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**ORIGINAL ARTICLE**

# Contents of Aerial Parts of *Salvia leriifolia* Benth

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**KEYWORDS**

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**ABSTRACT:** In the present study, we have reported the total phenolic content, total flavonoid content, antioxidant and antimicrobial activity of aerial parts of *Salvia leriifolia* extracts and fractions. Methanolic, n-hexane, chloroform, and ethyl acetate extracts were screened to analysis their antioxidant activities by four complementary test systems, namely DPPH free radical scavenging activity (RSA), total phenolic content (TPC), total flavonoid content (TFC), and ferrous ion cheating (FIC). In most cases the leaf extracts and ethyl acetate fraction had more activity. The methanolic extracts of leaf and flower showed considerable antimicrobial activity using disc diffusion method against *Escherichia coli*, *Streptococcus pneumonia*, *Acinetobacter*, *Serratia*, *Pseudomonas aeruginosa.* The extracts showed the highest activity against *P. aeruginosa* and *K. pneumonia*.

## INTRODUCTION

The genus of *Salvia* belongs to Nepetoideae subfamily of the Mentheae tribe from the Lamiaceae family, which covers numerous species and displays diverse biological activities emanated by the different components that allow many pharmaceutical and medicinal uses of the plant materials or extracts. Many diterpenes have been isolated from different species of Salvia. These compounds exhibited some bioactivity such as antioxidant activity [1], antimicrobial [2], anti-inflammatory, analgesic [3], antipyretic, hemostatic [4], hypoglycemic [5], and antitumor [6]. *Salvia leriifolia* Benth. (Local names such as Nuruozak and Jobleh), introduced in 1982 in Florica Iranica (Rechinger, 1982), grows exclusively in the south regions of Khorasan and Semnan provinces, Iran. In the past, people have used this plant as a blood clotting factor and anti-transpiration .In medieval times it was used to treat constipation, cholera, colds and various tabs and liver disorders [7]. Besides the *Salvia*'s extract was used to treat some diseases of the pharynx and larynx, as well as a mouth rinse .In recent years, the different pharmacologic activities of this plant, such as debilitation of morphine dependence, hypoglycemic, analgesic and anti-inflammatory, anticonvulsant, antiulcer effects, and antibacterial activities, were evaluated [3, 8-13].

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The oil of *S. leriifolia* shows strong antiproliferative activity against lines—large cell carcinoma COR-L23 and amelanotic melanoma C32 in comparison with vinblastine used as a standard [14]. Besides, it showed good antioxidant activity and cholinesterase inhibitory properties for acetylcholinesterase and butyrylcholinesterase [15]. Hence, *an S. leriifolia antioxidant property was* mentioned as a species for treating of neurodegenerative diseases such as Alzheimer disease [16].

Free radicals such as superoxide anion, and hydroxyl and peroxyl radicals, produced in biological systems and foods, are responsible for oxidation of cell lipids and DNA damage, and they may cause serious diseases [17]. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide and hydro peroxide, and thus inhibit the oxidative mechanisms that lead to degenerative diseases [18, 19]. The most widely used antioxidants are synthetic ones like tertbutyl hydroquinone (TBHQ) butylated hydroxyl toluene (BHT), butylated hydroxyl anisole (BHA), and propyl gallate (PG), but due to the toxicity and carcinogenic ability of these compounds their uses has been questioned [20]. Therefore, recently most attention has been focused on the isolation, characterization and utilization of effective and inexpensive antioxidants from natural products [21].

In this research we used a different fractionation method to screening of antioxidant and antimicrobial activity of plant extracts compared to previous studies, which usually used normal maceration method to obtain plants extracts; however there are no reports on the antioxidant and antimicrobial activity of the plant extract in literature. On the basis of these considerations, the objects of this research were determination of the total phenolic content, the total flavonoid content, and antioxidant activity using radical scavenging activity and ferrous ion chelating assays of aerial parts of *S. leriifolia* including leaf and flower extracts. Besides, antimicrobial activity of the plant extracts was screened.

