

## Phytochemical analysis and in vitro biological activity of *Betonica officinalis* and *Salvia officinalis* extracts

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### Abstract

The objectives of this study were to define the phenolic profile, antioxidant, antimicrobial and anti-inflammatory properties of extracts from *Betonica officinalis* and *Salvia officinalis* herbs used in traditional medicine. The results indicated that the extract inhibits scavenges stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) cation radicals. In the present study, *Salvia officinalis* was potent antibacterial agent against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa*. The extracts inhibited cyclooxygenase (COX-1) and denaturation of protein, which suggests these species might be a potential source of plant-derived anti-inflammatory substances. Our study, for the first time, revealed the antimicrobial and anti-inflammatory activity of the *Betonica officinalis* herb. The results of this study could be helpful to develop medicinal preparations and uses of this species as an antimicrobial, anti-inflammatory and wound healing agent.

**Key words:** HPTLC analysis, antioxidant, antimicrobial, anti-inflammatory, *Betonica officinalis*, *Salvia officinalis*

### Introduction

Medicinal plants have long been recognised as important sources of biological active compounds. *Betonica officinalis* L. (wood betony, Lamiaceae family, synonyms *Stachys officinalis* L.) is a perennial herb found in dry grassland, meadows and open woods in most of Europe, western Asia and North Africa. *B. officinalis* has been used in traditional medicine internally as tea, or externally as compresses or baths for treatment of disorders of the respiratory tract, gastrointestinal tract, nervous system, skin and gynecological problems [1]. *Stachys* species contain polyphenols such as tannins, phenolic acids, flavonoids, alkaloids trigonelline and stachydrine, betaine, volatile oils, and choline [2, 3].

*Salvia officinalis* L., also known as sage, is an herbaceous plant belonging to the Lamiaceae family. It is native to Mediterranean regions and has been used for culinary and

medical purposes. *Salvia* species are reported to have antioxidant and anti-inflammatory properties and their use as medicinal herb includes the treatment of body wounds [4-7]. The *in vitro* anti-inflammatory activity of several essential oils and solvent extracts of *Salvia* species was evaluated using the 5-lipoxygenase assay, indicating that essential oils exhibited better anti-inflammatory activity than solvents extracts [8].

The inflammatory processes are subjects to a wide range of diseases such as: rheumatoid arthritis, osteoarthritis, atherosclerosis a.s.o, that affects people worldwide. The treatment relies on steroidal and non-steroidal anti-inflammatory drugs which are found to have several adverse effects that include hirsutism, peptic ulceration, hyperglycemia, osteoporosis, and immunodeficiency related problems [9]. Therefore, the search for new anti-inflammatory drugs based on natural products represents an important field in drug discovery.

In order to search for effective natural antioxidants, antimicrobial and anti-inflammatory natural drugs, current study has selected three Romanian medicinal plants based on their ethno-pharmacological importance.

## Materials and methods

All chemicals and solvents were purchased from Sigma Chemical Company (Sigma Aldrich, Germany), Fluka (Switzerland), Roth (Carl Roth GmbH, Germany), Chemical Company (Romania) and deionized water was used for all the performed analysis (Millipore, Bedford, MA). Media Brain Heart Infusion Broth (BHI) was purchased from Fluka. HPTLC plates G60 F254, 100x100 mm (Merck, Darmstadt, Germany). Leaves and flowers of *Betonica officinalis* and *Salvia officinalis* were collected in 2014 from Piatra Neamt region, Romania and authenticated by a plant taxonomist. Plant material was dried in a well-ventilated room, in a single layer, protected from direct solar light and then powdered.

### Extraction procedure – ultrasound-assisted extraction

Ground plant material was extracted with solvent ethanol/water (50% and 70% v/v) in an ultrasonic apparatus (Elma Transsonic 460/H, frequency 35 kHz) for 1 h. The obtained extract was filtered under vacuum through No.1 Whatman filter paper, stored at 4 °C for further use. The herbal's mass concentration in the solvent was 100 g/L.

### Phytochemical characterization of the extracts

#### HPTLC identification

HPTLC chromatographic studies were done according to literature [10].

