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# EVALUATION OF POLYPHENOLIC PROFILE AND ANTIOXIDANT ACTIVITY FOR SOME *SALVIA* SPECIES

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**Abstract**

The polyphenolic composition and antioxidant capacity of some *Salvia* L. species (*S. aetiopsis*, *S. austriaca*, *S. sclarea*, *S nutans*, *S. verticilatta* and *S. nemorosa*) were the goals of this study. Analysis of polyphenols was performed by a HPLC/UV/MS method and was achieved on the ethanolic extracts obtained from aerial parts of the plants, collected from the spontaneous Flora of Republic of Moldova. The total polyphenolic, flavonoids and caffeic acid derivatives content were measured by spectrophotometric methods. *In vitro* antioxidant activity of these extracts was evaluated using DPPH (α,α-diphenyl-β-picrylhydrazyl), FRAP (ferric reducing antioxidant power) and HAPX (Haemoglobin/Ascorbate Peroxidase Activity Inhibition) methods. The polyphenolic profile of these species revealed the common components (caffeic acid, *p*-cumaric acid, isoquercitrin, hyperoside, luteolin, apigenin) and other non-common compounds (caftaric acid, chlorogenic acid, ferulic acid, rutin, quercitrin, quercetin, kaempferol). The highest antioxidant capacity was revealed by *S. verticillata*. All studied *Salvia* species contain significant amounts of polyphenolic compounds (22.25 - 118.75 mg GAE/g dry weight material plant) and may be used as important source of natural antioxidants along with the most famous *S. officinalis*.

**Rezumat**

Obiectivele acestui studiu au vizat evidenţierea profilului polifenolic şi capacitatea antioxidantă a unor specii de *Salvia* L. (*S. aetiopsis*, *S. austriaca*, *S. sclarea*, *S. nutans*, *S. verticilatta* şi *S. nemorosa*). Analiza polifenolilor a fost realizată prin metoda HPLC/UV/MS, pe extracte etanolice obţinute din părţi aeriene ale plantelor recoltate din flora spontană a Republicii Moldova. Concentraţiile de polifenoli totali, flavonoide şi derivaţi de acid cafeic au fost evaluate prin metode spectrofotometrice. Activitatea antioxidantă *in vitro* a fost evaluată utilizând metodele DPPH, FRAP şi HAPX (inhibarea activităţii hemoglobinei/ascorbat peroxidazei). Profilul polifenolic al acestor specii a evidenţiat componente comune (acid cafeic, acid *p*-cumaric, izoquercitrina, hiperozida, luteolina, apigenina), precum şi componente de diferenţiere a speciilor (acid caftaric, acid clorogenic, acid ferulic, rutozida, quercitrina, quercetol, kempferol). Cea mai bună activitate antioxidantă a prezentat *S. verticillata.* Speciile de *Salvia* studiate conţin cantităţi semnificative de compuşi polifenolici (22,25 - 118,75 mg GAE/g produs vegetal uscat) şi pot fi folosite ca surse importante de antioxidanţi naturali, alături de specia oficinală *S. officinalis*.

**Keywords**: *Salvia aetiopsis*, *S. austriaca*, *S. sclarea*, *S nutans*, *S. verticilatta*, *S. nemorosa*, polyphenols, DPPH, FRAP, HAPX

## Introduction

Polyphenols are secondary metabolites that are widely spread in the plant kingdom and are known for their antioxidative capacity, neutralizing free-radical responsible for cell damage. Thereby polyphenols may be utilized in prevention and treatment of a large array of free-radical mediated diseases. In recent decades, significant amounts of research have been carried out on natural polyphenol sources [1-4].

*Salvia* L. (*Lamiaceae*) is a large and diversified genus including approximately 90 species, of which almost 15 are largely distributed in South-eastern Europe, included *Salvia officinalis* (sage) which is the best known and most widely used medicinal plant [5].

Other *Salvia* species are important sources of active principles as well, but are less studied. *Salvia* species are generally known for their multiple pharmacological effects including antiproliferative, antiinflammatory, anti-nociceptive, antioxidant, antimicrobial, anti-mutagenic, anti-dementia, hypoglycaemic and hypo-lipidaemic effects [6].

