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Original Article

A Study of Flavonoid Composition and Antimicrobial Activity of *Scutellaria Altissima L.* from Different Floristic Regions of Bulgaria

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Abstract

Introduction: *Scutellaria* extracts and its constituents, especially the characteristic flavonoids such as baicalein and its glycoside baicalin, exhibit significant antimicrobial activity.

Aim: To perform a comparative analysis of flavonoid composition and antimicrobial activity of extracts obtained from aerial parts and roots of *Scutellaria altissima* from the regions of Mezek and Bachkovo, Bulgaria.

Materials and methods: 70% ethanol and aqueous extracts of aerial parts and roots of *S. altissima* were used. HPLC analysis of *S. altissima* extracts was performed. Microbiological tests were done on clinical isolates of *Streptococcus mitis, Staphylococcus aureus, Escherichia coli*, and *Candida albicans*. Minimal bactericidal and minimal bacteriostatic concentrations of *S. altissima* extracts were determined by the agar method.

Results: The ethanol extracts contain flavonoids approximately twice as much in comparison with the aqueous extracts. The baicalin content in the aerial parts of *S. altissima* from the region of Mezek is 5 times higher than that in the roots. *S. altissima* extracts have effective antimicrobial activity against *S. mitis* only. The minimal bactericidal concentration of ethanol extracts of *S. altissima* aerial parts and *S. altissima* roots is 2000 μg/ml and 8000 μg/ml at 24 hours, respectively. The bactericidal effect of aqueous extracts occurs at 48 hours at minimal bactericidal concentration of *S. altissima* aerial parts – 2000 μg/ml and of *S. altissima* roots – 6000 μg/ml.

Conclusions: The finding that extracts of Bulgarian *S. altissima* possess effective antimicrobial properties against *S. mitis* suggests that it can be used as a potential source for the development of natural antimicrobial agents to suppress oral pathogens and prevent some oral infections.

Keywords

baicalin, scutellarin, *S. mitis*, wogonoside

INTRODUCTION

The plant family Lamiaceae is one of the most used plants in traditional medicine and a subject of phytochemical research. One of the family members is the genus *Scutellaria* L. which includes about 350 species widespread in East Asia, Europe, and North America. Some species of *Scutellaria* are documented to have a broad range of medical properties.[1] According to some World Health Organization (WHO) monographs, *Scutellariae radix*, which consists of the dried roots of *Scutellaria baicalensis* Georgi (Baikal skullcap), possesses anti-hepatotoxic, anti-inflammatory, antioxidant, antimicrobial, antitumour, and antiviral activity.[2] Pharmacological studies have indicated that *Scutellaria* extracts and its constituents, especially neoclerodane type diterpenoids and the characteristic flavonoids, such as baicalein and its glycoside baicalin, exhibit significant antimicrobial activity against Gram-positive and Gram-negative bacteria, as well as against fungi.[3,4]

In vitro antibacterial activities of the extracts of *S. baicalensis* roots have been reported against cariogenic bacterial strains, including *Streptococcus mutans*, *Streptococcus sobrinus,* and *Streptococcus salivarius*.[5] Moreover, ethanolic extracts of *S. baicalensis* could also improve the antimicrobial activity of several antibiotics (gentamicin, ciprofloxacin, ceftriaxone, and penicillin G) against *S. aureus*.[6] This activity is associated with the presence of baicalein, one of the major flavonoids found in dried roots of *S. baicalensis*, which shows a synergistic effect with tetracycline against methicillin-resistant *S. aureus* (MRSA) and potentiates the effects of β-lactam antibiotics against MRSA.[7] The synergistic interactions of baicalin, the most bioactive flavonoid of *Scutellaria* spp., were also observed in combinations with oxytetracycline and tetracycline, enhancing its antimicrobial activity against *S. aureus*, including methicillin- and tetracycline-resistant strains.[8]

In addition, neo-clerodane diterpenoids isolated from several *Scutellaria* species, distributed in Bulgaria, have also shown substantial antimicrobial effect against Gram-positive bacteria (*S. aureus, Bacillus cereus,* and *Listeria monocytogenes*), as well as Gram-negative bacteria (*E. coli, Pseudomonas aeruginosa, P. fluorescens, Salmonella abony,* and *Aeromonas hydrophila*).[9]

S. altissima is one of the eight species widespread in the Bulgarian flora (**Fig. 1**). It is a perennial herb, up to 70 cm tall, its corolla is pale bluish with whitish lower lip. The species blooms from May to September. Its natural habitats are characterized by shady and stony places in mountain regions.[10]

In our previous study on phenolic composition of *S. altissima* growing in Bulgaria, we have established for the first time flavonoids similar to those found in *S. baicalensis*.[11]

The presence of the biologically active flavonoids baicalin and baicalein in *S. altissima*, native to Bulgaria, invokes a vested interest in the potential pharmacological benefits of this natural medicine resource.

