolites were isolated from the active antioxidant fractions; rosmarinic acid **(1)**, chlorogenic acid **(2)**, caffeic acid **(3)**, 4-hydroxybenzoic acid **(4)**, benzoic acid **(5)**, luteolin 7-O-glucoside **(6)**, bis-(2-ethylhexyl)benzene-1,2-dicarboxylate **(7)**, salvianolic acid A **(8)**, salvianolic acid B **(9)**, 7-acetylroyleanone **(10)**, 6,7-dehydroroyleanone **(11)**, ferruginol **(12)**, inuroyleanol **(13)**, 12-hydroxy-6,7-secoabieta-8,11,13-triene-6,7-dial **(14)**, ursolic acid **(15)**, oleanolic acid **(16)**, taraxasterol **(17)**, lupenone **(18)**, *β*-sitosterol **(19)**, and stigmasterol **(20)**. Rosmarinic acid, which was obtained from the aerial parts, was found to be the best antioxidant compound among the isolated secondary metabolites in DPPH free radical and ABTS cation radical scavenging, and CUPRAC assays (IC_{50}: 1.20±0.04 µg/mL, IC_{50}: 1.74±0.06 µg/mL, $A_{0.5}$: 1.22±0.02 µg/mL, respectively). Chlorogenic and caffeic acids, luteolin 7-O-glucoside, salvianolic acids A and B, and inuroyleanol exhibited also high antioxidant activity in the mentioned assays.

Keywords: *Salvia cerino-pruinosa*; isolation; secondary metabolite; antioxidant.

1. Introduction

Salvia L. is one of the largest genera of the family of Lamiaceae (Labiatae) and is widespread especially in the Mediterranean region, South-East Asia, Central and South America. *Salvia* (Sage)

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species have been used to treat colds, bronchitis, tuberculosis, menstrual and digestive disorders, as well as being used as spice and tea throughout the world since ancient times [1]. In Turkey, *Salvia* species are named "adaçayı" and are commonly used as herbal tea due to their antiseptic, stimulant, diuretic, and wound healing properties [2]. *Salvia* species are generally known for their several pharmacological effects, including antibacterial [3], antituberculous [4] antiviral, cytotoxic [5], antioxidant [6-8], and cardiovascular [9] activities.

In Turkey, the genus *Salvia* is represented by over 100 taxa, half of which are endemic. Turkish *Salvia* species have been investigated phytochemically and biologically since 1968; many new and known secondary metabolites were isolated from their roots and aerial parts [6,10-15]. Phytochemical studies on *Salvia* species indicated the presence of various secondary metabolites belonging mainly to terpenoids, flavonoids, phenolic acids, phenolic glycosides, and other groups [6,10-20]. While the aerial parts of *Salvia* species contain especially flavonoid-, terpenoid- and steroid-type secondary metabolites, their roots are rich in diterpenoids. Sesquiterpenes and sesterterpenes are rarely found in *Salvia* species [17,18].

In this first phytochemical and biological study on *S. cerino-pruinosa* Rech. f. var. *cerino-pruinosa*, an endemic species grown in Turkey, the aerial parts and roots were extracted with ethanol, and then these extracts were fractionated on silica gel columns. Since the aim of this work was the bioguided isolation of antioxidant active secondary metabolites, the antioxidant capacity of the fractions was evaluated using DPPH free radical and ABTS cation radical scavenging and cupric reducing antioxidant capacity (CUPRAC) assays. Twenty known compounds, rosmarinic acid **(1)**, chlorogenic acid **(2)**, caffeic acid **(3)**, 4-hydroxybenzoic acid **(4),** benzoic acid **(5)**, luteolin 7-O-glucoside **(6)**, bis-(2-ethylhexyl)benzene-1,2-dicarboxylate **(7),** salvianolic acid A **(8)**, salvianolic acid B **(9)**, 7-acetylroyleanone **(10)**, 6,7-dehydroroyleanone **(11)**, ferruginol **(12)**, inuroyleanol **(13)**, 12-hydroxy-6,7-secoabieta-8,11,13-triene-6,7-dial **(14)**, ursolic acid **(15)**, oleanolic acid **(16)**, taraxasterol **(17)**, lupenone **(18)**, *β*-sitosterol **(19)**, and stigmasterol **(20)**, were isolated from the active fractions. The antioxidant potential of the isolated compounds was also determined using the mentioned methods.

