

# Phytochemical Analysis and Antioxidant Activity of *Salvia chloroleuca* Aerial Extracts

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## Abstract

**Background:** *Salvia*, known as Maryam Goli in the Persian language, is an important genus that includes approximately 900 species in the Lamiaceae family. There are 58 *Salvia* species growing naturally in Iran, including *Salvia chloroleuca* Rech. f. and Allen., which grows wild in the northeastern and central parts of the country.

**Objectives:** This study was designed to determine the chemical composition, in vitro antioxidant activity, and total phenol content of various extracts of *S. chloroleuca*.

**Materials and Methods:** Dried aerial parts of the plant were crushed, then sequentially extracted with n-hexane, ethyl acetate, and methanol. The fractions of *S. chloroleuca* were subjected to silica gel column chromatography and Sephadex LH-20. The antioxidant activities of these extracts were measured by ferric reducing antioxidant power (FRAP), and the total phenolic contents of the extracts were evaluated using Folin-Ciocalteu reagent.

**Results:** The separation and purification processes were carried out using different chromatographic methods. Structural elucidation was on the basis of <sup>1</sup>H-NMR and <sup>13</sup>C-NMR spectral data, in comparison with that reported in the literature. The isolated compounds were salvigenin (1), luteolin (2), cirsiolol (3),  $\beta$ -sitosterol (4), and daucosterol (5). Ethyl acetate extract displayed the highest level of total antioxidants and total polyphenols compared to the other analyzed extracts (n-hexane and methanol). In the FRAP assay, ethyl acetate extract had the highest (230.4 ± 10.5) FRAP value, followed by methanol (211.4 ± 8.3) and n-hexane (143.4 ± 12.04). Total phenol contents were calculated to be 13.8 ± 0.3, 58.25 ± 0.05, and 43.48 ± 0.38 mg of gallic acid/100 g in the n-hexane, ethyl acetate, and methanol extracts, respectively.

**Conclusions:** The above-mentioned compounds were isolated for the first time from *S. chloroleuca*. The antioxidant activity of this plant could be in part related to isolated flavonoids and sterols. The results of this study indicated that *S. chloroleuca* could be an important dietary source of phenolic compounds with high antioxidant capacity.

**Keywords:** Lamiaceae, Phytochemical, Antioxidant, Flavonoid, Sterol, *Salvia chloroleuca*

## 1. Background

It is well known that free radicals, including reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced in vivo, such as hydroxyl radicals, superoxide anions, and hydrogen peroxide, are extremely reactive and transient chemical species (1). They can damage proteins, DNA, and lipids. It has been reported that imbalances between ROS formation and scavenging systems contributes to the pathogenesis of different conditions, including aging, Alzheimer's disease, diabetes mellitus, atherosclerosis, and hypertension (2). Antioxidants can inhibit or delay oxidative chain reactions in lipids, proteins, carbohydrates, and DNA (3). Several studies have proposed that natural antioxidants may be less toxic than synthetic ones, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (4). *Salvia* is an important genus that

includes approximately 900 species in the Lamiaceae family and is known as Maryam Goli in the Persian language. There are 58 *Salvia* species growing naturally in Iran, 17 of which are endemic. *Salvia* species have been used in traditional medicine throughout the world as tonics, anti-rheumatics, antimicrobials, and carminatives (5, 6). It has been speculated that *Salvia* originated in Afghanistan, although the highest number of species (about 250) are found in Mexico (7). Traditionally, crude extracts of *Salvia* species have been used for various purposes in foods, drugs, and perfumery (8). Several biological studies have reported considerable anti-inflammatory, antioxidant, antibacterial, and antitumor effects of this genus (9, 10).

*Salvia chloroleuca* Rech. f. and Allen. is one of Iran's endemic species. A review of the literature revealed no reports on the non-volatile constituents of the aerial part of *S. chloroleuca*. The chemical constituents of the essential

oil of *S. chloroleuca* have been investigated, and the main components were  $\alpha$ -pinene,  $\beta$ -pinene,  $\beta$ -caryophyllene, 1,8-cineole, and carvacrol (11). The structural elucidations of the isolated compounds were established according to <sup>1</sup>H-NMR and <sup>13</sup>C-NMR spectral data.

