

## Extraction and separation of Antioxidative Compounds from *Salvia leriifolia* Leaves

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### ABSTRACT

In this research, methanolic extraction of *Salvia leriifolia* leaves produced a higher yield and antioxidative activity than other organic solvents (ethanol, acetone, chloroform, n-hexane). The methanolic extract was reserved about 24 hours in the refrigerator and its precipitates were then separated. The extract was separated into 12 fractions by thin-layer chromatography (TLC). The highest yields were found in fractions with  $R_f$  values of 0.29, 0.54, 0.11 and 0.38 at 16.24%, 12.48%, 8.81% and 7.60%, respectively. All fractions and also whole methanolic extract and precipitates of methanolic extract had more antioxidative activity than the control based on the thiocyanate method. Whole methanolic extract, precipitates of methanolic extract and most separated fractions showed more antioxidative activity than  $\alpha$ -tocopherol. The fraction with  $R_f$  value of 0.29 at 16.24% yield and 85.61% antioxidative activity of synthetic antioxidant BHT based on thiocyanat method was chosen as the fraction with the highest yield and the highest antioxidative activity.

**Keywords:** Antioxidant activity, *Salvia*.

### INTRODUCTION

Incorporation of antioxidants in foods is one of the most effective means to retard the oxidation of lipids, increase the shelf life and the stability of lipids and lipid-containing foods, and preventing loss of sensory and nutritional quality. The most widely used antioxidants are synthetic ones like butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertbutylhydroquinone (TBHQ) and propyl gallate (PG). However, their use has been questioned because of possible toxic and carcinogenic components formed during their degradation [5, 8]. Because of these health concerns, natural antioxidants, such as tocopheroles and ascorbic acid, are widely used

as safe antioxidants. Nevertheless, they are not as effective as synthetic antioxidants and their manufacturing costs are high. Therefore, recently attention has been focused on the isolation, characterization and utilization of inexpensive and effective antioxidants from natural products [9].

The present study describes the extraction and separation of antioxidative components from *Salvia leriifolia* leaves and the characterization of the fraction having the highest yield and the most antioxidative activity. *Salvia leriifolia* grows in the southern and climatically warm and dry regions of Khorassan and Semnan provinces of Iran. This plant was introduced in Flora Iranica in 1982 [11]. Species of the genus *Salvia*, Labiatae, are generally known for their anti-

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oxidative activity [2, 6].

## MATERIALS AND METHODS

### Material

*Salvia leriifolia* leaves were obtained from Koohsorkh mountain to the south west of Nishabour city, Iran. After harvesting, the leaves were dried by indirect sunlight at room temperature for three days and then ground into a fine powder in a mill (Moulinex Depose-Brevete S.G.C.G., France). The ground powder was sealed in a plastic bag and stored at 4°C until used.

### Solvent Extraction

The leaf powder was separately extracted overnight with methanol, ethanol, acetone, chloroform or *n*-hexane (1:20 w/v), in a shaking incubator (Faraz Teb Tajhiz, Iran) at room temperature. The extracts were then filtered through Whatman #1 filter paper. The extraction was repeated twice overnight, and the combined filtrate was bleached with active carbon (1g active carbon : 5g leaf powder) for 15 min in a shaking incubator at room temperature and then filtered through a Whatman #1 filter paper to yield a light brown filtrate. The extract was then concentrated *in vacuo* below 45°C in a rotary evaporator (Heidolph vv1, Germany). This extract was stored for about 24 hours in a refrigerator and filtered to remove the precipitates. The combined filtrate and the precipitates were evaporated to near dryness *in vacuo* below 40°C and weighed to determine the yield [3, 12].

### Chromatographic Separation by Thin-Layer Chromatography (TLC)

An aliquot of 5% methanolic extract (2 µl) was streaked using a TLC applicator (CAMAG Ltd., Muttenz, Switzerland) on a precoated silica gel plate 2×10 cm, F<sub>254</sub>, 0.25

mm, (E. Merck, Darmstadt, Germany), which had been activated for 15 min at 100°C. The plate was developed in the ascending direction for 10 cm with the solvent system benzene: ethyl acetate: methanol: formic acid (65:15:17:3, by volume) (BEMF). The developed plate was then dried with a hair drier and visualization of the chromatogram was carried out under short-wavelength (254 nm) ultraviolet (UV) radiation.

To obtain sufficient quantities of the anti-oxidative components in *Salvia leriifolia* leaves, the 5% methanolic extract (100 µl) was streaked on a precoated silica gel plate (10×20 cm) and developed with the BEMF solvent system. Fractions with the same R<sub>f</sub> value were scraped and collected from 40 plates. Each fraction was isolated with methanol; then the combined extracts were filtered through a 0.45 µm membrane filter (Millipore, HVLP) and evaporated to near dryness *in vacuo* below 40°C. The residue was weighed to determine the yield of each fraction.

### Antioxidative Activity Determination

The antioxidative activity of all the organic solvent extracts was determined by AOCS Cd: 8-53 method by using 0.1 g of dried antioxidative extract. The extract was dissolved in 1 ml of the related solvent and then added to 100 ml refined and deodorized sunflower oil (Damoan factory, Fariman). The samples were held up to 10 days at 60°C and then their peroxide values (PV) measured [1].