2. Materials and Methods

2.1. General Experimental Procedures

1D- and 2D NMR spectra were obtained on Agilent Premium Compact 600 MHz instruments using TMS as an internal standard for chemical shifts. The Shimadzu 8040 LCMS-IT/TOF (LC-20 AD, SIL-20AC, DGU-20A3, CTO-20AC), Agilent GC-MS 7890 A, FTIR-ATR (Perkin Elmer Spectrum 100), UV-Visible (Perkin Elmer Lambda 25), Shimadzu Prep- HPLC-DAD, GL Sciences Preparative Columns (Inertsil ODS-3 10 μm, 10.0 mm × 250 mm), Shimadzu Scales (ATX224), Rotary Evaporator (Buchi L-100), Microplate Reader (Eon Biotek-960) were used as equipment. All chemicals used are of analytical purity and obtained from Sigma Aldrich.

2.2. Plant Material

Salvia cerino-pruinosa Rech. f. var. *cerino-pruinosa* was collected from Elazığ (Turkey) in May 2015 and identified by Dr. Mehmet Firat (Department of Biology, Faculty of Education, Yüzüncü Yıl University). A voucher specimen was deposited in the Herbarium of Yüzüncü Yıl University M. Firat 32538 (VANF) [19,20].

2.2. Extraction and Fractionation

The aerial parts and roots of *S. cerino-pruinosa* var. *cerino-pruinosa* were dried in the shade and then cut into small pieces. Both aerial parts (2000 g) and roots (1300 g) were macerated with ethanol at room temperature (10 L × 3). After filtration, the solvent was evaporated to dryness under vacuum. The extraction yields of the aerial parts (88 g extract) and roots (28 g extract) were 4.40% and 2.15%,

respectively. The aerial parts and roots ethanol extracts were fractionated on silica gel columns (5×150 and 2×100 cm, respectively) using petroleum ether (40-60°C) followed by a gradient of dichloromethane, acetone, methanol, and water then up to 50%. Eighty-eight fractions were obtained from the aerial parts and seventy-seven fractions from the roots. After combining similar fractions by TLC control, 14 fractions from the aerial parts, and 13 fractions from the roots were tested to determine their antioxidant activity (Table S1). TLC plates were visualized by spraying with cerium (IV) sulphate dissolved in 10% sulphuric acid following UV light checking. Silica gel and Sephadex LH-20 columns, preparative TLC, and preparative HPLC were used to isolate secondary metabolites from the active antioxidant fractions.

2.3. Preparative HPLC

Both aerial parts and roots polar fractions were obtained from silica gel columns using Acetone/MeOH (v/v, 9/1) solvent system. The most polar ones were fractionated on a C-18 column ($MeOH/H_2O$: 70/30 mobile phase). Further separations were carried out on preparative HPLC-DAD (Inertsil ODS-3 10 μm, preparative columns 10.0 mm × 250 mm) using mobile phase $MeOH/H_2O$: 50/50.

2.4. Antioxidant Activity Assays

DPPH free radical and ABTS cation radical scavenging, and cupric reducing antioxidant capacity (CUPRAC) assays were applied to determine antioxidant potential of the fractions, and secondary metabolites isolated from the aerial parts and roots ethanol extracts of *S. cerino-pruinosa* var. *cerino-pruinosa* [21-23].

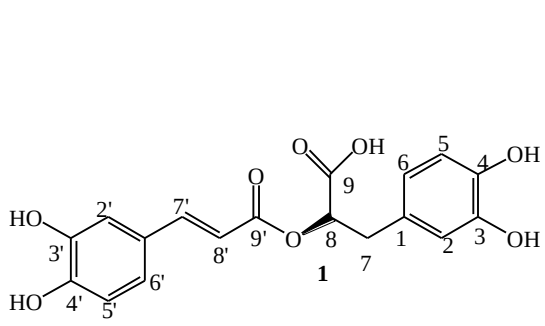
3. Results and Discussion

3.1. Antioxidant Activity of the Fractions

As shown in Table S1, the fractions obtained from the aerial parts' ethanol extract of *S. cerino-pruinosa* var. *cerino-pruinosa* exhibited higher antioxidant activity than those of the fractions obtained from its roots' ethanol extract. Some of the fractions exhibited better antioxidant activity than those of the standard compounds, butylated hydroxytoluene (BHT) and α-tocopherol (α-TOC). Fractions from the aerial parts and the roots showed the best antioxidant potential in DPPH free radical, ABTS cation radical scavenging, and CUPRAC assays which were used to isolate secondary metabolites that could be responsible from the activity.