## 2. Objectives

To our knowledge, there are no studies in the literature on the chemical composition of the non-volatile constituents of the aerial part of *S. chloroleuca*. Hence, the objective of the present study was to isolate and identify some of the phytochemical constituents of *S. chloroleuca*. The total phenol content and potential antioxidant activities of the extracts were also evaluated using the ferric reducing antioxidant power (FRAP) method.

## 3. Materials and Methods

### 3.1. Plant Materials

The aerial parts of *S. chloroleuca* were collected from the Shahrestanak region in Tehran province, Iran, in May 2013, and identified by Dr. Y. Ajanii. A voucher specimen (ACECR-244) was deposited at the herbarium of the medicinal plants research center in the faculty of pharmacy at Tehran University of Medical Sciences.

### 3.2. Extraction and Isolation

The air-dried, powdered aerial parts of *S. chloroleuca* (2.5 kg) were extracted successively with n-hexane (3 × 9 L), ethyl acetate (3 × 9 L), and methanol (3 × 9 L) by maceration at room temperature. A rotary vacuum evaporator was used to concentrate the solution. The n-hexane portion (8 g) was subjected to silica gel column chromatography (230-400 mesh) with chloroform, chloroform: ethyl acetate (8: 2, 5: 5), and ethyl acetate. After screening with TLC, fractions with similar compositions were pooled in order to yield five combined fractions (A-E). The C fraction (320 mg) was subjected to Sephadex LH-20 column chromatography with methanol to give ten fractions (C<sub>1</sub>-C<sub>10</sub>). For more purification, the C<sub>5</sub> (17 mg) portion was submitted to Sephadex LH-20 with methanol: ethyl acetate (6: 4) to give pure compound 4 (4 mg). The ethyl acetate (25 g) was subjected to silica gel column chromatography with chloroform, ethyl acetate: methanol (8: 2, 6: 4, 2: 8), and methanol to give six main fractions (A-F). Fraction D (3.3 g) was submitted to the silica gel column with ethyl acetate: methanol (8: 2, 5: 5) and methanol to yield 13 parts (D<sub>1</sub>-D<sub>13</sub>). For more purification, the D<sub>1</sub>, D<sub>6</sub>, and D<sub>12</sub> parts were subjected to Sephadex LH-20 with methanol: ethyl acetate (9:1) as eluent to give pure compounds 1 (28 mg), 2 (20 mg), and

3 (15 mg), respectively. The methanol extract (30 g) was successively chromatographed on the silica gel column with ethyl acetate, ethyl acetate: methanol (5:5, 2:8), methanol, and water, in order to gain eight fractions (A-H). Fraction G (500 mg) was subjected to silica gel column with ethyl acetate: methanol (5: 5, 1: 9) and methanol to yield ten fractions (G<sub>1</sub>-G<sub>10</sub>). G<sub>6</sub> (45 mg) was subjected to reverse-phase HPLC using a gradient solvent system consisting of water-methanol (20: 80, 40: 60, 80: 20) and methanol to give compound 5 (7 mg) (12).

### 3.3. Instruments and Chemicals

The <sup>1</sup>H and <sup>13</sup>C-NMR spectra were measured on a Bruker DRX-500 spectrometer with tetramethylsilane as an internal standard, and the chemical shifts are given in  $\delta$  (ppm). Spectrophotometric measurements were performed with a UV-VIS spectrophotometer 2.2. (double-beam) Specord 200 Analytik Jena GmbH, Germany. Silica gel (70 - 230 and 230 - 400 mesh, Merck) was used for column chromatography. Silica TLC was conducted on Merck F254 silica gel plates. The spots were detected by spraying an anisaldehyde-H<sub>2</sub>SO<sub>4</sub> reagent followed by heating (120°C for 5 minutes). HPLC was used on a Knauer model with Vertex (Knauer, Germany) column C18 (250 × 20 mm ID). The UV detector was a PDA, and the injection volume was 2 mL. Ferrous chloride was purchased from Merck (Darmstadt, Germany). Folin-Ciocalteu, gallic acid, and TPTZ (2, 4, 6-tripyridyl-s-triazine) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All solvents and chemicals used in the experiments were of analytical or HPLC grade.

