Quantitative Analysis of Polyphenols and Biological Activity of Sage Macerates

GABRIELA STANCIU1, SIMONA LUPSOR1, FLORIN AONOFRIESEI2, NICOLETA CALOTA3, ANTOANELA POPESCU4, RODICA SIRBU4
1Ovidius University of Constanta, Department of Chemistry and Chemical Engineering, 1 Universitatii Alley, 900470, Constanta, Romania
2Ovidius University of Constanta, Faculty of Natural Sciences and Agricultural Sciences I, Department of Natural Sciences, University Str. 900470, Constanta, Romania
3Ovidius University of Constanta, Faculty of Physical Education and Sport, Physical Therapy Department, 124 Mamaia Blvd., 900527, Constanta, Romania
4Ovidius University of Constanta, Department of Pharmaceutical Science, 6 Capitan Aviator Alexandru Serbanescu, Constanta, Romania

The total phenols concentration in two sage macerates has been estimated by Folin-Ciocalteau method, identified and quantified using HPLC-DAD method in order to assess the biological activity. The results for total phenols values of Folin Ciocalteau method indicate that Salvia officinalis L macerate S2, presents a higher amount of phenolic compounds than macerate S1. By HPLC-DAD method, six individual phenolic compounds were identified in sage macerates among which where cinnamic acid was found in highest concentration (652.478 mg/100g d.w. in S2 and 473.381 mg/100g d.w. in S1). The antioxidant activity of sage macerates was evaluated using DPPH Radical Scavenging test. Sage macerates exhibited high antioxidant activity, between 439.5 mg GAE/mL and 400 mg GAE/mL. Antibacterial activity of sage macerates was evaluated against 20 Gram positive and Gram negative bacterial strains isolated from clinical specimens. Both macerates showed significant but variable antibacterial activity with inhibition zones ranging from 4.97 mm (S2) to 7.28 mm (S1). The effect was stronger on Gram positive (Enterococcus, Staphylococcus) than Gram negative bacteria (Escherichia sp, Proteus sp, Klebsiella sp). Eleven metals concentrations were determined by AAS method in sage leaves; it has been found that Cd, Ni and Pb concentrations are below the detection limits.

Keywords: sage macerates, phenolic compounds, antioxidant activity, antibacterial activity, metals concentration

Comparative studies showed that the polyphenolic compounds from sage presents significant variations depending of the plant origin, extraction solvent and harvesting season [12].

Considering the data from scientific literature the following determinations using two samples of S. officinalis macerates (S1, S2) have been performed: i) the determination of the total polyphenolic content using the Folin-Ciocâlteu spectrophotometric method, ii) the identification and quantification of the main phenolic compounds using HPLC-DAD method, iii) the evaluation of the antioxidant capacity using DPPH Radical Scavenging test.

The resistance of many pathogens to available antibiotics has become a global problem and stimulated the interest in natural alternatives. Therefore, the study of the antimicrobial properties and determination of the chemical constituents responsible for the biological effects of various plant components has become a priority [13-16] to find new solutions in controlling pathogens and preventing infectious diseases and also in exploring a natural valuable source of antioxidants. In this context, the paper analyses the effect of sage macerates (S1, S2) against 20 bacterial strains isolated from clinical specimens.

Since the plants can accumulate some toxic compounds [17-20], sage leaves were tested to estimate the toxic metal concentration before the extraction. On the other hand, the content of other bioelements such as calcium, potassium, sodium, copper and zinc has also been evaluated.
Experimental part

Plant materials
Leaves of *Salvia officinalis* L. were collected in June 2018 from organic culture in Topraisar, Constanta County, Romania. The leaves were dried at ambient temperature until constant weight was achieved and grinded to obtain powder.