## MATERIALS AND METHODS

### *Chemicals*

1,1-Diphenyl-2-picrylhydrazyl (DPPH), α-tocopherol, 3-(2-pyridyl)-5,6-bis(4-phenylsulfonicacid)-1,2,4-triazine (ferrozine), and butylated hydroxyl toluene (BHT) were purchased from Sigma; ascorbic acid (AscA), and Ethylenediaminetetraacetic acid (EDTA) from Merck; $Na_2CO3$, and $FeSO_4{\cdot}7H_2O$ from BDH; Folin-Ciocalteu's reagent (FCR), gallic acid (GA) from Acros.

### *Plant material*

Plant parts of *S. leriifolia* including leaf and flower were collected in Jun 2014 from north of Sabzevar, northwestern Iran. A voucher species man of 201 was deposited at the Hakim Sabzevari University Herbarium (HSUH). The plant species were identified by Dr. Mozaffarian.

### *Microorganisms*

The test bacterial strains of nine gram-negative of *Pseudomonas aeruginosa ATCC27853, Escherichia coli ATCC25922, Staphylococcus coagulase, Citrobacter frurdii ATCC8090, Enterobacter aeruginosa ATCC21754, Agrobacterium tumefaciens ATCC33970, Acinetobacter baumannii ATCC19606, Serratia marcescens ATCC8100, Klebsiella pneumoniae ATCC13883* and one gram-positive bacterium of *Streptococcus pneumoniae ATCC 49619* were obtained from Microbiology Laboratory culture collection, Department of Biology, Faculty of Science, Hakim Sabzevari University and also Microbiology Laboratory of Sabzevar Medical Science University.

### *Solvent extraction*

The plant parts were dried by indirect sunlight at room temperature and then powdered in a mill. The ground powder was the sealed in a plastic bag and kept in cold place until used. The different plant parts powder were

separately macerated in methanol for 72 h and then filtered through Whatman no.1 filter paper. Afterward the extracts were concentrated under low pressure at 45 °C by a rotary evaporator (Buchi Rotavapor R-114). The concentrated extracts were dissolved in specified amount of methanol and then they washed using various solvents with different polarity including n-hexane, chloroform and ethyl acetate, respectively in triplicate. Finally the obtained extracts with each solvent were combined, concentrated at low pressure, and kept for the antioxidant assays.

***Determination of total phenolic content (TPC)***

The total phenolic content of the extracts were determined by the Folin-Ciocalteu method with some modification [22, 23]. Three rows of vials were prepared, a 0.5 ml of each extract (1000 µg/mL in methanol), 1.5 mL distillate water and 0.5 mL of FCR 10% were added to each vial. The mixture was shaken vigorously and released for 10 min. Then 1 ml of 5% sodium carbonate was added to each vial. After 120 min. the absorbance was read at 760 nm by the Photonix Ar 2015 UV- Vis. Spectrophotometer. The total phenolic content was calculated based on the standard curve of gallic acid (5-20 µg/ml). The results were expressed as mg of gallic acid equivalents per gram of dried extract (GAE).

***Determination of total flavonoid content (TFC)***

The aluminum chloride method procedure was used for the determination of the total flavonoids content of all extracts [24]. A 1 mL of each extract in concentration of 100 µg/mL was mixed with 1 mL of 2% methanolic aluminum chloride solution. After 30 min at room temperature and in the dark, the absorbance of all samples was determined at 415 nm. The total phenolic content was calculated based on the standard curve of rutin (10-160 µg/mL). The results were represented in mg of rutin equivalent per gram of dried extract.

