ECOLOGIA BALKANICA
2020, Vol. 12, Issue 2 December 2020 pp. 165-174

Antimicrobial Potential of Methanolic Extracts from Betonica bulgarica Degen et Neič. (Lamiaceae)

Toncho G. Dinev[1*], Nikolina V. Rusenova[2], Milena T. Tzanova[1], Neli H. Grozeva[3], Mariya A. Gerdzhikova[4], Plamen S. Stoyanov[5,6], Tsvetelina R. Mladenova[5], Georgi G. Beev[1]

1 - Trakia University, Faculty of Agriculture, Department of Biochemistry, Microbiology and Physics, Students' campus, 6000 Stara Zagora, BULGARIA
2 - Trakia University, Faculty of Veterinary Medicine, Department of Veterinary Microbiology, Infectious and Parasitic Diseases, Students' campus, 6000 Stara Zagora, BULGARIA
3 - Trakia University, Faculty of Agriculture, Department of Biology and Aquaculture, Students' campus, 6000 Stara Zagora, BULGARIA
4 - Trakia University, Faculty of Agriculture, Department of Plant Production, Students' campus, 6000 Stara Zagora, BULGARIA
5 - University of Plovdiv "Paisii Hilendarski", Faculty of Biology, Department of Botany and Methods of Biology Teaching, 24 Tzar Assen Str., 4000 Plovdiv, BULGARIA
6 - Medical University of Plovdiv, Faculty of Pharmacy, Department of Bioorganic Chemistry, 15-A Vasil Aprilov Blvd., 4002 Plovdiv, BULGARIA
[*]Corresponding author: dinev_sz@mail.bg

Abstract. *Betonica bulgarica* Degen et Neič. (syn. *Stachys bulgarica* Hayek) is a Bulgarian endemic plant included in Red Data Book of Bulgaria under the category "endangered". The aim of the present study is to provide data about the antimicrobial activity of *B. bulgarica* leaf, flower, seed, stem and root methanolic extracts against *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Bacillus cereus, Aspergillus ochraceus* 2002 IM-BAS, *Fusarium moniliforme* 394 FN-9, *Fusarium graminearum* 2294 IMI 155426 and *Penicillium verrucosum* 2003 NRRL F-143. Antimicrobial activity of the extracts was evaluated by agar well diffusion method. Root extracts of *B. bulgarica* exhibited the highest antibacterial activity against *S. aureus* and *B. cereus* with large zones of inhibition. All extracts demonstrated either low and statistically insignificant activity against *E. coli* or a lack thereof. As a whole, extracts of Ablanovo area (in Sinite kamani National Park) exerted the highest activity against *S. aureus, B. cereus* and *E. coli*. Leaf, flower, stem and root extracts of *B. bulgarica* showed either a lack of antifungal activity or low and statistically insignificant one.

Key words: antimicrobial activity, methanol extracts, *Betonica bulgarica*, *Stachys bulgarica*.

Introduction

Betonica bulgarica Degen et Neič. (syn. *Stachys bulgarica* Hayek) is a Bulgarian endemic plant included in Red Data Book of the Republic of Bulgaria under the category "endangered" (Genova, 2011). The known locations of this species are Balkan Range (Central and Eastern), Tracian Plain and

Sinite Kamani Natural Park near to Sliven (Grozeva et al., 2014, 2016; Gerdzhikova et al., 2015).

Betonica (*Stachys*) species are widely used in folk medicine and recently in official medicine (Bankova et al., 1999). They have anti-inflammatory, immunomodulatory, antimicrobial, anti-cancer and antioxidant properties (Khavani et al., 2005; Amirghofran et al., 2007; Salehi et al., 2007; Saeedi et al., 2008; Morteza-Semnani & Saeedi, 2009; Hajdari et al., 2010; Serbetci et al., 2010; Šliumpaitė et al., 2013; Jassbi et al., 2014; Tzanova et al., 2018). Nowadays, the more frequent use of plant extracts as natural food preservatives and safer alternative of antimicrobial agents brings about the necessity to study their antimicrobial activity (Mostafa et al., 2018). *B. bulgarica* is known to have a high content of polyphenols and flavonoids, which not only have antioxidant, but also antimicrobial activity (Bankova et al., 1999; Tzanova et al., 2018; Yakoub et al., 2018). Moreover, it is found that many species from *Betonica* (*Stachys*) genus are rich in essential oils, which also have antimicrobial activity (Skaltsa et al., 2003; Grujic-Jovanovic et al., 2004; Vundac et al., 2006; Salehi et al., 2007; Morteza-Semnani & Saeedi, 2009; Rusenova & Parvanov, 2009; Ebrahimabadi et al., 2010; Hajdari et al., 2011; Dimitrova-Dyulgerova et al., 2015). Because of that, even though there are not data about the antimicrobial activity of *B. bulgarica*, it could be assumed that it exists.