### 3.4. FRAP Assay

The potential antioxidant capacities of the different extracts were determined with the FRAP assay by measuring their abilities to reduce Fe<sup>3+</sup> to Fe<sup>2+</sup> (13). Briefly, 100 and 500  $\mu$ g/mL of each sample were added to 1.5 mL of the freshly prepared FRAP solution, which contained 2.5 mL of 10 mM TPTZ solution in 40 mM HCl. Next, 2.5 mL of 20 mM FeCl<sub>3</sub>.6H<sub>2</sub>O was diluted in 25 mL of 0.3 M sodium acetate buffer (PH = 3.6) and allowed to remain at room temperature for 90 min, then the absorbance of the solution was recorded at 595 nm. The standard curve was prepared using different concentrations of FeSO<sub>4</sub>.7H<sub>2</sub>O, and the absorbances were read for the samples. The antioxidant power was calculated as grams of FeSO<sub>4</sub>.7H<sub>2</sub>O equivalent per grams of sample.

### 3.5. Total Phenol Assay

The total phenol concentrations of the extracts were calculated spectrophotometrically using the Folin-Ciocalteu procedure with little modification (14).

Lyophilized extract (0.5 mL) was soluted in methanol, then added to 2.5 mL of Folin's reagent (diluted to 1: 10), and the solution was left at room temperature for 5 minutes. Finally, 2 mL of Na<sub>2</sub>CO<sub>3</sub> (7.5% v/v) was added to the mixture after 90 minutes of incubation. The sample absorbance was read at 760 nm, and the standard curve was prepared using various concentrations of gallic acid as the standard. Total phenol content was expressed as  $\mu\text{g}$  of gallic acid equivalent per mg of extract. The data presented are the averages of four measurements.

#### 4. Results

We reported the isolation and structural elucidation of salvigenin (5-hydroxy-4',6,7-trimethoxy flavone), luteolin (3',4',5,7-tetrahydroxyflavone), cirsiolol (5,3,4-trihydroxy 6,7-dimethoxyflavone),  $\beta$ -sitosterol, and daucosterol (Figure 1) on the basis of spectroscopic data. These compounds were isolated and described here for *S. chloroleuca* for the first time. The structures of the isolated compounds were established mainly by comparisons of their <sup>1</sup>H and <sup>13</sup>C-NMR spectral data with those reported in the literature, and the results were consistent with our previously published data (15-17). The complete <sup>1</sup>H and <sup>13</sup>C-NMR analyses of isolated flavonoids (1 to 3) and sterols (4 and 5) are summarized in Tables 1 and 2, respectively.

The total phenolic contents of the aerial parts of *S. chloroleuca* were measured as  $13.8 \pm 0.3$ ,  $58.25 \pm 0.05$ , and  $43.48 \pm 0.38$  mg gallic acid/100 g for the n-hexane, ethyl acetate, and methanol extracts, respectively. Among all extracts tested, ethyl acetate ( $230.4 \pm 10.5$ ) had the highest FRAP value, followed by methanol ( $211.4 \pm 8.3$ ) and n-hexane ( $143.4 \pm 12.04$ ). FRAP values were reported as the weight (g) of FeSO<sub>4</sub> in 100 g of plant extract.

