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# *IN VITRO* BIOLOGICAL ACTIVITY OF SECONDARY METABOLITES FROM *SESELI RIGIDUM* WALDST. ET KIT. (APIACEAE)

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The antioxidant, antimicrobial activity, total phenolic content and flavonoid concentration of *Seseli rigidum* Waldst. et Kit. were evaluated. Five different extracts of the aboveground plant parts were obtained by extraction with distilled water, methanol, acetone, ethyl acetate and petroleum ether. Total phenols were determined using the Folin-Ciocalteu's reagent, with the highest values obtained in the acetone extract (102.13 mg GAE/g). The concentration of flavonoids, determined by using a spectrophotometric method with aluminum chloride and expressed in terms of rutin equivalent, was also highest in the acetone extracts (291.58 mg RUE/g). The antioxidant activity was determined *in vitro* using DPPH reagent. The greatest antioxidant activity was expressed in the aqueous extract (46.15 µg/ml). *In vitro* antimicrobial activities were determined using a microdilution analysis method; minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) were determined. Methanolic extract had the greatest influence on bacilli (MIC at 0.0391 mg/ml), but the best antimicrobial effect had acetone and ethyl acetate extracts considering their broad impact on bacteria. According to our research, *S. rigidum* can be regarded as promising candidate for natural plant source with high value of biological compounds.

*Keywords:* Antioxidants – antimicrobial activity – flavonoids – phenols – *Seseli rigidum*

## INTRODUCTION

Plants with their secondary metabolites have been used by human since the early days to treat health disorders, infections and illness [27]. Although over time the natural products are replaced by synthetics, extremely difficult or economically infeasible chemical synthesis of pharmaceutical significant secondary metabolites have led to the fact that today most of these metabolites are isolated from cultivated or wild plants [8].

Harmful effects and various problems caused by oxidative stress and increased production of reactive oxygen species can greatly be prevented by entering antioxidants. On the other hand, plant secondary metabolites have significant antioxidant activity, which have great advantage compared to synthetic and do not induce side effects. In addition to substances with antioxidant activity, plants are also sources of

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antibiotic compounds. The uncontrolled use of commercial antimicrobial chemicals has led to resistance in both human and plant pathogenic organisms, hence to find new sources of antimicrobial substances is necessary [26].

In the present study we carried out the analysis of aerial parts of *Seseli rigidum* Waldst. et Kit., as potential sources of valuable constituents with biological activities. *S. rigidum* belongs to the family Apiaceae Lindley (Umbeliferae Juss.). This is a perennial gray or gray-green plant with large spindle-shaped roots and vertical stems branched in the form of shield, up to 50 cm high. Leaves are linear, twice pinnate shared with petiole expanded in leaf sheat. Flowers are small and white, arranged in an umbelliform inflorescence. *S. rigidum* inhabits calcareous substrates or ultramafic as facultative serpentinophyte in Balkan Peninsula and Romania [2, 15, 18].

Plants from the *Seseli* genus have recently been investigated, particularly due to the content and activity of essential oils. The main constituent of essential oils obtained from *S. peucedanoides* was α-pinene, ß-pinene and limonene [3]. Carotol, γ-terpinene, germacrene D and sabinene were found to be the most abundant compounds in *S. petraeum* and *S. andronakii* essential oils [25]. In case of *S. annuum*, germacrene, sabinene, ß-ocimene and limonene as main constituent was highly effective in inhibiting the growth of fungi [17], while according to Ozturk and Ercisli [19] trans-caryophyllene, ß-farnesene, eusarone and spathulenol were related to antimicrobial activity of *S. libanotis* essential oils. Several biological activities of the *Seseli* species were reported, including *in vivo* and *in vitro* anti-inflammatory, antinociceptive, antimicrobial and antioxidant activities [12, 16, 21].

To the best of our knowledge, phytochemical analyzes of *S. rigidum* was done only in the case of essential oil composition and activity, both in the whole plant, and their parts [14, 15, 23]. These studies have shown that α-pinene, camphene, ß-pinene and limonene are main constituent of the essential oil with potential antimicrobial and antioxidant activity.