3.2. *Isolation of the Secondary Metabolites*

Rosmarinic acid **(1)**, chlorogenic acid **(2)**, and luteolin 7-O-glucoside **(6)** were isolated from SCCAPE-65-69, caffeic acid **(3)** from SCCAPE-70-75, 4-hydroxybenzoic **(4)**, and benzoic **(5)** acids from SCCAPE-48-53, salvianolic acid A **(8)** and salvianolic acid B **(9)** from SCCAPE-80-85 fractions by preparative HPLC using $MeOH/H_2O$ (v/v, 50/50) mobile phase (Table S2). Rosmarinic acid **(1)** was also isolated from SCCAPE 54-60 and SCCAPE 76-79. Bis-(2-ethylhexyl)benzene-1,2-dicarboxylate **(7)** was obtained from both aerial part and root fractions (SCCAPE-54-60, SCCRE-45-50 and SCCRE-51-55) by preparative TLC (petroleum ether/dichloromethane:1/3 solvent system).



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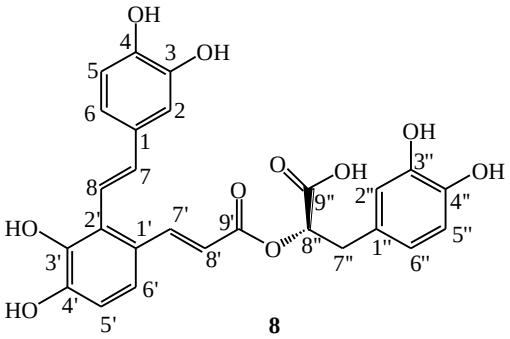

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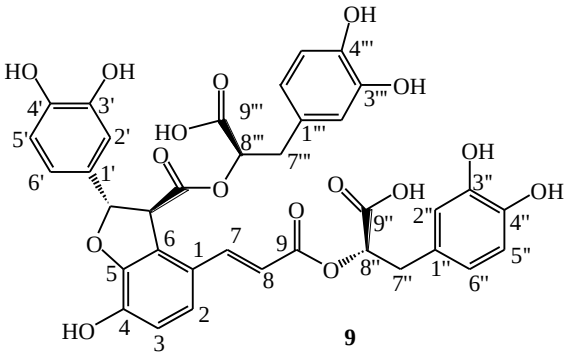

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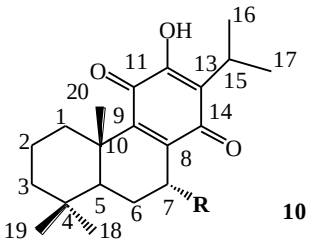
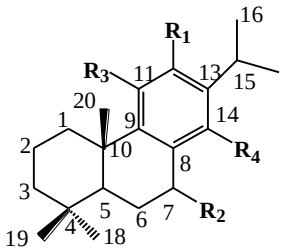


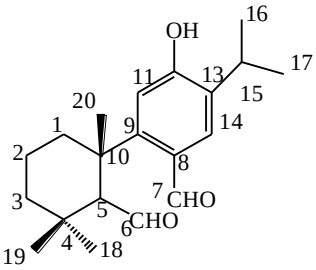
R

10 $COCH_3$

11 H, C_6-C_7: C = C



	R_1	R_2	R_3	R_4
12	OH	H	H	H
13	OCH_3	=O	OH	OH



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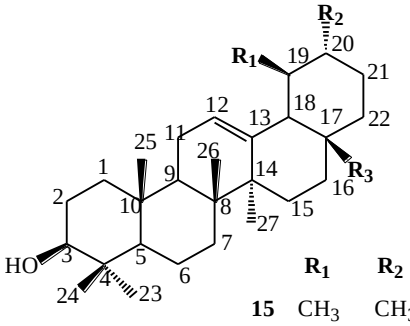
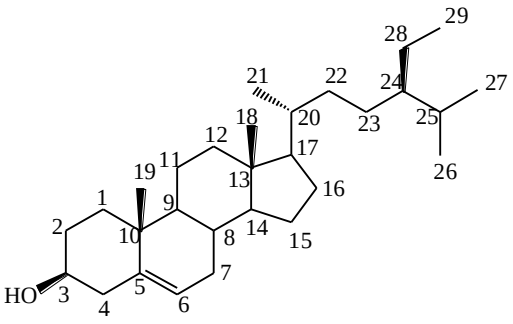


	R_1	R_2	R_3
15	CH_3	CH_3	CO_2H
16	H_2	$-(CH_3)_2$	CO_2H
17	CH_3	$=CH_2$	CH_3



19 C_{22}-C_{23}: C - C

20 C_{22}-C_{23}: C = C

Figure 1. Rosmarinic acid **(1)**, Salvianolic acid A **(8)**, Salvianolic acid B **(9)**, 7-Acetylroyleanone **(10)**, 6,7-Dehydroroyleanone **(11)**, Ferruginol **(12)**, Inuroyleanol **(13)**,12-Hydroxy-6,7-secoabieta-8,11,13-triene-6,7-dial **(14)**, Ursolic acid **(15)**, Oleanolic acid **(16)**, Taraxasterol **(17)**, *β*-Sitosterol **(19)**, and Stigmasterol **(20)**.