Chemicals
All used reagents for chemical determinations were of analytical reagent grade.
Gallic acid was purchased from Fluka (Buchs, Switzerland) and Folin-Ciocalteau reagent from Merck (Darmstadt, Germany). The solution of gallic acid (standard phenolic compound) $1 \times 10^{-2}$ mol×L$^{-1}$ was prepared by dissolving 0.1881 g of gallic acid in 100 mL ethanol. Folin-Ciocalteau reagent was diluted with distilled water 1:2 (V/V).

DPPH (2,2-difenil-1-picrililhidrazil) was purchased from Aldrich (Germany). The standard compound solution 0.0063% (1.268 mM) was prepared in a 200 mL calibrated flask by dissolving 0.0010 g of 2,2-difenil-1-picrililhidrazil in methanol.

Apparatus
The chromatographic determinations of phenolic compounds were performed with HPLC-DAD system Agilent 1200, with quaternary pump, DAD, auto sampler.
Spectrometric measurements were carried out using a UV-Vis JASCO V550 scanning spectrophotometer.
Metals concentration determinations by AAS were done using a ContrAA® 700 spectrometer. A Certipure multielement standard solution from Merck (1 mg/mL of each metal) was used for calibration.

Sample macerates
The ethanol 97º, used for maceration operations, was obtained by fermentation of grains.
The hydro-alcoholic macerate of *Salvia officinalis* L S1 has been obtained by maceration of 50 g powder of dry sage leaves with 200 mL ethanol 97º for 3 months.
In case of S2, 50 g powder of dry sage leaves was macerated for 3 months with 200 mL ethanol 97º and 300 mL aqueous distillate of sage leaves (named sage water). The sage water was prepared by distillation of a mixture of plant and water (1 kg of fresh sage leaves with 4 L of distilled water). The distillate (2 L of sage water with pH 4.02) was kept 40 days until reached pH 3.5. At this point, the sage water was ready to use to obtain macerate S2.
For metals concentration determination by AAS, 0.5 of powder of dry leaves of sage have been mineralized with 5 mL nitric acid and 40 mL deionized water to 120º C for 130 minutes, filtered in 50 mL volumetric flasks and filled up with water.

Identification and quantification of phenolic compounds by HPLC-DAD
The resulted extractive solutions were analysed by an adapted USP30 HPLC method [21] used for separation, identification and quantification of the phenolic compounds as previously described [14-16,19].
For separation it was used a Zorbax Eclipse XDB-C18 column: 250 mm, 4.6 mm; 5 µm (Agilent Technologies). The gradient of elution was phosphoric acid 0.1% in water (solvent A) and acetonitrile (solvent B) as presented in Table 1.

| Time (min) | Solvent A | Solvent B |
|-----------|-----------|-----------|
| 0-15      | 90        | 10        |
| 13        | 78        | 22        |
| 14        | 60        | 40        |
| 17        | 50        | 40        |
| 17.5      | 90        | 10        |
| 22        | 90        | 10        |

The operation parameters of chromatographic process were: the flow rate - 1.5 mL/min; the injection volume - 20 µL; the analysis time - 22 minutes.
The quantification of founded compounds was performed at 310 nm and 35ºC. The retention times and DAD spectra were compared to available authentic standards.
To register the retention time of standard, a mixture of standard solutions in 70% methanol having the following concentrations it was used: E - resveratrol = 37 mg/mL, Z - resveratrol = 0.22 mg/L, caffeic acid = 0.36 mg/mL, chlorogenic acid = 0.37 mg/mL, cinnamic acid = 0.58 mg/mL, vanillin = 0.42 mg/mL, gallic acid = 0.39 mg/mL, ferulic acid = 0.48 mg/mL, 3-methylgallic acid = 0.34 mg/mL, ellagic acid = 0.43 mg/mL, p-coumaric acid = 0.51 mg/mL (Table 2). Standard deviations of retentions time were obtained after statistical processing of the 6 injections (soft SPSS 10). The retention times were between 0.990±0.025 min for gallic acid and 15.867±0.007 minutes for cinnamic acid.