Some studies showed that these activities depended on essential oil, polyphenolcarboxilic acids (caffeic acid, chlorogenic acid, ellagic acid, gallic acid, rosmarinic acid), flavonoids and tannin composition [7-12].

The goal of this paper was to evaluate the polyphenolic content and the antioxidant activity of six *Salvia* L. species from spontaneous Moldavian Flora (*S. aetiopsis*, *S. austriaca*, *S. sclarea*, *S nutans*, *S. verticilatta* and *S. nemorosa*), using three *in vitro* model systems, DPPH, FRAP and HAPX (Haemoglobin/ Ascorbate Peroxidase Activity Inhibition). The results of our research could be useful as scientific information on these species as potential sources of antioxidants.

## Materials and Methods

*Experimental*

*Chemicals and Apparatus*. Ferulic, sinapic, gentisic, gallic acids, patuletin, luteolin were acquired from Roth (Karlsruhe, Germany); chlorogenic, *p*-coumaric, caffeic acids, rutin, apigenin, quercetin, isoquercitrin, quercitrin, hyperoside, kaempferol, myricetol, fisetin were purchased from Sigma (St. Louis, MO, USA); cichoric and caftaric acids were from Dalton (Toronto, ON, Canada). Sodium molybdate dihydrate, sodium nitrite, sodium hydroxide, sodium carbonate were purchased from Sigma-Aldrich (Steinheim, Germany). HPLC grade methanol, analytical grade ortho-phosphoric acid, hydrochloric acid, aluminium chloride, sodium acetate, ethanol and Folin-Ciocâlteu reagent were purchased from Merck (Darmstadt, Germany). $DPPH^{\cdot}$, $ABTS^{+}$ were obtained from Alfa-Aesar (Karlsruhe, Germany). Hydrogen peroxide, sodium ascorbate and bovine haemoglobin were purchased from Sigma-Aldrich (Steinheim, Germany). All spectrophotometric data were acquired using a Jasco V-530 UV-vis spectrophotometer (Jasco Int. Co. Ltd., Tokyo, Japan).

*Plant material and extraction procedure*. The aerial parts of the plants were collected in 2016, during the blooming period (June - July) from the North-eastern part of Republic of Moldova, Chişinău surroundings, N 47°02'58.58"; E 28°52'42.65". Voucher specimens (No. 220 - 225) were deposited in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Cluj-Napoca, Romania. The vegetal material was grinded to fine powder (300 μm) after air drying at room temperature. The material was extracted at 60°C (on a water bath) with 70% ethanol for 30 min. The supernatant was recovered after centrifugation at 4500 rpm for 15 min [13-15].

*HPLC Analysis*. The chemical determination of polyphenols was achieved using an Agilent Technologies 1100 HPLC Series system coupled with an Agilent 1100 mass spectrometer (LC/MSD Ion Trap VL) equipped with degasser, binary gradient pump, column thermostat, autosampler and detector. For the separation, a reverse-phase analytical column was employed. The detection of the compounds was performed on both UV and MS mode. Calibration curves were used for the 20 reference phenolic standards (chlorogenic, *p*-coumaric, caffeic, cichoric, caftaric, ferulic, sinapic, gentisic gallic acids, rutin, quercetin, isoquercitrin, quercitrin, hyperoside, kaempferol, myricetol, fisetin, patuletin, apigenin, luteolin), in 0.5 - 50 μg/mL range with good linearity ($R^2 > 0.999$) for a five point plots. The data were processed using ChemStation and DataAnalysis software from Agilent [16, 17].

*The total phenolic content* (TPC) of the extracts was determined by the Folin-Ciocâlteu method with some modifications. The results were expressed in gallic acid equivalents on dry material plant (GAE; mg/g dry weight material plant = d.w.) [16, 17].

*The quantitative determination of flavonoids* was performed using the spectrophotometric aluminium chloride method. The percentage of flavonoids was expressed in rutin equivalents (RE; mg/g d.w.) [16, 17].

*The caffeic acid derivatives content* was determined using the Arnow's spectrophotometric method. The phenolic acids content was expressed as caffeic acid equivalents (CAE; mg/g d.w.) [16, 17].