#### a. Flavonoids and polyphenolcarboxylic acids identification

1 g dry powder from each vegetal material was weighed accurately, and heated under reflux for 15 minutes with 10 mL methanol. The samples were cooled and filtered. The reference standards E1 (rutin, chlorogenic acid, hyperoside and caffeic acid) and E2 (rosmarinic acid) were weighed accurately and diluted with methanol (0.02% concentration of standard solution).

Aliquots (6 µL) of sample solutions, and aliquots (3 µL) of reference solutions were applied separately band wise (start position - 17 mm from both sides and 15 mm from the bottom, distance between bands – 6 mm, band length – 6 mm, delivery speed – 8 sec./µL) to silica gel 60 F254 – pre-coated HPTLC plates, 10x10 cm, using Camag Linomat IV automatic sample applicator. The plate was developed in a saturated vertical - developing chamber at room temperature (20-22°C) for 30 minutes, with ethyl acetate - anhydrous formic acid – acetic acid - water (100:11:11:26, v/v) as mobile phase. The development distance was 7 cm.

Before derivatization, the plate was heated at 105°C temperature for 10 minutes. The derivatization of the chromatogram was performed by spraying the plate with 1% β-aminoethyl diphenyl borate (NP) in methanol and 5% polyethylene glycol in ethanol. The documentation was carried out using CAMAG Reprostar 3 with digital video camera, on developed plate at 254 and 366 nm (Figure 1 a,b) and on derivatized plate 366 nm (Figure 1c).

#### b. Tannins identification

Samples: 0.5 g dry powder from each vegetal material was weighed accurately, and heated under reflux for 20 minutes with 10 mL ethanol 50% v/v. The samples were cooled and filtered. Each sample was concentrated to 2 ml, and applied. The reference standard (gallic acid, tannic acid) – E - was weighed accurately and diluted with methanol (0.02% concentration of standard solution).

Aliquots (7 μL) of sample solutions, and aliquot (4 μL) of reference solution were applied separately band wise (start position - 18.5 mm from both sides and 15 mm from the bottom, distance between bands – 7 mm, band length – 7 mm, delivery speed – 8 sec./μL) to silica gel 60 F254 – pre-coated HPTLC plates, 10x10 cm, using Camag Linomat IV automatic sample applicator. The plates were developed in a saturated vertical - developing chamber at room temperature (20-22°C) for 30 – 40 minutes, with toluene - ethyl acetate - anhydrous formic acid (12:12:2, v/v) as mobile phase. The derivatization of the chromatogram was performed by spraying the plate with 5% FeCl<sub>3</sub>, followed by heating of the plates at 100°C, 10 minutes. The documentation was carried out using CAMAG Reprostar 3 with digital video camera, on developed plates in white light (Figure 2).

#### Determination of phytochemical compounds

**Total polyphenols and flavonoids assessment.** The phenolic total content was determined by the Folin–Ciocalteu method [11]. Gallic acid (GAE) was used to calibrate the standard curve; total polyphenols contents were obtained from the regression equation of the gallic acid's calibration curve ( $y = 0.006x + 0.0197$ ,  $R^2 = 0.9973$ ) and expressed as Gallic acid equivalents (GAE) in mg/L of extracts.

The total flavonoid content was determined according to the aluminium chloride colorimetric method with few modifications [12]. Rutin was used as standard and the results were expressed as mg rutin equivalents (RE) /L of extract. Total flavonoid contents were obtained from the regression equation of the rutin's calibration curve ( $y = 0.0051x + 0.0286$ ,  $R^2 = 0.9966$ ).

**Determination of soluble sugar.** Total sugars were measured with phenol–sulfuric acid reagents spectrophotometrically at 480 nm according to Dubois [13]. The concentration of soluble sugar was determined against a standard curve prepared by using a glucose solution ( $y = 0.0235x + 0.1945$ ,  $R^2 = 0.9859$ ) and the results were expressed as mg glucose equivalents/L of extract.

#### Antiradical activity

The antiradical activity was measured using the DPPH and ABTS methods.