Figure 1. *Scutellaria altissima* (Mezek).

AIM

The aim of this study was to perform a comparative analysis of flavonoids composition in extracts obtained from aerial parts and root of *S. altissima* from different floristic regions of Bulgaria, as well as the extracts' antimicrobial activity against *S. mitis, S. aureus, E. coli*, and *C. albicans*.

MATERIALS AND METHODS

Plant collection and extraction

S. altissima was collected during flowering from Bachkovo and Mezek in June 2019. The taxonomic identity of the plant has been confirmed by the Bulgarian botanist prof. Rumen Mladenov. Voucher specimens (No. 062641) were deposited in the Herbarium of the University of Agriculture, Plovdiv, Bulgaria. The collected plant material was separated into roots and aerial parts, and then was dried and powdered. Samples of roots (15 g) and aerial parts (15 g) of each region were extracted with 300 ml 70% ethanol and distilled water for 3 hours using a magnetic stirrer at a temperature of 50°C. After filtration, ethanolic extracts were evaporated at 40°C and all samples were lyophilized. The solutions of lyophilizates were prepared in distilled water in working concentration of 1000 μg/ml, 2000 μg/ml, 3000 μg/ml, 4000 μg/ml, 6000 μg/ml, 8000 μg/ml and 10000 μg/ml.

Chemical analysis

Baicalin, baicalein, wogonin, wogonoside, scutellarin, and verbascoside were determined by a previously developed

and validated HPLC method.[11] The HPLC system was composed of a ProStar 230 solvent delivery module and photodiode array detector model 335 and Hitachi C18 AQ column (250×4.6 mm, 5 μm). A solvent system including deionized water (A) adjusted to pH 3.0 with phosphoric acid and acetonitrile:methanol 40:60 (B) was used in the following gradient modes: 0-25 min 75A / 25B – 10A / 90B; 25-27 min – 10A / 90B; 27-30 min 10A / 90B – 75A / 25B. The flow rate was 0.9 ml/min and detection was at 330 nm for verbascoside, scutellarin and 275 nm for baicalin, baicalein, wogonin, wogonoside. The compounds of interest in the extracts were identified through their retention times, as well as by comparing their absorption spectra with those of standard substances.

Microorganisms

Microbiological analysis were performed with *S. mitis, S. aureus, E. coli*, and *C. albicans* using 3 strains of each, isolated and identified via MALDI TOF mass spectrometer (bioMérieux) from clinical specimens in the Laboratory of Microbiology of St. George University Hospital, Plovdiv, Bulgaria.

Microbiological methods

The bacterial strains were cultivated in BD Columbia agar with 5% defibrinated sheep blood plates and for *C. albicans* in BD CHROMagar Candida. After 24 hours of incubation at 37°C, they were transferred into trypsin broth (0.5 McFarland densities), where after reaching exponential growth they were treated with the corresponding concentrations of the extracts tested. The treated cultures were incubated for 24 and 48 hours at 37°C. Non-treated cultures of the corresponding strains were used in triplicates as controls. Minimal bactericidal concentration (MBCC) and minimal bacteriostatic concentration (MBSC) were determined by microbial number according to colony-forming units – 10^3, 10^4, 10^5 in 5% blood agar or in Chromagar Candida (for *C. albicans*). MBSC was accepted in microbial count less than 10^5 (10^4 or 10^3) and MBCC was considered if no growth was visible on the culture medium treated with the corresponding concentration of each extract.

Statistical analysis

Statistical analysis was carried out using IBM SPSS 17.0. When needed statistical comparisons were made using Duncan's multiple range test. Means were considered significantly different at $p<0.05$. The results were presented as means ± standard deviation of triplicate experiments.

RESULTS

Chemical analysis

HPLC analysis was performed on 70% ethanol and aqueous extracts obtained from aerial parts and roots of *S. altissima* from Bachkovo and Mezek to compare the content of the major flavonoids (scutellarin, baicalin, wogonoside, baicalein, wogonin) and verbascoside.