*Antioxidant Activity Test*

*DPPH free radical method* is the most often used antioxidant assay. The absorbance was measured at 517 nm. The antiradical activity was expressed as $IC_{50}$ (μg/mL), that is the concentration of vegetal material required to cause a 50% DPPH inhibition [18].

*FRAP method* uses the reduction of the ferric to the ferrous ion in a complex formed with iron, of the radical 2,4,6-tripyridyl-s-triazine. Depending on the concentration of antioxidant compounds in the sample, the colour of the complex is changed and absorbance is measured at 593 nm. The results are expressed as mM Trolox equivalent/100 mL extract, on the basis of a calibration curve using a Trolox standard [19, 20].

*Haemoglobin/Ascorbate Peroxidase activity inhibition (HAPX) assay*. The haemoglobin ascorbate peroxidase activity (HAPX) assay was performed following the procedure described by Mot *et al*. [21]. Ascorbate (120 μM), peroxide (451 μM) and diluted extracts (5 μL) were mixed with met haemoglobin (6 μM) and the reaction was monitored at 405 nm, the wavelength specific for met haemoglobin. The extracts were diluted from the stock solutions as follows: 100-timed diluted *S. verticillata*, *S. aethiopis*, *S. austriaca* and 50% *S. sclarea*, *S. nutans*, *S. nemorosa*. An increase in the inhibition time reflects the antioxidant capacity of the tested extracts. The percentage of the inhibition time was transformed to caffeic acid equivalents (CAE) using a calibration curve ($R^2 = 0.99$) with caffeic acid standard solutions of 0 - 60 μM.

*Statistical Analysis*

The samples have been analysed in triplicate or more; the average of the relative SD and the correlation (the correlation matrices) have been calculated using the Excel software package.

## Results and Discussion

The HPLC profile of polyphenolic compounds (Table I) revealed the presence of 13 phenolic compounds in

the studied species. Five phenolic acids (caftaric, caffeic, chlorogenic, *p*-coumaric, ferulic acids), four flavonoid glycosides (hyperoside, isoquercitrin, rutin, quercitrin) and four flavonoidic aglycones (quercetin, luteolin, kaempferol and apigenin) were identified. Two flavonoidic aglycones, luteolin and apigenin were found in all the samples and the highest concentration was determined in *S. austriaca* (6379.9 ± 2.83 µg/g and 1214.6 ± 1.64 µg/g, respectively). Among flavonoid glycosides, isoquercitrin and rutin were determined in significant concentrations. Rutin, one of the most common flavonoid glycosides was detected in low amounts in all species (from 56.0 ± 0.10 µg/g to 367.8 ± 0.54 µg/g). Isoquercitrin was found in the largest amount in *S. sclarea* (2208.0 ± 1.80 µg/g). Caffeic acid was also identified in all samples and the highest concentration was found in *S. sclarea* (776.5 ± 1.12 µg/g), followed by *S. verticilatta* (741.5 ± 1.31 µg/g). For *S. nutans*, *p*-coumaric and ferulic acids were quantitated in significant concentrations (399.2 ± 0.25 µg/g and 207.39 ± 0.15 µg/g, respectively).