Because the chemical polymorphism of medicinal plants largely depends on various factors such as geographic conditions, collection time, vegetation phase, etc., the survey of medicinal flora present in various growing sites and countries is important part of plant studies (Dimitrova-Dyulgerova et al., 2015; Igwaran et al., 2017). The differences of the chemical constituent's content could lead to variations of antimicrobial activity of the cultivated plants, because many chemical constituents exert antimicrobial activity (Das et al., 2009).

The available literature is lacking data about the antimicrobial potential of extracts from different organs of *B. bulgarica*. This motivated the present study which aims to provide such findings from *B. bulgarica* leaf, flower, seed, stem and root methanolic extracts and to compare the antimicrobial activities of different populations of *B. bulgarica*.

Material and Methods

Plant material and extract preparation. Aerial parts of *Betonica bulgarica* were harvested from July to September in four locations from naturally growing populations in Bulgaria (Table 1; Figure 1). The roots were collected at the end of the vegetative period. The voucher specimens from studied populations are kept in the herbarium of the Agricultural University in Plovdiv (SOA). Plant material was air-dried in shade at room temperature and grounded in a mechanical grinder (final powder size less than 400 μm). The samples were stored in the dark and cool rooms at 16 – 18 °C prior to the analysis.

Table 1. Basic characteristics of the populations whence the plant materials of *Betonica bulgarica* were collected (by Tzanova et al., 2018).

Population No	Location, voucher number	North	East	Elev. m a.s.l.	Ecological conditions
1	Balkan Foothill Region, Lovnidol village, Pashova Livada area (SOA 062252)	42°59.079′	25°15.846′	368	Soil type – Cambisols (WRBSR, 2006). Herbaceous community dominated by *Festuca pratensis*. The terrain is slightly sloped (4° – 5°), non-eroded, facing south-west.

2	Balkan Foothill Region, Lovnidol village, Above Avdjiiski trap area (SOA 062253)	43°01.327′	25°15.154′	503	Soil type – Cambisols (WRBSR, 2006). Herbaceous community dominated by *Trifolium pratense* L. The terrain is very slightly sloped (2° – 3°), non-eroded, facing north-east.
3	Eastern Balkan Range, Sinite kamani Natural Park, Karandilska poliana area (SOA 062254)	42°42.688′	26°22.872′	972	Open meadow of the clifs northwest. Herbaceous community dominated by *B. bulgarica.* The terrain is slightly sloped (4° – 5°), non-eroded, facing north-east.
4	Eastern Balkan Range, Sinite kamani Natural Park, Ablanovo area (SOA 062255)	42°42.638′	26°17.262′	540	Soil type – Chromic Luvisols (WRBSR, 2006); Open meadow on the edge of a mixed deciduous forest comprising *Carpinus betulus L., Quercus robur L., Ulmus minor Mill., Fraxinus ornus L. and Crataegus monogyna Jacq.* The herbaceous community is dominated by *B. bulgarica*. The terrain is very slightly sloped (3° – 4°), non-eroded, facing south-east.