According to the data presented in **Fig. 2**, 70% ethanol was the optimal extragent for the flavonoids analysed by us. For example, the ethanol extracts obtained from the roots of *S. altissima* from both regions contained verbascoside and wogonin, which were not detected in the aqueous extracts. In general, aerial parts of *S. altissima* from the region of Mezek contained scutellarin (4800 μg/g) and wogonoside (4310 μg/g) about 2 times higher and also baicalin (57100 μg/g) about 5 times higher, compared to the roots, where higher amounts of their aglycones – baicalein (4580 μg/g) and wogonin (1630 μg/g) were detected. The highest content of scutellarin (8160 μg/g) was indicated in the ethanol extract of aerial parts obtained from the region of Bachkovo, while the highest levels of wogonoside (8265 μg/g) and wogonin (2980 μg/g) were found in the

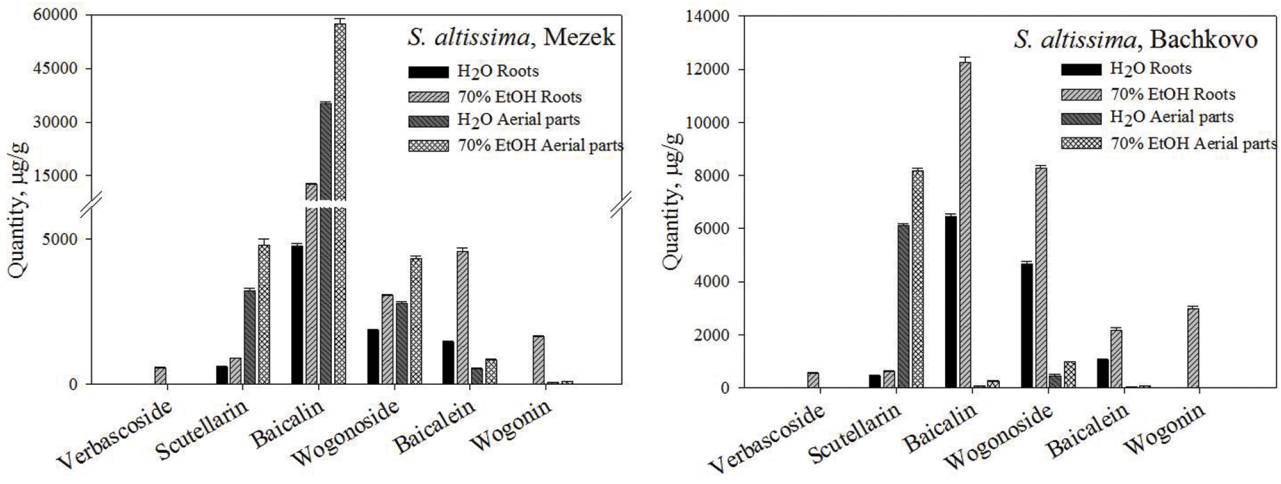

Figure 2. Quantities of bioactive compounds in extracts of *S. altissima* from different regions.

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roots. The quantified levels of flavonoids in the 70% ethanol extracts of aerial parts and roots of *S. altissima* for both regions were approximately twice as much as in the aqueous extracts.

Microbiological examinations

The results obtained showed that extracts from aerial parts and roots of *S. altissima* had effective antimicrobial activity against *S. mitis*, but no bacteriostatic or bactericidal effects were found for *S. aureus*, *E. coli*, and *C. albicans*. The antimicrobial activity of 70% ethanol extracts from roots and aerial parts of *S. altissima* against *S. mitis* is presented in **Table 1**. Bacteriostatic effect (BSE) and bactericidal effect (BCE) were manifested at 24 hours. The best antimicrobial activity was determined for the extract obtained from aerial parts of *S. altissima* from the region of Mezek, where BSE was observed at minimal concentration of 1000 μg/ml and BCE was established at MBCC 2000 μg/ml. MBSC and MBCC of *S. altissima* aerial parts extract from the region of Bachkovo were found at twice higher concentrations – 2000 μg/ml and 4000 μg/ml, respectively. Out of the two *S. altissima* root extracts, the sample of Mezek displayed better effect at MBSC – 6000 μg/ml and MBCC – 8000 μg/ml. The lowest activity was determined for the root extract from the region of Bachkovo, where MBSC was found at 8000 μg/ml, and a bactericidal effect was detected at the highest tested concentration – 10000 μg/ml. The BCE of all tested ethanol extracts was constant and irreversible at 48 hours of the cultivation. Antimicrobial activity of aqueous extracts from roots and aerial parts of *S. altissima* against *S. mitis* is shown in **Table 2**. At 24 hours after treatment no BSE or BCE were observed. The effect of aqueous extracts was manifested at 48 hours after treatment. The extracts obtained from aerial parts of *S. altissima* for both regions showed effect at the same tested concentrations: MBSC – 1000 μg/ml and MBCC – 2000 μg/ml for *S. altissima* (Mezek), respectively, and MBSC – 2000 μg/ml and MBCC – 4000 μg/ml for the plant extract from the region of Bachkovo. In the case of aqueous extracts of roots, BSE and BCE against *S. mitis* were again detected at higher tested concentrations: MBSC of 4000 μg/ml and MBCC – 6000 μg/ml of *S. altissima* (Mezek) and the BSE of the extract from the region of Bachkovo was found at MBSC of 6000 μg/ml, and the bactericidal effect was established at MBCC – 8000 μg/ml.