**Table I**

The HPLC analyses of polyphenolic compounds

| Compound | *m/z* value | $t_R$ ± SD min | *S. aetiopsis* µg/g d.w. | *S. austriaca* g/g d.w. | *S. sclarea* µg/g d.w. | *S. nutans* µg/g d.w. | *S. verticilatta* µg/g d.w. | *S. nemorosa* µg/g d.w. |
|---|---|---|---|---|---|---|---|---|
| Caftaric acid | 311 | 3.45 ± 0.05 | < 0.02 | < 0.02 | < 0.02 | < 0.02 | < 0.02 | NF |
| Caffeic acid | 179 | 6.25 ± 0.04 | < 0.02 | 615.0 ± 0.55 | 776.5 ± 1.12 | < 0.02 | 741.5± 1.31 | < 0.02 |
| Chlorogenic acid | 353 | 6.43 ± 0.05 | < 0.02 | NF | NF | < 0.02 | NF | < 0.02 |
| *p*-coumaric acid | 163 | 9.48 ± 0.08 | 44.1 ± 0.10 | 32.1 ± 0.07 | 74.2 ± 0.18 | 399.2 ± 0.25 | 55.7± 0.10 | 146.4 ± 0.22 |
| Ferulic acid | 193 | 12.8 ± 0.10 | 55.7 ± 0.19 | NF | 60.8 ± 0.16 | 207.4 ± 0.15 | NF | 182.1 ± 0.31 |
| Hyperoside | 463 | 18.60 ± 0.12 | < 0.02 | < 0.02 | < 0.02 | < 0.02 | < 0.02 | < 0.02 |
| Isoquercitrin | 463 | 20.29 ± 0.10 | < 0.02 | < 0.02 | 2208.0 ± 1.80 | 43.57 ± 0.08 | 713.1± 1.10 | < 0.02 |
| Rutin | 609 | 20.76 ± 0.15 | 115.4 ± 0.25 | 367.8 ± 0.54 | NF | 323.2 ± 0.26 | 56.0± 0.10 | 263.8 ± 0.45 |
| Quercitrin | 447 | 23.64 ± 0.13 | < 0.02 | < 0.02 | NF | NF | < 0.02 | NF |
| Quercetin | 301 | 27.55 ± 0.15 | NF | < 0.02 | NF | NF | NF | NF |
| Luteolin | 285 | 29.64 ± 0.15 | 39.8 ± 0.07 | 6379.9 ± 2.83 | 779.6 ± 1.10 | 88.2 ± 0.10 | 74.4± 0.13 | 67.5 ± 0.17 |
| Kaempferol | 285 | 32.48 ± 0.17 | NF | NF | NF | < 0.02 | NF | NF |
| Apigenin | 279 | 33.10 ± 0.15 | 48.1 ± 0.09 | 1214.6 ± 1.64 | 293.2 ± 0.23 | 312.8 ± 0.23 | 48.1 ± 0.16 | 57.9 ± 0.21 |

NF - not found, below limit of detection; Values are expressed as mean ± SD (n = 3).

The highest amount of total polyphenols was determined in *S. austriaca* (118.75 ± 2.04); the most abundant in caffeic acid derivatives was *S. verticillata* (39.14 ± 0.32 mg/g), while the content of flavonoids was highest in (10.33 ± 0.17 mg/g) *S. nutans* (Table II).

The quantitative composition of plant material could be influenced by the pedoclimatic conditions of the region where the plant grow. The different extraction methods and standards used for phenolic expression (tannic acid equivalents, TAE, rosmarinic acid equivalents, RAE, instead of gallic acid equivalents GAE) may result in a decrease or an increase in the total phenolic content values [10, 22].

Previous reports have revealed a range of 20.2 - 575 mg GAE/g d.w. for the total phenolic content in different *Salvia* species (S. *aethiopis*, *S. austriaca*, *S. glutinosa*, *S. pratensis*, *S. ringens*, *S. verticillata*) [9, 23]. Our results fall within the area of these limits. A lot of research was done on *S. officinalis* and the reported content of polyphenolic compounds was 86.4 mg TAE/g d.w. in the Iranian sample and 75.7 ± 0.26 mg RAE/g d.w.) in Romanian sample [7, 14].

**Table II**

The content of polyphenols

| Samples | TPC (mg GAE/g plant material) | Flavonoids (mg RE/g plant material) | Caffeic acid derivatives (mg CAE/g plant material) |
|---|---|---|---|
| *S. aetiopsis* | 22.25 ± 0.24 | 4.70 ± 0.14 | 17.22 ± 0.22 |
| *S. austriaca* | 118.75 ± 2.04 | 7.68 ± 0.16 | 24.39 ± 0.24 |
| *S. sclarea* | 46.00 ± 0.32 | 1.98 ± 0.04 | 26.003 ± 0.21 |
| *S. nutans* | 29.75 ± 0.28 | 10.33 ± 0.17 | 18.627 ± 0.24 |
| *S. verticillata* | 85.25 ± 0.64 | 1.67 ± 0.03 | 39.14 ± 0.32 |
| *S. nemorosa* | 40.25 ± 0.34 | 1.18 ± 0.04 | 28.66 ± 0.24 |

Values are expressed as mean ± SD.