The basic aim of this research was to screen *in vitro* radical scavenging, antioxidant properties and antimicrobial effects of the aqueous, methanolic, acetone, ethyl acetate and petroleum ether extract, as well as total phenolic content and flavonoid concentrations in aerial parts of *S. rigidum.* To the best of our knowledge, no data on antimicrobial and antioxidant activity, phenol and flavonoid content of these plants extracts have been provided so far.

## MATERIALS AND METHODS

### *Plant material*

In July 2012 aerial flowering parts of *Seseli rigidum* Waldst. et Kit. were collected from natural populations in the region of Homoljske planine mountain in eastern Serbia (position: 44° 17’ 951” N, 21° 32’ 626” E, altitude: 825 m, substratum: limestone). Collection of plant material was carried out by sampling of several aboveground flowering branches from 20 representative individuals of the population in the

first third of the flowering period. The voucher specimen of *S. rigidum,* determined by M. Stanković and D. Jakovljević, was deposited at the Herbarium of the Department of Biology and Ecology, Faculty of Science, University of Kragujevac. The collected plant material was air-dried in darkness at ambient temperature. The dried plant material from all individuals was cut up, mixed and stored in tightly sealed dark containers until needed.

### *Preparation of plant extracts*

Prepared plant material (10 g) as a powdered mixture from 20 sampled *S. rigidum* individuals was transferred to dark-colored flasks with 200 ml of solvent (distilled water, methanol, acetone, ethyl acetate and petroleum ether obtained from Zorka Pharma, Serbia), respectively and stored at ambient temperature. After 24 h, infusions were filtered by Whatman No. 1 paper and residue was re-extracted with equal volume of solvents. After 48 h, the process was repeated. Combined supernatants were evaporated to dryness under vacuum at 40 °C using a rotary evaporator.

### *Determination of total phenolic contents in the plant extracts*

The total phenolic content was determined using a spectrophotometric method [7]. The reaction mixture was prepared by mixing 0.5 ml of methanolic solution (1 mg/ml) of extract with 2.5 ml of 10% Folin-Ciocalteu's reagent (Fluka Chemie, Switzerland) dissolved in aqueous and 2.5 ml of 7.5% $NaHCO_3$. The samples were incubated at 45 °C for 15 min. The absorbance was determined at $\lambda_{max}$ = 765 nm. The samples were prepared in triplicate and the average value of absorbance was obtained. A blank was concomitantly prepared with methanol instead of extract solution. The same procedure was repeated for the gallic acid (Sigma Chemical Co., USA) and the calibration curve was construed. The total phenolic content was expressed in terms of gallic acid equivalent (mg of GAE/g of extract).

### *Determination of flavonoid concentrations in the plant extracts*

The concentration of flavonoids was determined using a spectrophotometric method [22]. The sample contained 1 ml of methanolic solution of the extract in the concentration of 1 mg/ml and 1 ml of 2% $AlCl_3$ (Fluka Chemie, Switzerland) solution dissolved in methanol. The samples were incubated for 1 h at ambient temperature. The absorbance was determined at $\lambda_{max}$ = 415 nm. The samples were prepared in triplicate and the average value of absorbance was obtained. The same procedure was repeated for the rutin (Sigma Chemical Co., USA) and the calibration curve was construed. Concentration of flavonoids in extracts was expressed in terms of rutin equivalent (mg of RUE/g of extract).

## *Evaluation of DPPH scavenging activity*

The ability of the plant extract to scavenge 1,1-dyphenyl-2-picrylhydrazyl (DPPH) free radicals was assessed using the method described by Kumarasamy et al. [11]. The stock solution of the plant extract was prepared in methanol to achieve a concentration of 1 mg/ml. Dilutions were made to obtain 10-fold serial concentrations (from 500 to 0.97 μg/ml). Diluted solutions (1 ml each) were mixed with 1 ml of DPPH (Sigma Chemical Co., USA) methanolic solution. After 30 min in darkness at ambient temperature, the absorbance was recorded at 517 nm. The control samples contained all the reagents except the extract. The percentage inhibition was calculated using the following equation: % inhibition = 100 × (A *control* – A *sample*)/A *control*). The $IC_{50}$ values (inhibitory dose that inhibited cell growth by 50%) were estimated from the % inhibition versus concentration sigmoidal curve, using a nonlinear regression analysis.