Identification and quantitative determination of the active constituents from samples macerates was done by comparing the chromatogram of standards mixture.

| Standards       | Retention time ± SD, minutes |
|-----------------|------------------------------|
| gallic acid     | 0.990 ± 0.025                |
| 3-0-methylgallic acid | 2.606± 0.008               |
| chlorogenic acid | 3.301 ± 0.015                |
| caffeic acid    | 4.598 ± 0.036                |
| vanillin        | 6.919 ± 0.051                |
| p-coumaric acid | 7.187 ± 0.019                |
| ferulic acid    | 8.385± 0.038                 |
| Z - resveratrol | 14.467 ± 0.017               |
| ellagic acid    | 15.303± 0.027                |
| Z - resveratrol | 15.751 ± 0.038               |
| cinnamic acid   | 15.867 ± 0.007               |

(standard deviation for six injections)
Total phenolic content (TPC)

The total phenols were estimated according to the Folin-Ciocalteau method [22-28]. The absorbance of the coloured phosphotungstic-phosphomolybdic complex was measured at 681 nm. Total phenols content of sage leaves macerate was expressed as mg of gallic acid equivalents per 100 grams of dry weight (mg GAE/100g d.w). All samples were performed in triplicate and the mean value was reported.

Calibration curve

In a series of 50 mL volumetric flasks volumes of 1 mL, 2 mL, 3 mL, 4 mL, 5 mL, 6 mL and 7 mL of gallic acid standard solution were introduced and after was added 1 mL of Folin-Ciocalteau-reagent 1:10 (V:V) and 1 mL of 20% (w/v) aqueous Na2CO3; after 10 min the volume was brought to mark with distilled water. After another 30 min. of incubation at 25°C the absorbance was measured at 681 nm.

The calibration curve was linear in the range of 0.68 - 4.76 mg GAE/L, (R² =0.9987) (Figure 1).

The characteristics of the obtained calibration curve are: Y = A + B* X; A = 0.1071; B = 0.0465; Correlation coefficient = 0.9987.

For TPC analysis, 10 mL of each macerate were transferred into a 25 mL volumetric flask and brought to the mark with the same solvent previously used for maceration operation.

To measure the total phenols content, 1 mL of previously diluted samples were added in 25 mL calibrated flasks each, then 1 mL Folin-Ciocalteau reagent 1:2 (V:V), 1 mL sodium carbonate solution 20% and the process was the same like those used for calibration (Table 5).

DPPH Radical Scavenging test

The process was the same like those used for calibration. The antioxidant capacity was evaluated using DPPH Radical Scavenging test. Gallic acid (GA) was used as standard to plot calibration curves and the results were expressed as equivalents (mg GAE) [14, 16, 27-32]. In 25 mL calibrated flasks different volume of gallic acid solutions were added, then 5 mL DPPH 0.063% (1.268 mM) in methanol, filled up to the mark with methanol and let in the dark, to the room temperature for 45 minutes before the absorbance registration at 530 nm versus methanol. Previously, the DPPH solution spectrum was recorded and the maximum absorbance was registered at 530 nm. The solutions absorbance' decrease due to the antioxidant capacity of standard compounds determined the downward allure of calibration curves.

The calibration curve with gallic acid as standard was linear in 0.68 - 4.08 mg GAE/L range and the correlation coefficient was 0.9988.

To measure the antioxidant capacity, 1 mL of each sample were added in 25 mL calibrated flasks, then 5 mL DPPH 1.268 mM in methanol, filled up to the mark with methanol and let in the dark, to the room temperature for 45 minutes before the absorbance registration at 530 nm using methanol as blank.
Atomic absorption measurements for metal concentrations

Atomic absorption measurements for metal concentrations were done with a spectrometer ContrAA® 700 using flame technique. The instrument setting and operational conditions were conducted in accordance with the manufacturers’ specifications. Therefore, a Certipure multielement standard solution from Merck (1 mg/mL of each metal) was used for calibration. The following performance parameters have been determined: concentration domain (µg/L) and correlation coefficients of the calibration curve ($R^2$), limits of detection (LOD), limits of quantitation (LOQ) (Table 3) [33-36].