DISCUSSION

The present study performs a comparative analysis of flavonoids composition and antimicrobial activity of aerial parts and roots of *S. altissima* obtained from the regions of Mezek and Bachkovo, Bulgaria. The data obtained from HPLC analysis indicated that the content of flavonoid com-

Table 1. Susceptibility of S. mitis to 70% ethanol extracts of *S. altissima*, 24 hours after treatment

Sample	Microorganism		
	Streptococcus mitis		
	Strain 1 MBSC/MBCC μg/ml	Strain 2 MBSC/MBCC μg/ml	Strain 3 MBSC/MBCC μg/ml
Aerial parts of *S. altissima* (Bachkovo)	2000/4000	2000/4000	2000/4000
Aerial parts of *S. altissima* (Mezek)	1000/2000	1000/2000	1000/2000
Root of *S. altissima* (Bachkovo)	8000/10000	8000/10000	8000/10000
Root of *S. altissima* (Mezek)	6000/8000	6000/8000	6000/8000

Table 2. Susceptibility of *S. mitis* to aqueous extracts of *S. altissima*, 48 hours after treatment

Sample	Microorganism		
	Streptococcus mitis		
	Strain 1 MBSC/MBCC μg/ml	Strain 2 MBSC/MBCC μg/ml	Strain 3 MBSC/MBCC μg/ml
Aerial parts of *S. altissima* (Bachkovo)	2000/4000	2000/4000	2000/4000
Aerial parts of *S. altissima* (Mezek)	1000/2000	1000/2000	1000/2000
Root of *S. altissima* (Bachkovo)	6000/8000	6000/8000	6000/8000
Root of *S. altissima* (Mezek)	4000/6000	4000/6000	4000/6000

pounds in *S. altissima* samples vary between geographical regions. Such variation of phytochemical constituents may be due to climatic conditions, e.g. water, intensity of sunlight, temperature conditions. However, the main bioactive flavonoids: baicalin, baicalein, wogonin, wogonoside, and scutellarin, although in different ratios and quantities, were found in all samples from aerial parts and roots of *S. altissima* from both regions. It is important to note that *S. baicalensis* radix is registered in the European Pharmacopoeia 8.0 because of the large content of baicalin in comparison with aerial parts.[12] Interestingly, our data indicated that baicalin was found to be present in the highest amount in the aerial parts of *S. altissima* (Mezek) rather than in the roots. Similar results were reported also by Makino et al., who found that baicalin content in the aerial parts of *Scutellaria lateriflora* (American skullcap) was higher than that in the roots.[13] The HPLC analysis also indicated that *S. altissima* flavonoids are extracted better in 70% ethanol extraction than in distilled water extraction. This may be the reason that *S. mitis* is more susceptible to the 70% ethanol extracts of *S. altissima* (Mezek) and in particular to those obtained from the aerial parts of the plant, while bacteriostatic and bactericidal effects of aqueous extracts occurs after prolonged treatment time. Our results can be compared with those obtained by other authors about the antibacterial properties of *Scutellaria* spp. extracts against *Streptococcus viridans* group. Duan et al. found bactericidal effect of ethanol extracts of *Scutellaria baicalensis* roots against *Streptococcus mutans*, *Streptococcus sobrinus*, and *Streptococcus salivarius* at concentrations of 1000 μg/ml, but no antibacterial effect of aqueous extracts even at the highest tested concentrations (10000 μg/ml).[5] In our study, the minimal bactericidal concentration of ethanol extract of *S. altissima* against *S. mitis* was achieved at higher concentrations – 2000 μg/ml, but antibacterial effect was found for all aqueous extracts tested by us.