**DPPH method** is based on the decrease of the 2,2-diphenyl-1-picrylhydrazyl (DPPH; Sigma-Aldrich) maximum absorbance at 519 nm in the antioxidant presence [14]. The decreasing of the DPPH radical absorption by the action of antioxidants could be used for measuring the antioxidative activity. The equivalent of the antioxidant capacity being expressed as Trolox equivalent:

$$TEAC_{\text{sample}} = C_{\text{Trolox}} \times f \times \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{Trolox}} - A_{\text{blank}}}$$

where  $A_{\text{blank}}$  represents the maximum absorbance of the blank solution,  $A_{\text{Trolox}}$  represents the maximum absorbance of Trolox stock solution,  $A_{\text{sample}}$  represents the absorbance of sample,  $f$  is the dilution factor of the sample,  $C_{\text{Trolox}}$  is the concentration of Trolox in μmol/ L.

The other used *method* was *ABTS* (2,2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic) radical cation scavenging [15]. Generation of radical cation (ABTS<sup>•+</sup>) involves the reaction between ABTS and potassium persulfate, resulting in production of the blue/green ABTS<sup>•+</sup> chromophore with a maximum absorption at 731 nm. In the presence of the antioxidants the pre-formed radical cation is reduced to ABTS, proportionally to the antioxidant activity. This assay is used to determine the total antioxidant activity. The ABTS<sup>•+</sup> radical scavenging ability was calculated according to the same equation as that of the DPPH assay.

#### **Antibacterial assay**

The hydroalcoholic vegetal extracts were tested *in vitro* for antibacterial activity, using diffusimetric method. The antimicrobial activity was tested against several human pathogenic strains, including Gram-positive and Gram-negative bacteria: *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25992 and, respectively, *Bacillus subtilis* 222 (from the Microbial Collection of MICROGEN, Department of Genetics, University of Bucharest).

Bacteria strains were incubated at 37°C for 24 h in liquid BHI media. The optical density of the bacterial cultures was measured at  $\lambda = 600$  nm using spectrophotometer ULTROSPEC 3000 (Pharmacia Biotech) and the bacterial suspension was adjusted at 0.5 with BHI liquid. 150  $\mu$ l bacterial suspension was incorporated in 4 ml BHI agar 7% and incorporated in BHI agar medium. After solidification we placed 10  $\mu$ l vegetal liquid extracts and ethanol 50% used as negative control and the plates were incubated at 37°C for 24h-48h. The antibiotic gentamicin was used as the positive control. The antibacterial activity was evaluated by measuring the diameter of the growth inhibition zone around the discs. All tests were performed in triplicate and the mean diameter of the inhibition zone was recorded.

#### **Evaluation of in vitro anti-inflammatory activity**

Anti-inflammatory activity of the extracts were evaluated by two assays: protein denaturation method and cyclooxygenase-1 inhibition.

**Inhibition of albumin denaturation** was performed as per Williams et al. with slight modifications [16]. The test mixture consisted of 1 mL bovine serum albumin (1% w/v) and 1 mL of test sample (1000, 500 and 250  $\mu$ g/mL). Diclofenac sodium was used as the reference drug. The samples were incubated at 37° C for 20 min and then heated at 60° C for 20 min. After cooling the samples, 1 mL phosphate buffer saline (pH 6.3) was added to each tube. Turbidity was measured spectrophotometrically at 660 nm. For control tests the distilled water was used instead of sample. The percentage inhibition of protein denaturation was calculated as follows:

$$\% \text{ inhibition} = (\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) / \text{Abs}_{\text{control}} \times 100$$

**Cyclooxygenase-1 assay** . The cyclooxygenase-1 (COX-1) activity was measured according to the literature with slight modifications [17]. The reaction system was incubated at 25 °C for 20 min, by sequential addition of the buffer, hematin, test solution (the plant extract or an inhibitor), and COX-1 into the system followed by mixing with arachidonic acid and N,N,N',N'-Tetramethyl-p-Phenylenediamine (TMPD). The initial velocity of the reaction was measured following the reaction of oxidation of TMPD at 611 nm for 60 s. The inhibitory effect of various concentrations of extracts and reference compound, ibuprofen was also expressed as percentage of enzyme activity inhibition. IC<sub>50</sub> indicating the concentration required to inhibit 50 % COX-1 activity was also calculated.