7-Acetylroyleanone **(10)** and 6,7-dehydroroyleanone **(11)** were isolated from the root fractions (SCCRE-18-24-(16-18)-9, SCCRE-25-28-(17-28)-7, respectively) by preparative TLC using petroleum ether/dichloromethane:1/2 solvent system. Ferruginol **(12)** were obtained from both aerial part and root fractions (SCCAPE-23-28-12**,** SCCRE-25-28-(17-18)-6) by preparative TLC using dichloromethane/acetone: 9.8/0.2 solvent system, inuroyleanol **(13)** from SCCRE-25-28-(17-18)-11) (preparative TLC, 100% dichloromethane), 12-hydroxy-6,7-secoabieta-8,11,13-triene-6,7-dial **(14)** from SCCRE-18-24 (16-18)-15 (preparative TLC, petroleum ether/dichloromethane: 1/6 solvent system).

Ursolic acid **(15)** and oleanolic acid **(16)** were isolated from several aerial and root parts fractions (preparative TLC, dichloromethane/acetone: 9/1 solvent system) [24], taraxasterol **(17)**, and lupenone **(18)** from the aerial part fractions (SCCAPE 41-47-(12)-3 and SCCAPE 41-47-(12)-1, respectively, preparative TLC, petroleum ether/dichloromethane: 1/3 solvent system), *β*-sitosterol **(19)** and stigmasterol **(20)** from several aerial part and root fractions (preparative TLC, petroleum ether/dichloromethane: 1/9 and dichloromethane/acetone: 9.8/0.2 solvent systems). As indicated in the literature, diterpenes (**10-14**) were isolated from the roots of *S. cerino-pruinosa* var. *cerino-pruinosa* and other compounds from its aerial parts (Table S2).

3.3. Structure Elucidation

Twenty known secondary metabolites were isolated from the antioxidant active fractions: seven phenolic acids (rosmarinic acid **(1)** [25], chlorogenic acid **(2)** [26], caffeic acid **(3)** [25], 4-hydroxybenzoic acid **(4)**, benzoic acid **(5)** [27], salvianolic acid A **(8)** [28], salvianolic acid B **(9)** [16]), a flavone (luteolin 7-*O*-glucoside **(6)** [29]), one phthalate ester (bis-(2-ethylhexyl)benzene-1,2-dicarboxylate (7) [24]), five abietane-type diterpenes (7-acetylroyleanone **(10)** [30], 6,7-dehydroroyleanone **(11)** [31], ferruginol **(12)** [32], inuroyleanol **(13)** [33],12-hydroxy-6,7-secoabieta-8,11,13-triene-6,7-dial **(14)** [34]), four triterpenes (ursolic acid **(15)**, [24], oleanolic acid **(16)** [24, taraxasterol **(17)** [35], lupenone **(18)** [36]), two steroids (*β*-sitosterol **(19)** [24], stigmasterol **(20)** [37]) (Figure 1). Their structures were elucidated by spectroscopic methods (UV, IR, ^{1}H- and ^{13}C-NMR (APT), HMQC, HMBC, Mass). The spectroscopic data of the isolated compounds were compared with those given in the literature (Figure S1-S93).

3.4. Antioxidant Activity of the Isolated Secondary Metabolites

In this study, CUPRAC method for ferruginol, and 6,7-dehydroroyleanone, ABTS and CUPRAC methods for inuroyleanol were used for the first time to determine the antioxidant activity while DPPH, ABTS, and CUPRAC were applied for the first time for 7-acetylroyleanone, 12-hydroxy-6,7-secoabieta-8,11,13-triene-6,7-dial, and bis-(2-ethylhexyl)benzene-1,2-dicarboxylate (Table 1).

The antioxidant capacity of the isolated compounds was determined by DPPH free radical and ABTS cation radical scavenging, and CUPRAC assays (Table 2). Among the tested compounds, rosmarinic acid exhibited the best antioxidant capacity in DPPH (IC_{50}: 1.20±0.04 µg/mL), ABTS (IC_{50}: 1.74±0.06 µg/mL), and CUPRAC methods ($A_{0.5}$: 1.22±0.02 µg/mL). Besides, chlorogenic acid and caffeic acid, luteolin 7-*O*-glucoside, salvianolic acids A and B, and inuroyleanol showed significant antioxidant activity in the tested assays.