It is thought that the antimicrobial properties of *S. baicalensis* are due to the flavonoids baicalin and its aglycone baicalein. Their antibacterial mechanism of action has been suggested to be related to the inhibition of cell wall synthesis by damaging the peptidoglycan structure.[8,14] In addition, there are many published data on the synergistic interactions of these flavonoids, increasing the efficacy of β-lactam antibiotics. Baicalin and baicalein have been shown to inhibit bacterial efflux pump overexpression, thereby increasing cell membrane permeability to the antibacterial agent and reducing antibiotic resistance.[7,8,15]

Based on our findings, the extracts of *S. altissima* contain the main bioactive compounds baicalin and baicalein responsible for the therapeutic properties of *S. baicalensis*, but plant extracts also contain other concomitant substances (e.g.,diterpenes, phenylethanoid glycosides, iridoid glycosides, polysaccharides, and other compounds) which may increase or decrease the effect of the main active compounds. This may be the reason for the stronger antimicrobial activity of substances isolated in pure form, compared to plant extracts and probably the reason why the tested *S. altissima* extracts were found to be inactive against *S. aureus*, *E. coli*, and *C. albicans* even at the highest concentrations. For instance, Chen et al. have found that sub-inhibitory concentrations of baicalein alone effectively prevent *S. aureus* biofilm formation and improve antibiotic permeability.[16] A similar effect was also reported for baicalin that decreased biofilm formation and the quorum-sensing system by inhibiting the efflux pump in *Staphylococcus saprophyticus*.[17] Furthermore, Serpa et al. demonstrated the antifungal properties of baicalein against *Candida* spp., as well as the synergistic action of baicalein and fluconazole against *C. albicans*.[18] In view of this, further research should focus on studying the influence of the individual bioactive flavonoids of *S. altissima* as synergistically acting antimicrobial agents. In spite of this, the extracts of *S. altissima* tested by us exhibit significant antimicrobial effect against *S. mitis*.

CONCLUSIONS

The flavonoid composition in different plant parts of *S. altissima* varies as affected by geographical region. The pharmacologically active flavonoids: baicalin, baicalein, wogonoside, wogonin, and scutellarin are contained in all examined plant parts of *S. altissima* with notably higher baicalin content in the aerial parts of *S. altissima* from the region of Mezek. The 70% ethanol extracts of *S. altissima* exhibit a significantly greater antimicrobial effect against *S. mitis*, while the effect of aqueous extracts occurs after prolonged treatment time. *S. mitis* is more susceptible to the extracts obtained from the aerial parts than to the extracts obtained from the roots of *S. altissima.* The effective antimicrobial properties of extracts of Bulgarian *S. altissima* against *S. mitis* suggests its potential as a source for the development of natural antimicrobial agents for the suppression of oral pathogens and prevention of some oral infections.

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Изучение флавоноидного состава и антимикробной активности *Scutellaria Altissima L.* из разных флористических регионов Болгарии

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Резюме

Введение: Экстракт шлемника и его составляющие, особенно характерные флавоноиды, такие как байкалеин и его гликозид байкалин, проявляют значительную противомикробную активность.

Цель: Провести сравнительный анализ флавоноидного состава и антимикробной активности экстрактов, полученных из надземных частей и корней шлемника высокого из районов Мезек и Бачково, Болгария.

Материалы и методы: Использовали 70% этанол и водные экстракты надземной части и корней *S. altissima*. Был проведен ВЭЖХ-анализ экстрактов *S. altissima*. Микробиологические исследования проводились на клинических изолятах *Streptococcus mitis, Staphylococcus aureus, Escherichia coli* и *Candida albicans*. Минимальную бактерицидную и минимальную бактериостатическую концентрации экстрактов *S. altissima* определяли агаровым методом.

Результаты: Спиртовые экстракты содержат примерно в два раза больше флавоноидов по сравнению с водными экстрактами. Содержание байкалина в надземных частях *S. altissima* из района Мезек в 5 раз выше, чем в корнях. Экстракты *S. altissima* обладают эффективной противомикробной активностью только в отношении *S. mitis*. Минимальная бактерицидная концентрация этанольных экстрактов надземной части *S. altissima* и корней *S. altissima* составляет 2000 μg/ml и 8000 μg/ml через 24 часа соответственно. Бактерицидное действие водных экстрактов проявляется через 48 часов при минимальной бактерицидной концентрации надземной части *S. altissima* – 2000 μg/ml и корней *S. altissima* – 6000 μg/ml.

Заключение: Обнаружение того, что экстракты болгарского *S. altissima* обладают эффективными противомикробными свойствами против *S. mitis*, позволяет предположить, что его можно использовать в качестве потенциального источника для разработки природных противомикробных агентов для подавления оральных патогенов и предотвращения некоторых оральных инфекций.

Ключевые слова

байкалин, скутелларин, *S. mitis*, вогонозид