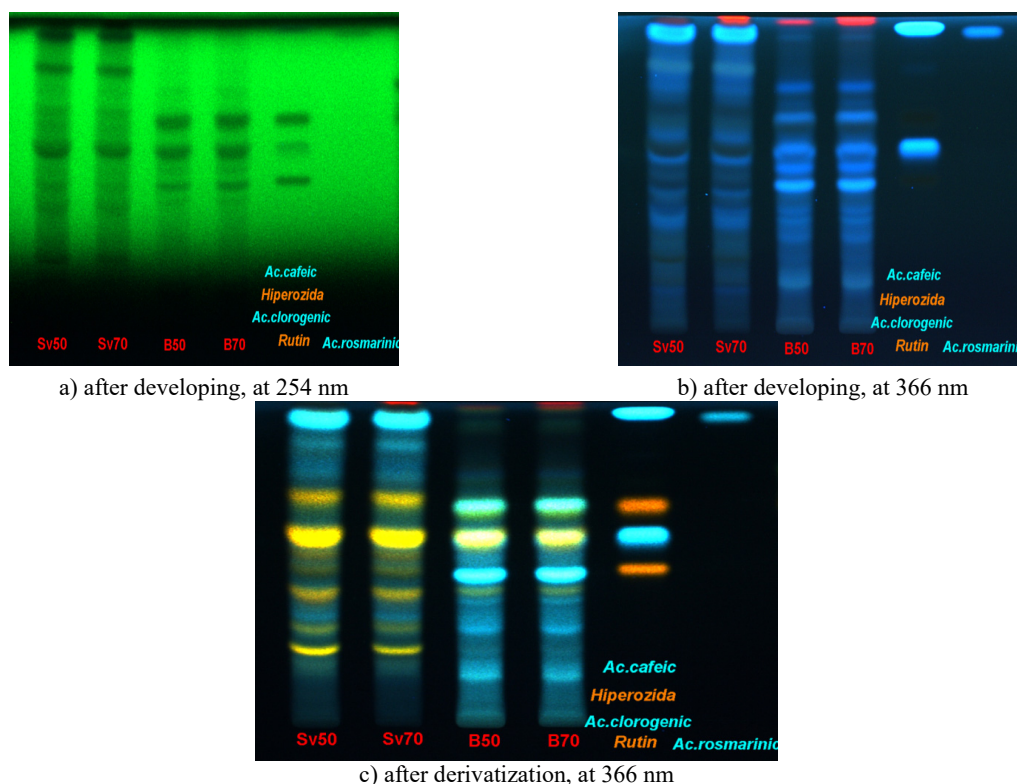
### Statistical analysis

The measurement was performed in triplicate, and for statistical processing Excel 2007 was used. The results were expressed as means  $\pm$  SD and evaluated by Student's *t*-test and  $p < 0.05$  was considered as statistically significant.

### Results and Discussions

In plants, phenolic and flavonoid compounds have exhibited antioxidant, antimicrobial, antiviral, and anti-inflammatory activities [18, 19]. Meantime, polysaccharides have attracted growing scientific interests for their ability to exert marked effects on immune system function and inflammation [20, 21].

High-performance thin-layer chromatography (HPTLC) was used to identify the compounds responsible for the antioxidant, antibacterial and anti-inflammatory activities. The qualitatively phytochemical study performed by thin layer chromatography revealed that the selected medicinal plants – *S. officinalis* (sage, aerial part) and *B. officinalis* (aerial part) have a diverse content in phenolic substances (flavonoids and polyphenolcarboxylic acids). After developing at UV 254 nm, flavonoids and polyphenolcarboxylic acids show dark gray spots (Figure 1a), while at UV 366 nm shows blue to strong blue fluorescent spots with different colours intensity (Figure 1b), at distinct *R<sub>f</sub>* values. After derivatization, polyphenolcarboxylic acids show blue fluorescent spots, while flavonoids show orange yellow to green spots with different colours intensity (Figure 1c), at distinct *R<sub>f</sub>* values. The HPTLC images (Figure 1 a-c) indicate that all sample constituents were clearly separated. The reference substances were identified at the following *R<sub>f</sub>* values: rutin - 0.40, chlorogenic acid - 0.54, hyperoside - 0.64, caffeic acid - 0.93, and rosmarinic acid - 0.92. HPTLC phenolic profiles are similar for both types of ethanolic extracts (50% and 70% v/v).