## *Determination of antimicrobial activity*

### *Suspension preparation*

Bacterial suspensions were prepared by the direct colony method. The turbidity of initial suspension was adjusted by comparing with 0.5 McFarland's standard for bacteria [1]. Initial bacterial suspensions contained about $10^8$ colony forming units (CFU)/ml. 1 : 100 dilutions of initial suspension were additionally prepared into sterile 0.85% saline for Gram-negative bacteria, and 1 : 10 for Gram-positive bacteria.

### *Microdilution method*

Antimicrobial activity was tested by determining the minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) by using microdilution method with resazurin (Alfa Aesar GmbH & Co., Germany) [20]. The 96-well plates were prepared by dispensing 100 μl of nutrient broth, Mueller-Hinton broth (Liofilchem, Italy) into each well. A 100 μl from the stock solution of tested extracts (concentration of 40 mg/ml) was added into the first row of the plate. Then, twofold, serial dilutions were performed by using a multichannel pipette. The obtained concentration range was from 20 mg/ml until needed. A 10 μl of diluted bacterial suspension was added to each well to give a final concentration of $5 \times 10^5$ CFU/ml. Finally, 10 μl of resazurin solution was added to each well inoculated with bacteria. Resazurin is an oxidation–reduction indicator used for the evaluation of microbial growth. It is a blue non-fluorescent dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. The inoculated plates were incubated at 37 °C for 24 h. MIC was defined as the lowest concentration of tested substance that prevented resazurin color change from blue to pink.

Minimum microbicidal concentration (MMC) was determined by plating 10 μl of samples from wells, where no indicator color change was recorded, on nutrient agar medium. At the end of the incubation period the lowest concentration with no growth (no colony) was defined as minimum microbicidal concentration.

Tetracycline (Sigma Chemical Co., USA) dissolved in nutrient liquid medium, was used as positive control. Stock solutions of crude extracts were obtained by dissolving in DMSO (Centrohem, Serbia) and then diluted into Mueller-Hinton broth to achieve a concentration of 10% DMSO. Solvent control test was performed to study the effects of 10% DMSO on the growth of microorganism. It was observed that 10% DMSO did not inhibit the growth of microorganism. Also, in the experiment, the concentration of DMSO was additionally decreased because of the twofold serial dilution assay (the working concentration was 5% and lower). Each test included growth control and sterility control. All tests were performed in duplicate and MICs were constant.

### *Data analysis*

Data were presented as average value from three different analyses of extract samples ± standard deviation. Statistical analyses and calculating of equivalents were performed using SPSS statistical software package (SPSS for Windows, ver. XII, 2004). For comparison between samples, data was analyzed by the Student's *t*-test and the one-way analysis of variance (ANOVA).

## RESULTS

In order to determine biological activity of *S. rigidum,* wild growing in eastern Serbia, the analysis of extracts obtained from aerial parts of plants was performed. To achieve the most effective extraction, we used various solvents with diverse polarity and analyzed five different extracts.

The results of total phenolic content of the examined plant extracts are presented in Fig. 1. *S. rigidum* is characterized by the concentration of total phenolic compounds in the range from 69.00 to 102.13 mg GAE/g. The highest phenolic content was found in the acetone extract (102.13 mg GAE/g), followed by ethyl acetate (81.33 mg GAE/g) and metanolic extract (76.62 mg GAE/g).

The summary of quantities of flavonoids identified in the tested extracts is shown in Fig. 1. For *S. rigidum*, flavonoid concentrations ranged from 6.10 to 35.62 mg RUE/g. The highest concentrations of flavonoids were obtained in acetone extract (35.62 mg RUE/g), similar with methanolic (34.66 mg RUE/g) and followed with ethyl acetate extract (23.8 mg RUE/g). According to results, *S. rigidum* have shown high contents of phenols and flavonoids. However, may be noted that concentration of these substances depends on the polarity of the used solvent.