***DPPH radical scavenging activity (RSA)***

The antioxidant activity of the extracts was measured on the basis of the scavenging activity of the stable DPPH free radical according to the method which reported previously with some modification [22, 25, 26]. The plant parts extracts with different concentrations (20, 40, 80 and 160 µg/mL) were added to a methanolic solution of DPPH. The reaction mixtures were shaken vigorously and then kept in the dark for 90 min. The absorbance of the resulting solutions was read at 517 nm against the blank (without DPPH radical). All tests were run in triplicate and the mean values calculated with using the following equation. BHT and α-tocopherol were used as a positive control.

$$\mathrm{RSA}(\%) = \left[\frac{(\mathrm{A_{Control}} - \mathrm{A_{Sampel}})}{(\mathrm{A_{Control}})}\right] \times 100$$

Where $A_C$ is the absorbance of the control (DPPH solution and solvent) and $A_S$ is the absorbance of the sample (DPPH solution and extract solution).

***Ferrous ion chelating ability (FIC)***

The ability of the extracts to chelate of iron (II) was assessed [27, 28]. In brief, a 50 µL (2 mM) of $FeSO_4$ was added to 1 mL of each extract in methanol at 600 µg/mL and 2 mL of distilled water. After 5 min incubation, the reaction was initiated by addition of 100 µL (5 mM) ferrozine to the mixture. After 10 min incubation period, the absorbance was read at 562 nm. All measurements were done in triplicate. EDTA and ascorbic acid were used as positive controls. The ratio of inhibition of ferrozine–$Fe^{2+}$ complex formation was calculated as follows:

$$\mathrm{inhibition}\% = \left[\frac{\mathrm{A_{Control}} - \mathrm{A_{Sampel}}}{\mathrm{A_{Control}}}\right] \times 100$$

Where $A_{Sampel}$ is the absorbance of ferrozine, ferrous ion, and extract mixture; and $A_{Control}$ is the control absorbance (ferrozine, ferrous ion, and solvent).

***Disc diffusion assay (DD)***

DD assay was carried out to analysis the antimicrobial activity of the extracts [29, 30]. The suspensions containing $10^8$ CFu/mL were loaded on a sterile cotton swabs and streaked over the dried surface of Mueller-hinton agar medium plates for vaccination. The 6 mm in diameter discs were impregnated with 30 µL of the extracts (100 mg/mL) and then placed on the inoculated agar. The plates were incubated at 37 °C for 24 h. Antimicrobial activity was determined by measuring the diameter of inhibition zone (diz) against the microorganisms. Chloramphenicol and vancomycin were used as the positive control. The values of inhibition levels were reported in percentage (%). The inhibition level was measured by dividing the inhibition zone diameter of the essential oils with that of the antibiotic (positive control) as following shown:

$$\text{Inhibition level (\%)} = \frac{\text{Inhibition zone diameter of sample}}{\text{Inhibition zone diameter of the antibiotic}} \times 100$$

Antimicrobial activity was categorized as strong for inhibition level ≥ 70%, moderate for inhibition level 50-70%, and weak for inhibition level < 50% [31].

**STATISTICAL ANALYSIS**

All measurements were carried out in triplicate and expressed as the average of three measurements ± standard deviation. Calculations and construction of curves were performed using MS Office Excel, 2013.

**RESULTS**

***Total phenolic content (TPC) and total flavonoid content (TFC)***

In this study we tried to determine the total phenolic content of different parts of *S. Leriifolia*. Among analyzed plant extracts, high total phenolic content was detected for flower ethyl acetate fraction of the plant by 83.85 ± 1.82 GAE/g, and the lowest total phenolic content was detected in n-hexane fraction of leaf with 12.68 ± 3.57 GAE/g (Table 1).

According to results in Table 1, the leaf of *S. Leriifolia* showed the highest total flavonoid content (TFC) in ethyl acetate fraction with 354.84 ± 2.85 mgRU/g and the lowest ones for chloroform fraction of leaves with 9.75± 0.37 mgRU/g. For the flower fractions maximum TFC was measured for ethyl acetate fraction with 193.26 ± 3.6 mgRU/g (Table1).