**Fig. 1 (a-c).** HPTLC chromatogram of flavonoids and polyphenolcarboxylic acids in *S. officinalis* and *B. officinalis*



Tracks: 1- Sv (sage); 2 – E1 (rutin, chlorogenic acid, hyperoside, caffeic acid);  
 3 – E2 (rosmarinic acid); 4 – B (betony);

The tannins of the analyzed vegetal materials were identified, according to the reference substances, in white light, after the chromatogram spraying with the identification reagent (Figure 2). An intense spot with a specific colour of tannins was noted in sage sample, at *Rf* value of 0.33. Some blue-gray spots (possibly other tannins), but weaker, were identified at *Rf* values of 0.1, 0.17 and 0.46. Some very weak spot (*Rf* 0.47 and 0.53) were visualized in betony sample.

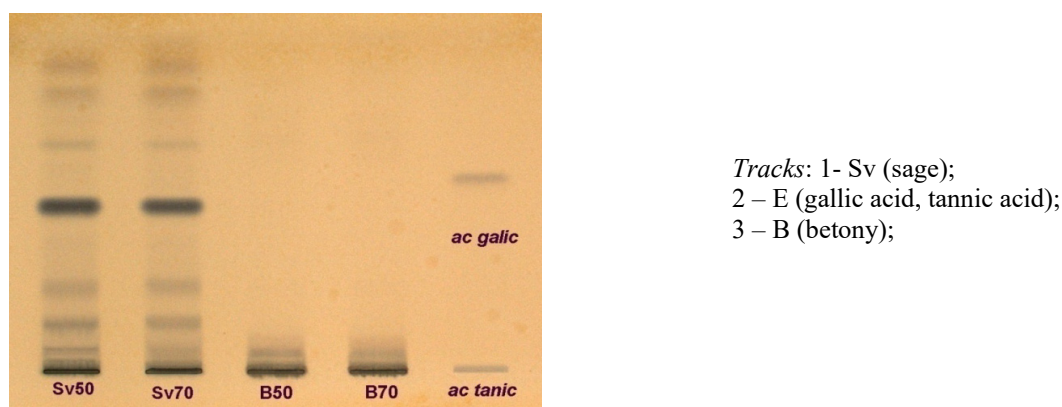


Fig. 2. HPTLC chromatogram of tannins in *S. officinalis* and *B. officinalis*

The highest total phenolic content (TPC) was exhibited by the *S. officinalis* hydroalcoholic extract (1072.9±10.7 mg GAE/L), followed by the *B. officinalis* hydroalcoholic extract (869.7± 18.2 mg GAE/L). The highest TFC was exhibited by the *S. officinalis* hydroalcoholic extract (267.3±4.9mg QE/L), too (Table 1).

Table 1. Bioactive compounds analysis of the hydroalcoholic extracts

Bioactive compounds	Sample	
	<i>Betonica officinalis</i>	<i>Salvia officinalis</i>
TPC (mg GAE/L)	869.7± 18.2 <sup>a</sup>	1072.9±10.7 <sup>c</sup>
TFC (mg QE/L)	64.5± 1.5 <sup>a</sup>	267.3±4.9 <sup>c</sup>
Sugars (mg Gluc/L)	122.1± 6.1 <sup>a</sup>	75.4±3.6 <sup>c</sup>