Difusimetric evaluation of sage macerates (S1 and S2) activity

Twenty bacterial strains, both Gram positive and Gram negative (Table 4), were tested against two hydro-alcoholic macerates (S1, S2).

Results and discussions

Phenolic compounds separation, identification and quantification

From all the available authentic standard used for determinations, only six individual phenolic compounds it were found in both sage macerates (S1, S2) (Table 5).

| Phenolic compound | S1 | S2 |
|-------------------|----|----|
| Chlorogenic acid  | 5.067 | 0.789 |
| Gallic acid       | 18.810 | 2.153 |
| Caffeic acid      | 15.858 | 1.816 |
| Camphor acid      | 18.991 | 18.991 |
| Ferulic acid      | 0.626 | 0.626 |
| p-Coumaric acid   | 18.564 | 18.564 |
| Total phenols     | 873.553 | 873.553 |

Table 5

Contents (mg/100g d.w.) and weight percentages (wt%) of individual phenolic compounds of tested sage macerates (S1, S2) determined by HPLC-DAD
Atomic absorption measurements for metal concentrations

Plants can accumulate heavy metals, which can be harmful to the human body or may cause serious health problems for humans [4, 5]. Therefore, the metal concentrations determinations for consumption plants are an important issue in order to secure the quality of both human and animal life.

The metals concentrations in the studied plant material are presented in Table 8.

Total phenolic content (TPC)

The obtained values in case of TPC analysis indicates that *Salvia officinalis* L leaves have a high amount of phenolic compounds comparable to other literature data [4, 6, 8, 11].

Table 6
THE WEIGHT PERCENTAGES (%) OF TOTAL POLYPHENOLS AND OF MAJOR PHENOLIC COMPOUNDS DETERMINED IN SAGE SAMPLES

| No. | Sample | TPC mg GAE/100g d.w. |
|-----|--------|----------------------|
| 1   | S1     | 1280                 |
| 2   | S2     | 1425                 |

The results for TPC indicates that *Salvia officinalis* L macerate S2, presents a higher amount of phenolic compounds than macerate S1, due to the different solvent used in maceration process.

The difference between the values of phenolic compounds determined by HPLC-DAD and TPC in case of tested sage macerates (S1 and S2) is due to the presence of other phenolic acids that were not determined in the applied HPLC-DAD conditions.

**DPPH Radical Scavenging test**

Results of free radical scavenging activity of both sage macerates are given in Table 7.

Table 7
THE ANTIOXIDANT ACTIVITY OF *SALVIA OFFICINALIS* L MACERATES

| No. | Sample | DPPH mg GAE/100g d.w. |
|-----|--------|-----------------------|
| 1   | S1     | 460                   |
| 2   | S2     | 493.5                 |

Data in the table show that the DPPH scavenging activities increase with the amount of the phenolic content found in studied samples.

The results indicate that both samples have significantly antioxidant activities and the differences are given by the particularity of each sage macerate which in case of sage macerate S2 is higher comparing to sage macerate S1. The findings were in agreement with the previous results of phenolic compounds quantification and identification.

From all metals considered for analysis, the concentrations of three metals (Cd, Ni and Pb) were found below the detection limit, that means there are not toxic metals in the studied sage samples. Except copper and zinc, all others found minerals are in considerable amount; this is normal given their role in living organisms.

Potassium and calcium are essential microelements for all higher plants and they were found in relatively large quantities in sage leaves.

Potassium, calcium, magnesium and sodium play specific important roles for plants and human body and the results indicate that sage leaves are a noticeable source of these essential microelements.

Based on the obtained results, the metal concentration from sage leaves expressed in mg/100g d.w. are in the range of permissible limit [33-36] and decrease in the order: K>Ca>Mg>Fe>Na>Mn>Zn>Cu.