#### 5. Discussion

Column chromatography of the crude extract from the aerial part of *S. chloroleuca* led to the isolation and identification of three flavonoids (salvigenin, luteolin, and cirsiolol) and two steroids ( $\beta$ -sitosterol and daucosterol). These compounds were isolated for the first time from this plant. There was a significant correlation between total phenolic content and FRAP value, and this result is compatible with previous studies on *Salvia* (18). Ethyl acetate extract exhibited the highest total phenol and FRAP, which may be due to the existing flavonoids in this portion, while the n-hexane portion had the lowest total phenol and FRAP. The species of *Salvia* are characterized by 6-hydroxyflavones and 6-methoxyflavones. Luteolin and salvigenin are the major flavones distributed in the extract

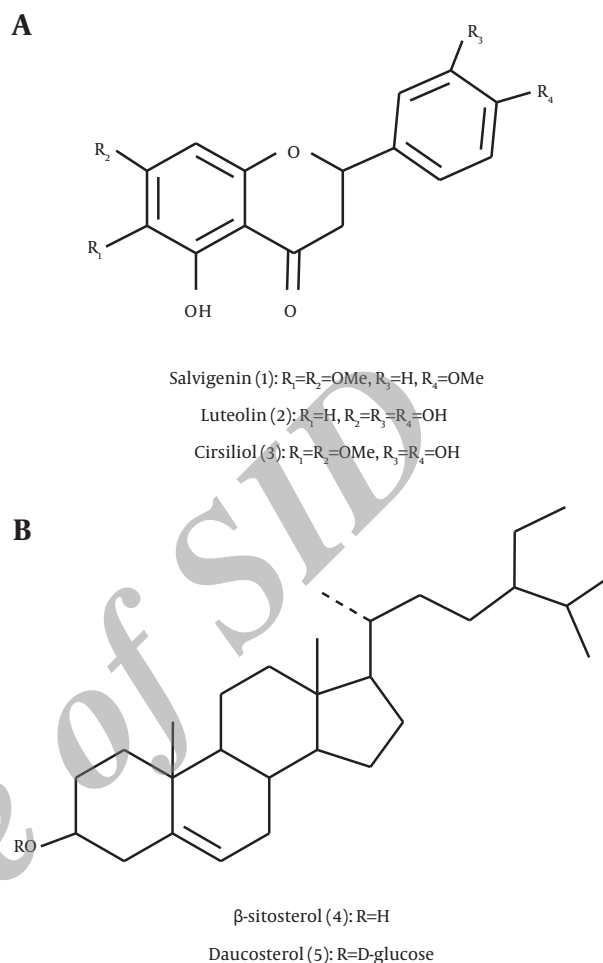


Figure 1. Structure of Isolated Compounds From the Aerial Part of *S. chloroleuca*

from the aerial part of *Salvia* (5). The potential antioxidant activities of the aerial part of *Salvia* species have previously been demonstrated with various methods (18). The antioxidant ability of polyphenolic compounds is derived from their ability to prevent lipid peroxidation and redox reactions (19). Flavonoids are classes of phenolic compounds that exhibit antioxidant activities (20). Therefore, it is reasonable to assume that the observed antioxidant activity of *S. chloroleuca* extract in our study could be in part related to its flavonoid content. These phenolic compounds can act as hydrogen donors, reducing agents, and free-radical quenchers (21). It has been demonstrated that flavonoids prevent oxidation of low-density lipoprotein (LDL), and they reduce thrombotic tendencies (22). The consumption of flavonoids in dietary plants may also decrease the risk of death from coronary heart disease in humans (23). Consequently, the antioxidant activities of phenolic com-

**Table 1.** <sup>1</sup>H and <sup>13</sup>C-NMR Data of Isolated Flavonoids From the Aerial Part of *S. chloroleuca*.  $\delta$  in ppm, J in Hz

Position	Salvigenin		Luteolin		Cirsiliol	
	$\delta_H$	$\delta_C$	$\delta_H$	$\delta_C$	$\delta_H$	$\delta_C$
2		163.5		164.4		164.6
3	6.93 (s)	103.3	6.67 (s)	103.3	6.90 (s)	103.1
4		182.2		182.1		182.5
5		152.6		161.9		152.5
6		131.8	6.19 (d, J = 2.05)	99.2		131.5
7		158.6		164.4		159.0
8	6.95 (s)	91.6	6.44 (d, J = 2.05)	94.2	6.74 (s)	91.8
9		152.6		157.7		153.0
10		105.1		104.1		105.4
1'		122.7		122.0		121.8
2'	8.07 (d, J = 9)	128.3	7.39 (d, J = 2.2)	113.7	7.45 (d, J = 2.5)	113.9
3'	7.12 (d, J = 9.05)	114.5		146.1		146.2
4'		162.4		149.9		150.3
5'	7.12 (d, J = 9.05)	114.5	6.88 (d, J = 8.3)	116.4	6.91 (d, J = 2.5)	116.3
6'	8.07 (d, J = 9)	128.3	7.42 (dd, J = 2.2, J = 8.3)	119.3	7.46 (dd, J = 2.5, J = 8)	119.4
5-OH	12.88		12.9			
6-Me		60.0				60.4
7-Me		56.4				56.8
4'-Me		55.6				