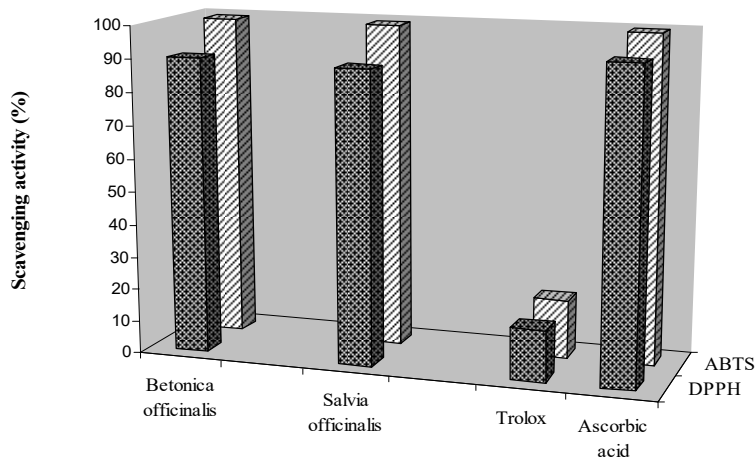
were: TPC = total phenolic content; TFC = total flavonoids content.

\*Values expressed as mean ± standard deviation obtained from 3 measurements. For each group of compounds (within each table line), different letters (a, b or c) represent significant differences at  $p < 0.05$ .

The antioxidant property of the extracts was investigated by two biochemical assays: DPPH and ABTS assays (Figure 3). DPPH and ABTS are commonly used as effective assays to evaluate the antioxidant capacity of plant extracts. Unlike the reactions with DPPH radical, which involves proton transfer, the reaction with ABTS radicals involves electron transfer process. As is shown in Figure 3, the *B. officinalis* extract followed by *S. officinalis* extract showed the highest DPPH and ABTS radical scavenging abilities, comparable to that of Ascorbic acid used as reference antioxidant drug.

The highest polyphenolic amount found in *S. officinalis* was in accordance with previous studies [7, 22, 23]. Regarding the quantitative results reported by the above

mentioned authors, however these results cannot be compared to the levels obtained in this work due to the units used to express results (mg/L). The antioxidant activity of *B. officinalis* and *S. officinalis* was previously reported and was related to the content of phenolic compounds [4, 5, 6, 23, 24].



**Figure 3** Radical scavenging activity of *Betonica officinalis* and *Salvia officinalis* by DPPH and ABTS methods, with Trolox and Ascorbic acid as positive controls

Antibacterial activity of extracts of *B. officinalis* and *S. officinalis* were carried out against a range of Gram-positive and Gram-negative microorganisms responsible for different food borne diseases and/or for the spoilage of contaminated products. Only the extract of *S. officinalis* exhibited zone of inhibition against all the bacteria tested, which were smaller when compared to zones of inhibition produced by the commercial antibiotic, gentamicin (10 µg/mL). *Betonica officinalis* extract showed moderate activity against *B. subtilis* and *P. aeruginosa*, but did not show any activity against other bacterial strains. These findings are of great significance, especially in the case of *S. aureus* and *B. cereus* that are well-known for being resistant to numerous antibiotics (Table 2). The results in Table 2 revealed that Gram (+) bacteria (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*) are more sensitive to the plant extracts than Gram (-) bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). These findings are of great significance, especially in the case of *S. aureus* and *B. cereus* that are well-known for being resistant to numerous antibiotics.

**Table 2.** Antibacterial activities of extracts against tested microorganisms based on inhibition zone diameters (mm).

Microorganism	Extracts		Antibiotic
	<i>Betonica officinalis</i>	<i>Salvia officinalis</i>	
<i>Staphylococcus aureus</i> ATCC 29213	-	12±0.5	21±1.2
<i>Staphylococcus epidermidis</i> ATCC 12228	-	9±0.4	19±0.9
<i>Bacillus subtilis</i> 222	4±0.1	14±0.8	21±1.1
<i>Escherichia coli</i> ATCC 25992	-	8±0.2	23±1.3
<i>Pseudomonas aeruginosa</i> ATCC 27853	8±0.3	10±0.7	22±1.5

-: No zone of inhibition

Due to the presence of flavonoids and phenolic compounds, a number of plants have been reported to also possess antimicrobial properties [25]. Generally, Gram (-) bacteria are more resistant than Gram (+) bacteria. The same characteristics were observed in other antimicrobial studies of plant extracts against pathogenic bacteria [26, 27]. Flavonoids can retard the growth of microorganisms by inhibiting their nucleic acid synthesis, cytoplasmic membrane function and energy metabolism [28]. Therefore, the antibacterial activity exhibited by these extracts could be attributed to the presence of specific phenolic compounds in their composition and to the possible existence of synergistic effects with others non phenolic compounds present in the extracts. Antimicrobial activities of *S. officinalis* hidroalcoholic extract have also been reported by other authors [29].