7-Acetylroyleanone (IC_{50}: 12.17±0.16 µg/mL), 6,7-dehydroroyleanone (IC_{50}: 16.84±0.18 µg/mL), ursolic acid (IC_{50}: 20.04±1.04 µg/mL), taraxasterol (IC_{50}: 18.93±0.11 µg/mL) and lupenone (IC_{50}: 19.64±10.03 µg/mL) indicated high activity in DPPH free radical scavenging, benzoic acid (IC_{50}: 13.29±0.39 µg/mL) and ferruginol (IC_{50}: 6.61±0.27 µg/mL) in ABTS cation radical scavenging, and 4-hydroxybenzoic acid ($A_{0.5}$: 18.83±0.11 µg/mL), oleanolic acid ($A_{0.5}$: 11.72±0.06 µg/mL), and *β*-sitosterol ($A_{0.5}$: 22.17±0.98 µg/mL) in CUPRAC methods (Table 1).

Table 1. Antioxidant activity of the isolated compounds (**1-20**)

Compounds number	Compounds	Antioxidant Activity*		
		IC_{50} (µg/mL)	IC_{50} (µg/mL)	$A_{0.5}$(µg/mL)
		DPPH	ABTS	CUPRAC
1	Rosmarinic acid	1.20±0.04	1.74±0.06	1.22±0.02
2	Chlorogenic acid	6.40±0.04	3.32±0.06	13.98±0.13
3	Caffeic acid	3.52±0.07	1.94±0.04	7.12±0.05
4	4-Hydroxybenzoic acid	28.03±1.51	365.63±6.32	18.83±0.11
5	Benzoic acid	72.53±0.12	13.29±0.39	75.30±1.74
6	Luteolin 7-*O*-glucoside	5.75±0.04	3.59±0.06	3.79±0.04
7	Bis-(2-ethylhexyl)benzene-1,2-dicarboxylate	>1000	280.50±3.81	213.80±5.70
8	Salvianolic acid A	4.06±0.04	2.66±0.07	4.76±0.01
9	Salvianolic acid B	4.31±0.03	3.51±0.03	13.90±0.07
10	7-Acetyl royleanone	12.17±0.16	27.64±1.05	35.55±1.02
11	6,7-Dehydroroyleanone	16.84±0.18	55.73±6.33	28.56±1.04
12	Ferruginol	33.80±2.20	6.61±0.27	49.83±2.02
13	Inuroyleanol	2.21±0.06	5.11±1.13	7.12±0.02
14	12-Hydroxy-6,7-secoabieta-8,11,13-triene-6,7-dial	>1000	54.94±0.27	72.40±1.10
15	Ursolic acid	20.04±1.04	104.44±8.06	178.09±5.02
16	Oleanolic acid	45.02±1.31	275.73±67.11	11.72±0.06
17	Taraxasterol	18.93±0.11	75.26±2.19	173.55±5.43
18	Lupenone	19.64±10.03	75.98±1.84	40.66±1.20
19	*β*-Sitosterol	72.11±1.94	154.34±2.12	22.17±0.98
20	Stigmasterol	>1000	312.75±4.92	172.48±1.15
	α-TOC	9.94±0.12	17.20±0.65	9.34±0.05
	BHT	59.12±1.12	12.93±0.84	5.12±0.02

*: Values expressed are means ± S.D. of three parallel measurements, and values were calculated according to a negative control.

Yener et al. [7] reported that luteolin 7-*O*-glucoside, rosmarinic, chlorogenic, caffeic, 4-hydroxybenzoic, and benzoic acids showed similar antioxidant activity in the three mentioned antioxidant methods, as indicated in this study. The antioxidant activity of inuroyleanol was determined using superoxide anion radical and DPPH free radical scavenging activity, and *β*-carotene bleaching assays. As reported in the present study, inuroyleanol exhibited high activity [38].

A literature survey showed that 6,7-dehydroroyleanone showed higher DPPH free radical scavenging effect than ABTS cation radical scavenging activity [38], ferruginol possessed high ABTS cation radical scavenging effect [15], *β*-sitosterol, stigmasterol, lupenone, ursolic and oleanolic acids, and taraxasterol exhibited moderate antioxidant activity in DPPH, ABTS and CUPRAC assays [40,41] as indicated in the current work.

As a result, the mentioned antioxidant secondary metabolites isolated from *S. cerino-pruinosa* var. *cerino-pruinosa* have potential to be used in pharmaceutical, cosmetic and food industries.

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Conflict of interest statement

We declare that we have no conflict of interest.

Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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