**Table 1.** Total phenolic content and total flavonoid content and Values of DPPH scavenging activity of flowers and leaves of *Salvia leriifolia*

| Plant part | Fraction | TPC (mgGAE/g) | TFC (mgRU/g) | DPPH $IC_{50}$ (µg/mL) |
|---|---|---|---|---|
| Flower | n-hexane | 18.39 ± 0.84* | 23.80 ± 2.01 | 160.22 ± 19.78 |
| | Chloroform | 14.61 ± 0.41 | 22.96 ± 0.27 | 96.78 ± 7.47 |
| | Ethyl acetate | 83.85 ± 1.82 | 193.26 ± 3.6 | 25.08 ± 1.53 |
| | Methanolic | 36.37 ± 1.46 | 20.47 ± 2.19 | 39.98 ± 0.21 |
| Leaves | n-hexane | 10.35 ± 0.41 | 87.40 ± 0.57 | 225.93 ± 10.65 |
| | Chloroform | 16.05 ± 0.39 | 9.75 ± 0.37 | 61.57 ± 1.27 |
| | Ethyl acetate | 75.53 ± 0.81 | 354.84 ± 2.85 | 23.64 ± 0.81 |
| | Methanolic | 31.38 ± 1.79 | 109.38 ± 2.88 | 48.19 ± 0.07 |
| Standards | α-tocopherol | - | - | 55.81 ± .98 |
| | BHT | - | - | 17.08 ± .465 |

***** Values are presented as means ± SD (n=3).

### *Antioxidant activity: RSA and FIC*

As shown in Table 1, the highest RSA was obtained for ethyl acetate fraction of leaf and flower of *S. Leriifolia* with $IC_{50}$ of 23.64 ± 0.81> 25.08 ± 1.53 respectively. As it was expected, radical scavenging activity of each extract increased by concentration. For the other fractions the activity was in order of methanolic, chloroform and n-hexane extracts respectively.

The highest ferrous ion chelating ability was observed for n-hexane fraction of flower and leaf of *S. Leriifolia* at 600 µg/ml concentration with 77.65±3.82 and 48.74±3.43 percent respectively. The fractions showed better FIC ability than positive controls of α-tocopherol and BHT with amounts of 43.75±3.23 and 16.21±1.46 percent. However the ability of EDTA with 92.01±1.23 percent was more than the fractions to chelate ferrous ions.

### *Antimocrobial activity*

The antimicrobial activities of the methanolic extracts of leaf and flower from *S. leriifolia* against one Gram-positive and 9 Gram-negative bacteria strain are shown in Table3. According to the results, the leaf and flower extracts of *S. leriifolia* showed strong antimicrobial activity compared to positive control of vancomaycine against *P. aeruginosa* (inhibition level of 185.2 and 150.6%), *S. pneumonia* (inhibition level of 92.4 and 85.4%). The flower also exhibited strong activity against *K. pneumonia* with inhibition level of 125.9%. A moderate activity was observed for leaf extract against *S. coagulase* (61.7%). The flower extract prevented the growth of *E. coli* and *A. baumannii* moderately with inhibition level of 51.8 and 58.0% respectively.

**Table 2.** Antimicrobial activities of the methanolic extracts of Salvia leriifolia aerial parts using disc-diffusion assay (each disc was impregnated by 30**µ**l of extract at 100 mg/ml)