**Difusimetric evaluation of sage macerates activity**

Generally, all strains were more or less sensitive to both macerates of sage (Table 9). However, the extent to which the individual bacteria responded to macerates was variable. In this respect, there was also a significant difference of the ability of macerates to inhibit Gram positive and Gram negative bacteria. The effect of macerates was generally weaker on Gram negative bacteria compared to Gram positive. In the case of Gram negative bacteria, the most pronounced inhibitory effect had macerate S1 (4 mm) compare to the effect was observed in case of S2 macerate (3.66 mm). *Enterococcus* strains proved to be the most sensitive, illustrated by the growth inhibition area between 9.25 (S1) and 5.5 mm for S2. Our results were comparable to other findings showing significant inhibitory effect of sage components on *enterococci* [37].
Enterococci can cause a range of infectious diseases (urinary tract infections, bacteraemia, endocarditis), and they are regarded as opportunistic pathogens in the hospital environment [38]. Therefore, sage components might be viewed as valuable alternatives to antibiotics against environmental [38]. Therefore, sage components might be regarded as opportunistic pathogens in the hospital environment [38]. Therefore, sage components might be viewed as valuable alternatives to antibiotics against Enterococcus and Staphylococcus while the growth of Gram negative bacteria was less affected by both macerates.

Conclusions

Our results showed that the total phenols concentration in sage macerates (S1 and S2) estimated by Folin-Ciocâlteau method is as following: the sage macerate S2 contains a higher amount of phenolic compounds than S1.

The difference between the values of total phenolic compounds determined by HPLC-DAD and TPC is linked to the presence of other phenolic acids that were not determined in the applied HPLC-DAD conditions.

The antioxidant capacity determined by DPPH indicates that for both samples is significantly high and the differences can be attributed to the maceration procedure.

The metal concentration from sage leaves expressed in mg/100 g d.w. are in the range of permissible limit and decrease in order: K>Ca>Mg>Fe>Na>Mn>Zn>Cu.

Sage macerates exhibit variable inhibitory effect on the bacterial growth depending on the macerate type and bacterial species. Most sensitive groups were Enterococcus and Staphylococcus while the growth of Gram negative bacteria was less affected by both macerates.

Enterococci can cause a range of infectious diseases (urinary tract infections, bacteraemia, endocarditis), and they are regarded as opportunistic pathogens in the hospital environment [38]. Therefore, sage components might be viewed as valuable alternatives to antibiotics against especially vancomycin resistant strains [39]. Important effects of growth inhibition were also recorded in especially vancomycin resistant strains [39]. Important effects of growth inhibition were also recorded in Staphylococcus strains, when large inhibition zones were recorded, ranging from 5.76 mm (S2) to 8.61 mm (S1). Significant effects of sage have also been observed against Enterococcus and Staphylococcus while the growth of Enterococcus and Staphylococcus groups was also recorded in the applied HPLC-DAD conditions.

The metal concentration from sage leaves expressed in mg/100 g d.w. are in the range of permissible limit and decrease in order: K>Ca>Mg>Fe>Na>Mn>Zn>Cu.