The results for antioxidant activity of *S. rigidum* extracts are shown in Table 1. Parallel to the examination of the antioxidant activity of the plant extracts, the values

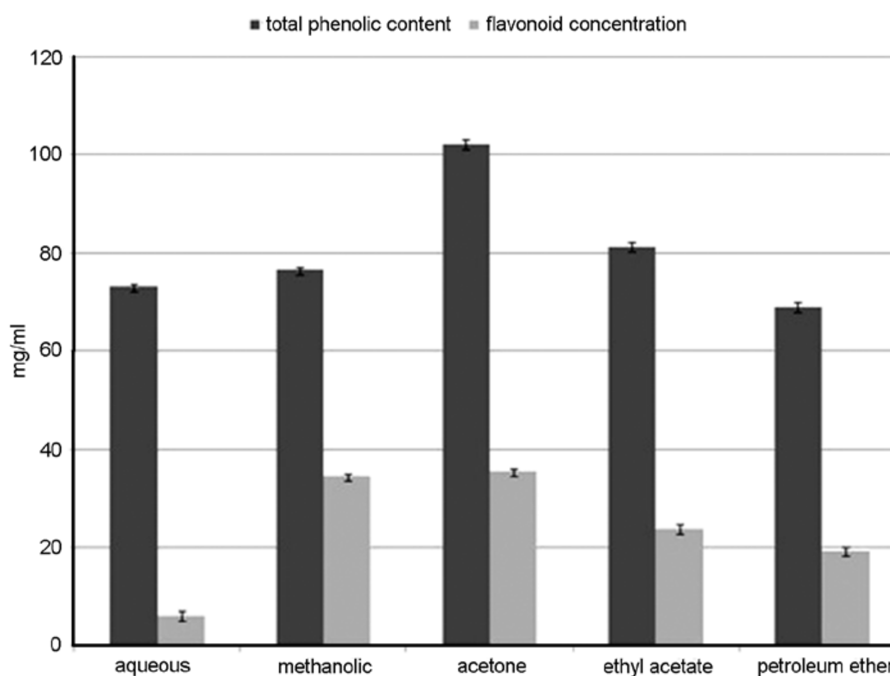


*Fig. 1.* Total phenolic content expressed in terms of gallic acid equivalent (mg of GAE/g of extract) and flavonoid concentrations expressed in terms of rutin equivalent (mg of RUE/g of extract) in different *Seseli rigidum* extracts. Each value is the average of three analyses ± standard deviation

*Table 1*
Values[1] of antioxidant (DPPH scavenging) activity of *Seseli rigidum* extracts

| Extract | $IC_{50}$ values (μg/ml) |
|---|---|
| Aqueous | 46.15 ± 1.04 |
| Methanolic | 98.95 ± 1.11 |
| Acetone | 1118.25 ± 1.55 |
| Ethyl acetate | 1123.42 ± 1.18 |
| Petroleum ether | 1436.45 ± 1.72 |

[1]Each value is the average of three analyses ± standard deviation.

*Table 2*
Values[1] of antioxidant (DPPH scavenging) activity of substances obtained for comparison with the values of *Seseli rigidum*

| Substances | $IC_{50}$ values (μg/ml) |
|---|---|
| Chlorogenic acid | 11.65 ± 0.52 |
| *Ginkgo biloba* | 33.91 ± 1.11 |

[1]Each value is the average of three analyses ± standard deviation.

*Table 3*

Antibacterial activities of *Seseli rigidum* extracts outgrowth against tested bacteria