pounds in *Salvia* extract are probably part of the reason they are valuable in the prevention and treatment of heart disease. Luteolin and salvigenin have been used in some countries as food additives to prevent lipid peroxidation (24-26). Salvigenin occurs throughout the Lamiaceae family, especially in the *Salvia* genus, although it has been identified in other *Achillea* species. Salvigenin possesses a wide range of biological activities, including antioxidant, antibacterial, antitumor, and immunomodulatory activities; inhibition of the growth of malarial parasites; and vasorelaxant effects in isolated rat aortas (27-29). Cirsiliol has previously been isolated from other *Salvia* species. It is the most potent inhibitor of arachidonate 5-lipoxygenase, an enzyme responsible for leukotriene biosynthesis (5, 30).

In conclusion, the results of this study indicated that *S. chloroleuca* could be an important dietary source of phenolic compounds with high antioxidant capacities. With

**Table 2.** <sup>1</sup>H and <sup>13</sup>C-NMR Data of Isolated Sterols From the Aerial Part of *S. chloroleuca*.  $\delta$  in ppm, J in Hz

Position	$\beta$ -sitosterol		Daucosterol	
	$\delta_H$	$\delta_C$	$\delta_H$	$\delta_C$
1		37.4		37.4
2		31.6		30.2
3	3.52 (m, 1H)	71.8	3.48 (1H, m)	78.5
4		42.3		39.2
5		140.9		140.9
6	5.34 (s, 1H)	121.9	5.33 (m, 1H)	121.9
7		31.8		31.8
8		31.8		31.8
9		50.2		50.2
10		36.1		36.8
11		21.1		21.1
12		39.8		39.8
13		42.2		42.2
14		56.7		56.8
15		24.3		24.3
16		28.3		28.3
17		56.1		56.1
18	0.68 (s, 3H)	11.8	0.65 (s, 3H)	11.8
19	1.01 (s, 3H)	19.8	1.01 (s, 3H)	19.3
20	0.92 (d, 3H, J = 6.5)	36.3		36.3
21		18.8	0.91 (d, 3H, J = 6.2)	18.9
22		34.1		34.1
23		26.1		26.2
24		45.8		45.9
25		29.1		29.2
26	0.83 (m, 3H)	19	0.82 (d, 3H, J = 6.6)	19.0
27	0.84 (m, 3H)	19.4	0.80 (d, 3H, J = 6.6)	19.8
28	0.84 (m, 2H)	23.1		23.2
29	0.84 (m, 3H)	12.0		12.0
G-1			4.23 (d, 1H, J = 7.7)	102.5
G-2			3.03 (m, 1H)	75.3
G-3			3.08 (m, 1H)	78.6
G-4			3.05 (m, 1H)	71.6
G-5			3.05 (m, 1H)	78.1
G-6			3.65 (m, 2H)	62.5

regard to the presence of natural antioxidants with valuable biological activities, such as flavonoids and sterols, it is proposed to evaluate the effect of *S. chloroleuca* extract on

important ailments, including cardiovascular disease and Alzheimer's.

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## Footnotes

**Authors' Contribution:** Study concept and design, Iraj Salimikia, Hamid Reza Monsef-Esfahani, Ahmad Reza Gohari; analysis and interpretation of data, Ahmad Reza Gohari, Mehrnoosh Salek; drafting of the manuscript, Iraj Salimikia, Ahmad Reza Gohari; critical revision of the manuscript for important intellectual content, Ahmad Reza Gohari, Iraj Salimikia.

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