Sage macerates exhibit variable inhibitory effect on the bacterial growth depending on the macerate type and bacterial species. Most sensitive groups were Enterococcus and Staphylococcus while the growth of Gram negative bacteria was less affected by both macerates.
22. DOBRINAS, S., STANCIU, G., LUPSOR, S., Journal of Science and Arts, 2, no. 38, 2017, p. 321.
23. SIRBU, R., STANCIU, G., TOMESCU, A., CADAR, E., Rev. Chim. (Bucharest), 70, no. 4, 2019, p. 1197.
24. POPOV, I. N., LEWIN, G., Photosintesitized chemiluminiscence. Its medical and industrial applications for antioxidizability tests. In: A. M. Garcia-Campana, WRG Baeyens (Eds) Chemiluminiscence in Analytical Chemistry, Marcel Decker Inc., New York, Basel, 2001.
25. STANCIU, G., LUPSOR, S., SIRBU, R., Journal of Science and Arts, 1, no. 38, 2017, p. 141.
26. STANCIU, G., MIHAIESI, M., L. LUPSOR, S., Scientific Study & Research, VII, no. 4, 2006, p. 799.
27. STANCIU, G., ROTARIU, R., POPESCU, A., TOMESCU, A., Rev. Chim. (Bucharest), 70, no. 4, 2019, p. 1173.
28. STANCIU, G., LUPSOR, S., TOMESCU, A., SIRBU, R., Rev. Chim. (Bucharest), 70, no. 2, 2019, p. 373.
29. SIRBU, R., MUSTAFA, A., TOMESCU, A., STANCIU, G., CADAR, E., Mat. Plast., 56, no. 1, 2019, p. 148.
30. SIRBU, R., STANCIU, G., CADAR, E., TOMESCU, A., CHERIM, M., Rev. Chim. (Bucharest), 70, no. 3, 2019, p. 835.
31. CHERIM, M., SIRBU, R., TOMESCU, A., POPA M.F., CADAR, E., Mat. Plast., 56, no. 3, 2019, p. 179.
32. NICOLESCU, C.M., BUMBAC, M., OLTEANU, R.L., ALECU, G.C., BOBOACA-MIHAESCU, D.N., NECULA, C., RADULESCU, C., Journal of Science and Arts, 46, no.1, 2019, p. 201.
33. ***Jurnalul oficial al Uniunii Europene, Reg. CE 1881/2006, 58, no. 3, 2006, p. 3.
34. ***Jurnalul oficial al Uniunii Europene, Dir. 2002/46/CE, 36, no. 13, 2002, p. 39.
35. ***Jurnalul oficial al Uniunii Europene, Reg. CE 161/2011, L 296, 2011, p. 29-30.
36. ***Jurnalul oficial al Uniunii Europene, Dir. CE 46/2002, 58, no. 3, 2006, p. 3.
37. COWEN, M.M., Clin. Microbiol. Rev., 12, no. 4, 1999, p. 564.
38. MAATHUIS F.J.M., J. Exp. Bot., 65, no. 3, 2014, p. 849.
39. EL ASTAL, Z.Y., ASHOUR, A., KERRIT, A.A.M., Pak. J. Med. Sci., 21, 2, 2005, p. 187.
40. ELIOPOULOS, G.M., GOLD, H.S., Clin. Infect. Dis., 33, 2001, p. 210.
41. ZOMORODIAN, K., MOEIN, M., PAKSHIR, K., KARAMI, F., SABAHI, Z., Journal of Evidence-Based Complementary & Alternative Medicine, 22, no. 4, 2017, p. 770.
42. ARORA, D., KAUR, J., Intern J Antimicrob Agents, 12, 1999, p. 257.
43. OKEMO, P., MWATHA, W., CHHABRA, S., FABRY, W., Afr. J. Sci. Tech., 2, 2001, p. 113.
44. MADAMOMBE, I., AFOLAYAN, A., Pharmaceut. Biol., 41, 2003, p. 199.
45. INOUYE, S., TAKIZAWA, T., YAMAGUCHI, H., J Antimicrob Chemother., 47, 2001, p. 565.
46. NITTA T, ARAI T, TAKAMATSU H, INATOMI, Y., MURATA, H., IINUMA, M., TANAKA, T., ITO, T., ASAI, F., IBRAHIM, I., NAKANISHI, T., WATABE, K., J Health Sci., 48, 2002, p. 273.
47. GORDON, R.J., LOWY, F.D., Clin Infect Dis., 46, 2008, p. S350.
48. BAJPAI, V.K., RAHMAN, A., KANG, S. C., Int. J. Food Microbiol., 125, no. 2, 2008, p. 117.

Manuscript received: 4.11.2019