| | | Inhibition zone diameter (mm) and Inhibition level (%) | | | |
|---|---|---|---|---|---|
| **Gram- negative** | | **Leaf** | **Flower** | **Positive Control[a]** | **Negative Control[b]** |
| ***Pseudomonas aeruginosa* (-)** | mm | 15.0±0.0[c] | 12.3±0.5 | 8.1±0.2 | 6.0±0.0 |
| | % | 185.2 | 150.6 | - | - |
| ***Escherichia coli* (-)** | mm | 8.7±1.7 | 9.5±0.5 | 18.3±0.0 | 6.0±0.0 |
| | % | 47.4 | 51.9 | - | - |
| ***Staphylococcus coagulase* (-)** | mm | 10.1±1.6 | Na[d] | 16.3±1.1 | 6.0±0.0 |
| | % | 61.7 | - | - | - |
| ***Citrobacter frurdii* (-)** | mm | - | - | na | 6.0±0.0 |
| | % | na | na | - | - |
| ***Enterobacter aerogenes* (-)** | mm | - | - | 21.5±0.5[e] | 6.0±0.0 |
| | % | na | na | - | - |
| ***Agrobacterium tumefaciens* (-)** | mm | - | 8.7±0.9 | 17.3±0.5[f] | 6.0±0.0 |
| | % | na | 50.3 | - | - |
| ***Acinetobacter baumannii* (-)** | mm | 9.3±1.8 | 12.3±3.3 | 21.2±0.9 | 6.0±0.0 |
| | % | 43.9 | 58.0 | - | - |
| ***Serratia marcescens* (-)** | mm | 10.2±0.5 | 7.3±0.6 | 47.5±2.1[g] | 6.0±0.0 |
| | % | 21.5 | 15.4 | - | - |
| ***Klebsiella pneumoniae* (-)** | mm | - | 10.7±0.9 | 8.5±1.3 | 6.0±0.0 |
| | % | na | 125.9 | - | - |
| **Gram-positive** | | | | | |
| ***Streptococcus pneumoniae* (+)** | mm | 13.3±1.2 | 12.3±0.8 | 14.4±0.5 | 6.0±0.0 |
| | % | 92.4 | 85.4 | - | - |

a: Vancomicin 30 µg; b: Solvent; c: Diameter of inhibition zone (mm) including disk diameter of 6 mm, data are mean ± SD for three replicates;d : non-active; e,f: Chloramphenicol 30 µg.

## DISCUSSION

### *Total phenolic content (TPC) and Total flavonoid content (TFC)*

Phenolic compounds are one of the important groups of natural compounds found in plants and known to inhibit some molecular targets in inflammatory responses and act as primary antioxidants or free radical exterminator [8]. A previous study on TPC of eight *Salvia* species from Turkey, namely *S. aethiopis, S. candidissima, S. limbata, S. microstegia, S. nemorosa, S. pachystachys, S. verticillata*, and *S. virgate*, the amount of total phenolic content was highest for *S. verticillata* 167.1 mgGAE/g, followed by *S. virgate* 101.2 mgGAE/g, *S. candidissima* 100.3 mgGAE/g, and S. *microstegia* showed the lowest TPC of 50.3 mgGAE/g [32]. Bahadori et al. reported the TPC of methanolic extract of *S. urmiensis* with 109.25±10.5 mgGAE/g and 102.54 ± 8.2 mgGAE/g for n-hexane extract of *S. hydrangea* [33]. On the other hand, several studies have been carried out on the *Salvia* species and their TPC has been found to be between 41-134 mgGAE/g [34, 35]. A comparison of previous studies with the present study illustrated that there was no significant different between the TPC of *Salvia* species.

Flavonoids as one of the most diverse and wide spreading group of natural compounds are perhaps the most important natural phenols. These compounds possess a broad spectrum of chemical and biological activity including radical scavenging activity, coronary heart disease prevention, hepatoprotective, anti-inflammatory, and anticancer activities due to the presence of hydroxyl groups [36, 37]. The TFC of *S. officinalis* was 0.436 mgQE/g, (mg of quercetine equivalents per gram of dried extract) [38], which was lower than that of *S. leriifolia*; and TFC of ethyl acetate extract from *S. urmiensis* was obtained for 170.5mgQE/g [33].