| Species | Plant extracts | | | | | | | | | | Tetracycline | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Aqueous | | Methanolic | | Acetone | | Ethyl acetate | | Petroleum ether | | | |
| | MIC[1] | MMC[2] | MIC | MMC | MIC | MMC | MIC | MMC | MIC | MMC | MIC | MMC |
| [4]*Salmonella enterica* | >20 | >20 | >20 | >20 | >20 | >20 | >20 | >20 | >20 | >20 | 1.953 | 3.906 |
| [4]*Salmonella typhimurium* | >20 | >20 | >20 | >20 | >20 | >20 | >20 | >20 | >20 | >20 | / | / |
| [4]*Proteus mirabilis* | >20 | >20 | 20 | 20 | 20 | 20 | 10 | 20 | 20 | 20 | 500 | 500 |
| [3]*Proteus mirabilis* ATCC 12453 | >20 | >20 | 20 | 20 | 10 | 10 | 10 | 10 | 20 | 20 | 125 | 125 |
| [4]*Escherichia coli* | >20 | >20 | >20 | >20 | >20 | >20 | >20 | >20 | >20 | >20 | 1.953 | 3.906 |
| [3]*Escherichia coli* ATCC 25922 | >20 | >20 | >20 | >20 | >20 | >20 | 20 | >20 | >20 | >20 | 0.976 | 3.906 |
| [4]*Pseudomonas aeruginosa* | >20 | >20 | 20 | >20 | 20 | >20 | 20 | >20 | 20 | >20 | 500 | 1000 |
| [3]*Pseudomonas aeruginosa* ATCC 27853 | >20 | >20 | 20 | >20 | 20 | >20 | 20 | >20 | >20 | >20 | 7.812 | 62.5 |
| [3]*Bacillus subtilis* IP 5832 | 2.5 | 5 | 0.0391 | 0.0781 | 0.0781 | 2.5 | 0.1563 | 2.5 | 0.1563 | 10 | >0.5 | >0.5 |
| [4]*Bacillus cereus* | 2.5 | 2.5 | 0.0391 | 0.0391 | 0.0391 | 0.3125 | 0.0391 | 0.0391 | 0.0781 | 0.0781 | >0.5 | 3.906 |
| [3]*Bacillus pumilus* NCTC 8241 | 2.5 | 2.5 | 0.0391 | 0.0391 | 20 | 20 | 10 | 20 | >20 | >20 | >0.5 | >0.5 |
| [4]*Staphylococcus aureus* | 5 | >20 | 2.5 | 10 | 1.25 | 10 | 2.5 | 20 | 10 | 10 | 0.976 | 15.62 |
| [3]*Staphylococcus aureus* ATCC 25923 | 5 | >20 | 1.25 | 20 | 0.625 | 10 | 2.5 | 20 | 5 | 20 | 0.244 | 1.953 |

[1]Minimum inhibitory concentration (MIC) and [2]minimum microbicidal concentration (MMC) values are given as µg/ml for plant extract and µg/ml for antibiotic (tetracycline); / means not determined; [3]standard strains; [4]clinical isolates.

for well-known medicinal plant *Ginkgo biloba* and chlorogenic acid (Sigma Chemical Co.) were obtained and compared to the values of the antioxidant activity (Table 2). In the extracts obtained from *S. rigidum* antioxidant value ranged from 1436.45 to 46.15 µg/ml. The highest DPPH radical neutralizing abilities were manifested in aqueous extract of the plant, which at a concentration of 46.15 µg/ml neutralizes 50% of free radicals. The methanolic extract also showed significant antioxidative activity (98.95 µg/ml), while the activity of other extracts was significantly lower. When these values are compared with antioxidant activity of *Ginkgo biloba* standardized extract ($IC_{50}$ = 33.91 µg/ml), it can be noticed that aqueous extract of *S. rigidum* possess strong antioxidant activity, similar to *Ginkgo biloba.* In the case of values obtained from chlorogenic acid as standardized substance ($IC_{50}$ = 11.65 µg/ml), aqueous extract of plant have about four times less activity then the pure standard antioxidant. Having in mind purity of substances that used as standard and the fact that plant extracts are mixture of a great number of components, aqueous extract of *S. rigidum* can be regarded promising antioxidant.

The extracts of *S. rigidum* showed antibacterial potential in wide spectar of values (Table 3). The concentrations of extracts that were defined as MIC values ranged from 0.0391 mg/ml up to more than 20 mg/ml (the same for MMC). Statistically proved, it is obvious that all the extracts influenced much better on Gram-positive bacteria than on Gram-negative bacteria. On Gram-positive bacteria the influence was the best by acetone and ethyl acetate extracts, especially on *Proteus mirabilis* and *P. mirabilis* ATCC 12453 strains (values at 10 mg/ml). When it went to Gram-negative bacteria, the influence was strong and better on bacilli than on staphylococci, and the lowest values were at 0.0391 mg/ml. Here, the extract that stood out was methanolic extract. Generally observed, the weakest influence had the aqueous extract.

## DISCUSSION

The secondary metabolites (total phenolics and flavonoid concentration), antioxidant and antimicrobial activity of five different extracts from *S. rigidum* were investigated for the first time. As the conventional extraction methods is associated with the small extraction yields and recovery of active compounds, extraction from the source, as the first step in the medicinal plants analysis, remains crucial [10].

According to our results, *S. rigidum* have shown high contents of phenols and flavonoids. However, it may be noticed that concentration of these substances depends on the polarity of the used solvent. High concentrations in acetone, ethyl acetate and methanolic extracts are the result of high solubility of these metabolites in the solvents, which is consistent with their chemical characteristics [4]. In previous studies, the greatest quantity of total phenolic compounds, including flavonoids, was achieved in acetone extract [5].