Compounds that have radical scavenging activities may thus be expected to have therapeutic potentials for several inflammatory processes. As part of the investigation of biological activity of *Betonica officinalis* and *Salvia officinalis* hidroalcoholic extracts, the *in vitro* anti-inflammatory activity was studied by protein denaturation and cyclooxygenase (COX-1) inhibition. The results are summarized in Table 3.

**Table 3.** Anti-inflammation activity of hidroalcoholic extracts from *Betonica officinalis* and *Salvia officinalis*

Extracts/standards	Protein denaturation	COX-1
	IC <sub>50</sub> , mg/mL	inhibition IC <sub>50</sub> , µg/ml
<i>B. officinalis</i>	11.7±0.6	49.2±1.2
<i>S. officinalis</i>	5.0±0.3	19.1±0.8
Diclofenac sodium	0.108±0.01	-
Ibuprofen	-	3.12±0.2

The results showed that the diclofenac sodium and tested extracts inhibited the heat-induced protein denaturation in a concentration dependent manner. Evaluating the anti-inflammatory potential of selected herbal extracts was performed also by using the colorimetric ovine cyclooxygenase-1 (COX-1) assay. The IC<sub>50</sub> of the extracts showed potent inhibitory activity for *S. officinalis* (IC<sub>50</sub> 19.1±0.8 µg/ml) and moderate activity for *B. officinalis* (IC<sub>50</sub> 49.2±1.2 µg/ml). However, the inhibition of COX-1 activity of all extracts was lower than that of ibuprofen.

Denaturation of proteins is a well documented cause of inflammation and rheumatoid arthritis. Mechanism of denaturation probably involves alteration in electrostatic, hydrogen, hydrophobic and disulphide bonding [30, 31]. From the results of present study it can be stated that hidroalcoholic extracts of *B. officinalis* and *S. officinale* are capable of controlling the production of auto antigen and inhibits denaturation of protein in inflammatory disease. A candidate that can inhibit denaturation of protein may be a suitable anti-inflammatory agent.

Cyclooxygenase (COX) is the key enzyme in the synthesis of prostaglandins, prostacyclins and thromboxanes which are involved in which are involved in vascular, pain and inflammatory processes. Inhibition of cyclooxygenase activity is the mechanism by which nonsteroidal anti-inflammatory drugs (NSAIDs) exert their analgesic, antipyretic, anti-inflammatory, and antithrombotic effects [32]. The most recent studies present the selective COX-1 inhibition and their relevance to human pathology such as cancer, neuro-inflammation, fever and pain [33]. All the evaluated medicinal plants in this study showed



COX-1 inhibitory activity and other pharmacological activities. Phytochemicals, in particular phenolics, were observed in the all tested extracts. It is previously reported that many flavonoids and some polyphenols are known to inhibit some molecular targets of pro-inflammatory mediators in inflammatory responses. They also act as antioxidants by scavenging radicals and thereby attenuate the inflammatory process [34]. According to Chi et al., anti-inflammatory activity of flavonoids is mediated by the inhibition of the arachidonic acid metabolizing enzymes, as well as by their antioxidant properties [35].

Salvianolic acid B, a bioactive compound present in *Salvia spp.*, has been reported to have anti-inflammatory and anti-oxidative effects [36]. Caffeic and rosmarinic acids present in *B. officinalis* and *S. officinalis* have been also reported the anti-inflammatory ability of in animal model [37]. Our results supported previous work showing that a hydroalcoholic extract of *S. officinalis* produced anti-inflammatory effects *in vivo* [38].

In this study, we report for the first time the anti-inflammatory and antibacterial activities of *Betonica officinalis*.

In conclusion, this study suggests that the studied herbal extracts may be of value for application in human and animal health and have provided a potential source for development of novel therapeutic agents.

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