Alongside with the traditional methods used for the characterization of the antioxidant capacity (DPPH and FRAP), another new physiologically relevant method was applied here - the haemoglobin ascorbate peroxidase activity inhibition (HAPX) assay, based on the enzymatic properties of haemoglobin to reduce hydrogen peroxide in the presence of antioxidants *via* the high valent form of the iron, ferryl [18]. *S. verticilatta* presents the highest antioxidant capacity determined by DPPH (42.923 ± 4.81 µg/mL), FRAP (8044 ± 4.81 mmol Trolox/mg) and HAPX (1074.3 ± 836 mg CAE)/g) (Table III). After *S. verticillatta*,

*S. nemorosa* followed *S. sclarea* by appear to have a good antioxidant capacity according to all three methods. *S. nutans*, *S. austriaca* and *S. aetiopsis* have the lowest antioxidant actvity. For *S. verticillata* there is a close correlation between the antioxidant capacity with the total polyphenolic content and with the caffeic acid derivatives content. The antioxidant capacity of *S. sclarea* and *S. nemorosa* are well correlated with the poyphenolic content, flavonoid and caffeic acid derivatives content (exception for the correlation of *S. sclarea* with the flavonoid content). For *S. austriaca* there is a correlation between FRAP method and caffeic acid derivatives content. Even if this extract has the highest total polyphenolic content and very high content of flavonoids, it does not have the highest antioxidant capacity measured by DPPH and HAPX. Also a lack of correlation is found for *S. nutans* between antioxidant capacity and the content of flavonoids. In previous studies the $IC_{50}$ value of DPPH radical scavenging was reported to range from 7.7 to 192 µg/mL for *S. officinalis* extracts [10]. For the Romanian sample $IC_{50}$ was 81.12 ± 1.87 µg/mL and the HAPX value was 237.61 ± 26.35 mg RAE/g [14]. Overall, one may note an excellent correlation (coefficients at ~ 0.8 or higher) between HAPX, FRAP and caffeic acid derivative contents, respectively. Hence, the higher FRAP or HAPX results in *S. verticillata* and *S. sclarea* may be assigned to higher contents of caffeic acid derivatives. On the other hand, all three parameters showed much weaker correlation coefficients with DPPH, flavonoids or total polyphenolics. In fact, the correlation coefficients between HAPX/FRAP/caffeic and DPPH or flavonoids are negative (r = -0.3 ~ -0.7). On the other hand, DPPH correlates reasonably well with the flavonoid content (r ~ 0.8). These general trends are in reasonable agreement with previous observations when comparing parameters obtained from various methods of measuring the antioxidant and pro-oxidant reactivities [24].

**Table III**

The antioxidant activity of *Salvia* species

| Samples | DPPH $IC_{50}$ (µg/mL) | FRAP (mmol Trolox/mg d.w.) | HAPX (mg CAE)/g |
|---|---|---|---|
| *S. aetiopsis* | 158.76 ± 0.82 | 1399 ± 5.01 | 6.31 ± 0.21 |
| *S. austriaca* | 123.14 ± 0.70 | 2066 ± 4.81 | 8.88 ± 1.56 |
| *S. sclarea* | 97.67 ± 0.56 | 2791 ± 4.81 | 146.66 ± 23.69 |
| *S. nutans* | 158.03 ± 0.88 | 1546 ± 4.81 | 62.2 ± 7.31 |
| *S. verticillata* | 42.923 ± 0.23 | 8044 ± 4.81 | 1074.3 ± 836.16 |
| *S. nemorosa* | 80.09 ± 0.6 | 2797 ± 4.81 | 91.2 ± 10.34 |
| Quercetin | 5.62 ± 0.33 | - | - |
| BHT | 16.2 ± 0.42 | - | - |

All assays were performed in triplicate

## Conclusions

The polyphenolic profile and the antioxidant activity for six *Salvia* species were evaluated in order to complete scientific data related to *Salvia* genus that may be important sources of natural antioxidants. During the phytochemical screening, there were revealed significant differences, both qualitative and especially quantitative, between these species. *S. verticilatta*, rich in polyphenols, had the higher antioxidant activity, by all the three used methods. *S. austriaca*, with high content of luteolin and apigenin, was less antioxidant. A good antioxidant activity for *S. sclarea* and *S. nemorosa* could be related to the high content of caffeic acid derivatives. Overall, the studied *Salvia* species could be considered important natural sources of active principles, in special polyphenols.

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