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

R. Farhoosh[1], H. Purazrang[1], M. H. H. Khodaparast[1], M. Rahimizadeh[2], and S. M. Seyedi[2]

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 α–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-

[1] Ferdowsi University of Mashhad, Faculty of Agriculture, Food Science and Technology Department, Mashhad, Islamic Republic of Iran.
[2] Ferdowsi University of Mashhad, Faculty of Science, Chemistry Department, Mashhad, Islamic Republic of Iran.

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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 the 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_{254}, 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 antioxidative 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_f 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 (Damoon 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

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

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

[a] The antioxidative activity of extract has been stated in terms of peroxide valve.
[b] 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 α–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 induction 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. α–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-

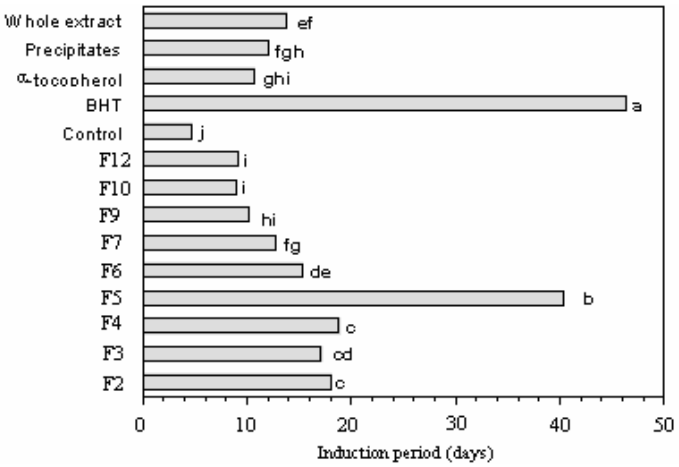

Figure 1. Induction period of the components extracted from *Salvia leriifolia* leaves by methanol. The components with the same letters are not significantly different at $P < 0.05$.

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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 α-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 α-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, α-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 [a]	Color	Yield (%) [b]
1	0.03	Deep blue	Ng
2	0.06	Deep blue	6.93 de[c]
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

[a] Solvent system = benzene: ethyl acetate:methanol:formic acid (65:15:17:3, by volume).
[b] Based on 100 µl of 5% methanolic extract. Ng: negligible.
[c] Means within a column with the same lower case letters are not significantly different at $P < 0.05$.

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Table 3. Antioxidative activity of the components extracted from methanolic extract in comparison with α-tocopherol and BHT.

Components	Antioxidative activity (%) in terms of	
	α-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.

REFERENCES

1. AOCS. 1989. *Peroxide Value (acetic acid-chloroform) Official method Cd*: 8-53. AOCS, Champaign, I11.
2. Cuppett, S. L. and Hall, C. A. 1998. Antioxidant Activity of the Labiatae. Adv. Food Nut. Res. **42**: 245-271.
3. Duh, Pin-Der, Yeh, Dong-Bor and Yen, Gow-Chin. 1992. Extraction and Identification of an Antioxidative Component from Peanut Hulls. *J. Amer. Oil Chem. Soc*. **69**: 814-818.
4. Economou, K. D., Oreopoulou, V. and Thomopoulos, J. 1991. Antioxidant Activity of Some Plant Extracts of the Family Labiatae. *J. Amer. Oil Chem. Soc*. **68**: 109-113.
5. Ito, N., Fukushima, S. and Tsuda, H. 1985. Carcinogenicity and Modification of the Carcinogenic Response by BHA, BHT, and Other Antioxidants. *Crit. Rev. Toxicol*. **15**: 109-150.
6. Jimenez, J., Risco, S., Ruiz, T. and Zarzuelo, A. 1996. Hypoglycemic Activity of *Salvia lavandulifolia*. *Planta Med*. **4**: 260-262.
7. Kikugawa, K., Kunugi, A., and Kurechi, T. 1990. Chemistry and Implications of Degradation of Phenolic Antioxidants. In: "*Food Antioxidants*". (Ed.): Hudson, B. J. F. Elsevier Science Publishers Ltd., England, pp. 65-99.
8. Maeura, Y., Weisburger, J. H., and Williams, G. 1984. Dose-dependent Reduction of N-2-fluorenylacetamide-induced Liver Cancer and Enhancement of Bladder Cancer in Rats by Butylated Hydroxy Toluene. *Cancer Res*. **44**:1604-1608.
9. Moure, A., Cruz, J. M., Franco, D., Dominquez, J. M., Sinerio, J., Dominquez, H. and Nunez, M. J. 2001. Natural Antioxidants from Residual Sources - a Review. *Food Chem*. **72**: 145-171.
10. Osawa, T., and Namiki, M. 1981. A Novel Type of Antioxidant Isolated from Leaf Wax of Eucalyptus Leaves. *Agric. Biol. Chem*. **45**: 735-739.
11. Rechinger KH. 1982. *Flora Iranica, No. 150, Labiatae*, Akademische Druck-V. Verlagsanstalt, Graz-Austria, pp 439- 440.
12. Wu, J. W., Lee, M. H., Ho, C. T., and Chang, S. S. 1982. Elucidation of the Chemical Structures of Natural Antioxidants Isolated from Rosemary. *J. Amer. Oil Chem. Soc*. **59**: 339-345.

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استخراج و جداسازي تركيبات آنتي اكسيداني از برگ گياه نوروزك
(Salvia leriifolia)

ر. فرهوش، ه. پورآذرنگ، م. ح. ح. خداپرست، م. رحيمي زاده و س. م. سيدي

چكيده

در اين تحقيق، استخراج متانولي برگ گياه نوروزك در مقايسه با ساير حلالهاي آلي (اتانول، استون، كلروفورم و هگزان نرمال)، بازده و فعاليت آنتي اكسيداني بيشتري را پديد آورد. عصاره متانولي به مدت ٢٤ ساعت در يخچال نگهداري و سپس رسوبات آن جدا گرديد. عصاره متانولي گياه با روش كروماتوگرافي لايه نازك (TLC) به ١٢ فراكسيون تفكيك شد. بيشترين ميزان بازده به ترتيب به فراكسيونهاي داراي R_f معادل ٠/٢٩ , ٠/٥٤ , ٠/١١ و ٠/٣٨ با مقادير ١٦/٢٤ , ١٢/٤٨ , ٨/٨١ و ٧/٦٠ درصد تعلق داشت. تمام فراكسيونها به انضمام عصاره كامل متانولي و رسوبات عصاره متانولي بر اساس روش تيوسيانات داراي فعاليت آنتي اكسيداني بيش از نمونه شاهد بودند. عصاره كامل متانولي، رسوبات عصاره متانولي و اكثر فراكسيونهاي جداسازي شده، فعاليت آنتي اكسيداني بيشتري از آلفا- توكوفرول نشان دادند. فراكسيون داراي R_f معادل ٠/٢٩ با ١٦/٢٤ درصد بازده و فعاليت آنتي اكسيداني ٨٥/٦١ درصد آنتي اكسيدان سنتزي BHT بر مبناي روش تيوسيانات به عنوان پربازده ترين و فعالترين فراكسيون آنتي اكسيداني برگزيده شد.

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