### *Antioxidant activity: RSA and FIC*

RSA is an important biological activity of secondary metabolites, because of the disadvantageous role of free radicals in foods and in biological systems. Antioxidant compounds can change the purple color of DPPH, as a stable radical, to yellow color [39]. The methanolic extracts of five *Salvia* species growing in Iran namely *S. multicaulis, S. verticillata, S. lachnocalyx, S. mirzayanii*, and *S. macrosiphon* were analyzed for their antioxidant activities by DPPH assay, the results showed the $IC_{50}$ range of 386.9 to 2743.05 µg/mL respectively [40]. The result comparison exhibited the ability of ethyl acetate extract of *S. leriifolia* to scavenge DPPH radical is more than the mentioned species. The $IC_{50}$ of *S. euphratica* and *S. sclarea* were reported 20.7 ± 1.22 and 23.4 ± 0.97 µg/mL, however the polar extracts of *S. aethiopis* did not show any radical-scavenging activity in this assay [41].

Most of the plant extracts participate with the constitution of ferrous and ferrozine complex, suggesting that they can capture ferrous ion forming a more stable complex than ferrozine [42]. Transition metals such as iron by generating hydroxyl radicals through Fenton reaction can motivate and precipitate lipid peroxidation by decomposing lipid hydro peroxides into alkoxyl and peroxyl radicals therefore drive the chain reaction of lipid peroxidation. *S. miltiorrhiza* extract could not chelating ferrous ion [43]. However chloroform and acetone extracts of *S. sclarea* exhibited 31.6 and 37.7% iron binding capacity, respectively [44].

The correlation between free radical scavenging and total phenol and flavonoid content suggested that almost the free radical scavenging of *S. Leriifolia* is contributed to phenolic compounds. The antioxidative activity of polyphenols is generally ascribed to their hydroxyl groups [45]. Therefore, the phenolic content of plants may contribute directly to their antioxidant action. Plants belonging to the Lamiaceae family are rich of

polyphenolic compounds. The major phenolic compounds are rosmarinic acid, carnosic acid, salvianolic acid and its derivates carnosol, rosmanol, epirosmanol, rosmadial and methyl carnosate which identified in the extracts of sage [46, 47]. Among these, rosmanol is a major constituent of many *Salvia* species and has strong antioxidant activities because these groups cause phenols to more easily donate hydrogen atoms to activate free radicals to interrupt the chain reaction of antioxidation [36].

***Antimicrobial activity***

Antimicrobial activity along with the antioxidant effectiveness of extracts is one of the most examined features, that important for control of human and animal diseases of microbial origin. Numerous reports suggest strong antimicrobial activities of a broad spectrum of extracts, especially for plants belonging to the *Lamiaceae* family [48, 49]. Antimicrobial activity of the methanol extracts of the eleven *Salvia* species including *S. aegyptiaca, S. aethiopis, S. atropatana, S. eremophila, S. hypoleuca, S. limbata, S. nemorosa, S. santolinifolia, S. sclarea, S. syriaca,* and *S. xanthocheila* were measured on 6 different Gram-negative and Gram-positive bacteria. *S. eremophila, S. limbata, S. santolinifolia* and *S. sclarea* were the most active plants and inhibited the growth of all tested microorganisms [50]. Tenore et al. studied the *in vitro* antimicrobial activity of the essential oil of *S. lanigera* against 14 bacteria species was evaluated and showed an interesting activity against the Gram (+) pathogens, but in the Gram (–) bacteria, the sample indicated a significant activity only against *E. coli, S. typhi Ty2* and *K. pneumonia* Antimicrobial activity could be mainly due to the presence of phenolic compounds, such as thymol and carvacrol [49].

## CONCLUSIONS

The highest total phenolic content and total flavonoid content were obtained for ethyl acetate fraction of leaf and flower of *S. Leriifolia*. Moreover, the ethyl acetate extract was shown to possess a remarkable radical scavenging activity even more than vitamin E as a positive control. So the presence of the highest phenolic and flavonoid compounds in leaves and flowers cause the maximum antioxidant activity. Our data indicate that *S. leriifolia* methanolic extracts shows a wide range of antimicrobial activity against referenced strains, especially in Gram-negative bacteria.

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