Antioxidative activity determination of all separated fractions, whole methanolic extract and methanolic precipitates was carried out by the thiocyanate method [10]. In brief, a mixture of 4 mg sample in 4 ml absolute ethanol, 4.1 ml of 2.52% linoleic acid in absolute ethanol, 8 ml of 0.05 M phosphate buffer (pH 7.0), and 3.9 ml of water was placed in a vial (φ = 38 mm, h = 75mm) with a screw cap and then placed in an oven at 40°C in the dark. To 0.1 ml of this solution

**Table 1.** Yield and antioxidative activity of *Salvia leriifolia* leaf extracts with various solvents.

Solvents	Yield (%)		Activity (PV) <sup>a</sup>	
Methanol	15.6	a <sup>b</sup>	4.03	a
Ethanol	8.8	b	4.33	b
Acetone	6.3	c	4.28	b
Chloroform	5.7	c	4.54	c
<i>n</i> -hexane	4.7	c	4.62	d

<sup>a</sup> The antioxidative activity of extract has been stated in terms of peroxide value.<sup>b</sup> Means within a column with the same lower case letters are not significantly different at  $P < 0.05$ .

was added 9.7 ml of 75% ethanol and 0.1 ml 30% ammonium thiocyanate. Precisely 3 min after the addition of 0.1 ml of 0.02 M ferrous chloride in 3.5% hydrochloric acid to the reaction mixture, the absorbance was measured at 500 nm and subsequently every 24 hours until the absorbance reached maximum. The control and standards were subjected to the same procedures as the sample except that for the control, only the solvent was added, and for the standards, a 4 mg sample was replaced with 4 mg of  $\alpha$ -tocopherol and BHT.

The antioxidative activity or percentage of inhibition of lipid peroxidation in percent was calculated using the following equation:

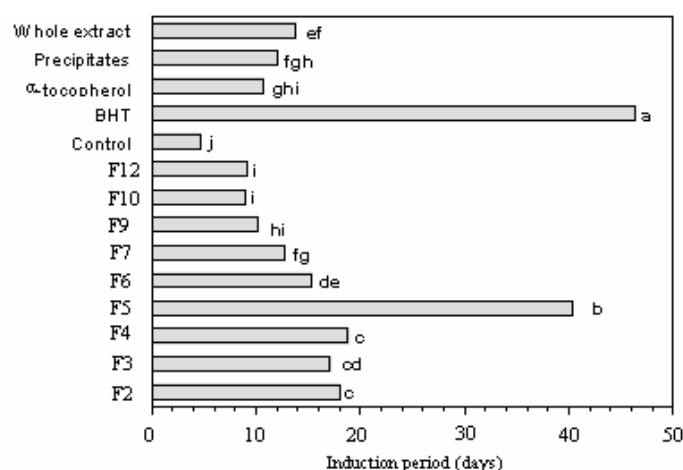
$$A = [(t - t_c) / (t_s - t_c)] \times 100$$

where A is antioxidative activity, t is induc-

tion period of the mixture with the new antioxidant,  $t_c$  is induction period of the mixture without antioxidant (control), and  $t_s$  is induction period of the mixture with the standard (e.g.  $\alpha$ -tocopherol and BHT) [7].

### Statistical Analysis

All determinations were carried out in three replicates and data were subjected to analysis of variance. Analysis of variance was performed using the ANOVA procedure. Statistical analyses were performed according to the MSTATC software. Significant differences between means were determined by Duncan's multiple range tests. P values less than 0.05 were consid-

**Figure 1.** Induction period of the components extracted from *Salvia leriifolia* leaves by methanol. The components with the same letters are not significantly different at  $P < 0.05$ .



ered statistically significant.

## RESULTS AND DISCUSSION

The yields and antioxidative activities (based on PV) of different organic solvent extracts from *Salvia leriifolia* leaves are shown in Table 1. The results indicate that the yield of extracts increased with increasing polarity of the solvent. The efficiency of the solvents for the extraction was in the order of methanol>ethanol>acetone= chloroform>n-hexane. This is in agreement with the report of Economou *et al.* [4] that methanol is a widely used and effective solvent for the extraction of antioxidants. Among the five organic solvents, methanolic extracts exhibited the highest yield and the lowest PV or the strongest antioxidative activity. In addition, the methanolic extracts were easily powdered. Therefore, we focused on the use of methanolic extracts from *Salvia leriifolia* leaves in the following research.

With the BEMF solvent system, the methanolic extract separated on TLC into twelve UV-distinct bands. The characteristics of all fractions are shown in Table 2. The highest yields were found in fractions with  $R_f$  values of 0.29, 0.54, 0.11 and 0.38 at

16.24%, 12.48%, 8.81% and 7.60%, respectively. The yield of the other fractions was not significantly different ( $P < 0.05$ ) from the fraction with  $R_f$  value of 0.38.