Due to their potential health benefits, plant-derived antioxidants have gained considerable importance [24]. The model of scavenging the stable DPPH radical is a

widely used method to evaluate the free radical scavenging ability of various samples [13]. Analyzing the *in vitro* radical scavenging properties of *S. rigidum,* it may be noted that the aqueous extract has the highest DPPH radical neutralizing capacity. In previous studies of antioxidants in case of the used solvents, aqueous extracts have shown good activity [22].

The results published in a previous paper, also, revealed high metabolite content and activity of *Seseli* plants. In the study of Matejić et al. [16] methanolic extracts of tree *Seseli* taxa from Serbia were investigated. According to these authors, *S. pallasii, S. libanostis* ssp. *libanotis,* and *S. libanotis* subsp. *intermedium* exhibited good antioxidant and antimicrobial activity, as well as high phenolic and flavonoid content. Comparing these tree taxa with *S. rigidum,* major differences can be noticed. Although methanolic extracts in both studies contained a similar concentration of secondary metabolites, significantly higher phenolic content and flavonoid concentration was obtained in *S. rigidum* acetone extract. When it came to the antibacterial activity, their testing showed MIC for *E. coli* ATCC 25922 already at 0.78 mg/ml. In Gram-positive bacteria they also used *Staphylococcus aureus* ATCC 25923 and the MIC results were 0.78 and 3.125 mg/ml. This strain has been influenced by extracts used in our antibacterial testing very similarly. The MIC results we noted for *S. aureus* ATCC 25923 were 0.625, 2.5 mg/ml, and so on. They also used *Pseudomonas* and *Bacillus,* but different strains than ours, and the results varied. Although, according to our results, the methanolic extract had the greatest influence on bacilli (MIC at 0.0391 mg/ml), the best antimicrobial effect was measured in the case of acetone and ethyl acetate extracts with respect to their broad impact on bacteria. In addition, aqueous extract of *S. rigidum* showed significantly better antioxidant activity, compared to the three studied plant taxa.

The results obtained by Stojković et al. [23] on the essential oil content of *S. rigidum,* showed the values presented in µl/ml. They used the same strain of *E. coli* (ATCC 25922) and the result for MIC was 50 µl/ml and MMC at 100 µl/ml. They also observed that the best influence was on bacilli (in their case *Bacillus subtilis* ATCC 10707), which is the same in our investigation. However, flower essential oil tested, according to these authors, has low capacity for free radicals scavenging.

El-Abgar et al. [6] have carried a study of the antioxidant activity of several well-known and worldwide used medicinal plants. The methanolic extracts of *Matricaria chamomilla, Thymus vulgaris* and *Salvia officinalis* showed antioxidant activity with $IC_{50}$ values of 65.8, 54.2 and 17.7 µg/ml, respectively. In the study of Koksal et al. [9] aqueous extract of *Mellisa officinalis* displayed effective antioxidant activities with $IC_{50}$ = 31.4 µg/ml. When comparing the values for antioxidant capacity of aqueous extract from *S. rigidum* ($IC_{50}$ = 46.15 µg/ml) with these plants widely used in human consumption and folk medicine, potential use of this plant can be considered.

*S. rigidum* extracts expressed strong antioxidative activity and significant antibacterial influence. To achieve the greatest activity, aqueous is the most efficient solvent in case of antioxidance. However, when it comes to the antibacterial activity, the aqueous extract of *S. rigidum* generally had the weakest influence. This could be due to the complexity of the substances which cause the different biological activities of

this plant. For concentrations of phenols, flavonoids, also antibacterial influence, the best solvents to be used are acetone and ethyl acetate.

Preliminary screening of different extracts from *S. rigidum* carried out in this study contribute to the biology of this species. Furthermore, the results have possible practical importance, as the starting point for the development of further research of phenological dynamics and interpopulation variability of secondary metabolites, together with the development of method of cultivation. Based on our results, *S. rigidum* can be regarded as a novel candidate with high value of compounds with promising biological activities. Overall, we suggest further detail studies of the composition and activities, as well as development of cultivation process in order to preserve the natural population.

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