Except for the fractions 1, 8 and 11, which had a negligible yield, the induction period of all fractions as well as the control, standards (BHT and  $\alpha$ -tocopherol), whole extract and precipitates were determined by the thiocyanate method (Figure 1). All fractions and also the whole extract and precipitates had induction periods of significantly more than the control, therefore, all extracted components from *Salvia leriifolia* leaves had antioxidative activity. Fractions 7, 9, 10, 12 and precipitates were not significantly different from  $\alpha$ -tocopherol, but the other fractions and the whole extract had a better antioxidant properties. In comparison with BHT, only fraction 5 was slightly different. Therefore, fraction 5 with  $R_f$  value of 0.29 was the most active fraction (85.61% of BHT) and also had the highest yield (16.24%) among all extracted components. Table 3 indicates the antioxidative activity of all the components mentioned in terms of two standards,  $\alpha$ -tocopherol and BHT.

Therefore, it is necessary that we consider these natural sources of antioxidant occurring in our own country and carry out more research in this field due to several factors

**Table 2.** The characteristics of different fractions of methanolic extract from *Salvia leriifolia* leaves on silica gel plate.

Fractions	$R_f$ value <sup>a</sup>	Color	Yield (%) <sup>b</sup>
1	0.03	Deep blue	Ng
2	0.06	Deep blue	6.93 de <sup>c</sup>
3	0.11	Deep blue	8.81 c
4	0.16	Deep yellow	7.52 de
5	0.29	Yellow to deep brown	16.24 a
6	0.38	Deep blue	7.60 d
7	0.42	Deep blue	6.43 e
8	0.49	Slight blue	Ng
9	0.54	Deep blue	12.48 b
10	0.64	Slight blue	6.41 e
11	0.67	Slight blue	Ng
12	0.73	Slight blue	6.84 de

<sup>a</sup> Solvent system = benzene: ethyl acetate:methanol:formic acid (65:15:17:3, by volume).

<sup>b</sup> Based on 100  $\mu$ l of 5% methanolic extract. Ng: negligible.

<sup>c</sup> Means within a column with the same lower case letters are not significantly different at  $P < 0.05$ .

**Table 3.** Antioxidative activity of the components extracted from methanolic extract in comparison with  $\alpha$ -tocopherol and BHT.

Components	Antioxidative activity (%) in terms of	
	$\alpha$ -tocopherol	BHT
Whole extract	149.18	21.82
Precipitates	122.95	17.99
F12	73.77	10.79
F10	70.49	10.31
F9	90.16	13.19
F7	132.79	19.42
F6	175.41	25.66
F5	585.25	85.61
F4	232.79	34.05
F3	204.92	29.98
F2	219.67	32.13

that contribute to its importance: (a) the abundant and widespread use of such additives in the foods, drug, cosmetic and hygienic products industries, (b) increasing use of alternatives to synthetic antioxidants by using safer natural ones, and (c) importation and hard currency-consumption of such items for our country. *Salvia leriifolia* is a native plant that is readily available in Iran and has good antioxidative potential, thus it has good potential as an alternative for synthetic antioxidants. However, this plant grows in the wild and efforts should be made to select plants with high antioxidant activity and domesticate these as agricultural crops. In addition, more research is needed to determine the antioxidant characteristics and structural identification of active fractions, particularly fraction 5, in this plant.

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## استخراج و جداسازی ترکیبات آنتی اکسیدانی از برگ گیاه نوروزک (*Salvia leriifolia*)

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### چکیده

در این تحقیق، استخراج متانولی برگ گیاه نوروزک در مقایسه با سایر حلالهای آلی (اتانول، استون، کلروفرم و هگزان نرمال)، بازده و فعالیت آنتی اکسیدانی بیشتری را پدید آورد. عصاره متانولی به مدت ۲۴ ساعت در یخچال نگهداری و سپس رسوبات آن جدا گردید. عصاره متانولی گیاه با روش کروماتوگرافی لایه نازک (TLC) به ۱۲ فراکسیون تفکیک شد. بیشترین میزان بازده به ترتیب به فراکسیونهای دارای  $R_f$  معادل ۰/۲۹، ۰/۵۴، ۰/۱۱ و ۰/۳۸ با مقادیر ۱۶/۲۴، ۱۲/۴۸، ۸/۸۱ و ۷/۶۰ درصد تعلق داشت. تمام فراکسیونها به انضمام عصاره کامل متانولی و رسوبات عصاره متانولی بر اساس روش تیوسیانات دارای فعالیت آنتی اکسیدانی بیش از نمونه شاهد بودند. عصاره کامل متانولی، رسوبات عصاره متانولی و اکثر فراکسیونهای جداسازی شده، فعالیت آنتی اکسیدانی بیشتری از آلفا-توکوفرول نشان دادند. فراکسیون دارای  $R_f$  معادل ۰/۲۹ با ۱۶/۲۴ درصد بازده و فعالیت آنتی اکسیدانی ۸۵/۶۱ درصد آنتی اکسیدان سنتزی BHT بر مبنای روش تیوسیانات به عنوان پربازده ترین و فعالترین فراکسیون آنتی اکسیدانی برگزیده شد.