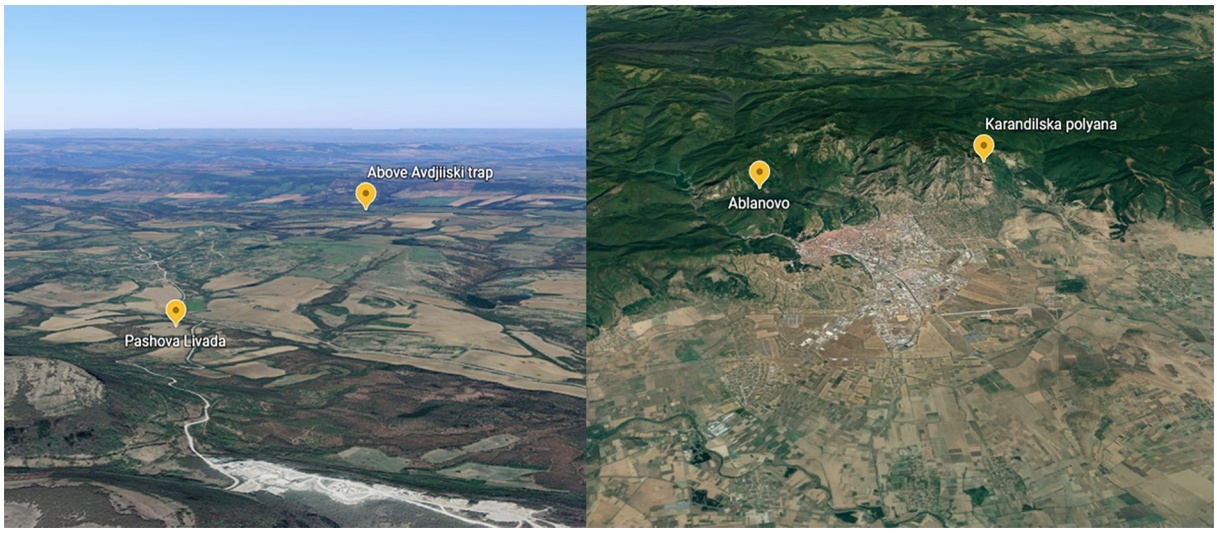

Fig. 1. Map of the four locations of *Betonica bulgarica* populations.

The target compounds were extracted by Soxhlet method, for 8h. As solvent was used methanol in the ratio of plant material: solvent 1:10. After filtration through 0.45 µm membrane, the extracts were concentrated by rotary vacuum evaporator at 30°C (Hossain & Rahman, 2015).

Tested microorganisms. In this study were included reference bacterial strains (*Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922) and a clinical bacterial isolate (*Bacillus cereus*). The fungi

strains were *Aspergillus ochraceus* 2002 IMBAS, *Fusarium moniliforme* 394 FN-9, *Fusarium graminearum* 2294 IMI 155426 and *Penicillium verrucosum* 2003 NRRL F-143. The bacterial strains were stored at -20 °C. Prior to use they were restored by trypticase soy blood agar (Himedia, India). The fungal strains were grown on Potato-glucose agar (glucose 20.0 g, potatoes 200.0 g, yeast extract 2.0 g, agar 20.0 g., pH 5.6).

Antimicrobial activity. Antimicrobial activity of the extracts was evaluated by agar well diffusion method described by Velichkova et al. (2018). In brief, for measuring of antibacterial activity, inoculums were prepared in saline corresponding to 0.5 of the McFarland standard (1.5x 10^8 CFU/mL) from 24 h bacterial colonies incubated on trypticase soy blood agar. 20 mL of Mueller Hinton agar (Himedia, India) was poured in every *Petri* dish. The wells were formed with a sterile 6 mm cork borer after pre-application of the inoculum with a sterile cotton swab. The wells were filled with 100 µL of the extracts. Positive control with gentamicin at a concentration of 12.5 µg/mL and negative with methanol was performed. The plates were incubated at 37 °C for 24 h under aerobic conditions.

For measuring of antifungal activity, 72 h old fungal cultures were grown on Potato glucose agar. 20 mL of Potato glucose agar was poured in every *Petri* dish. After solidification, 0.1 mL inoculum of the fungal strains (1-2 x 10^4 CFU/mL) was introduced on the agar plate surface and the wells were made by sterile cork borer of size 6.0 mm. The wells were filled with 0.1 mL of the methanol extract. An incubation period of 3-7 days at 23°C was maintained.

Antimicrobial activity was evaluated by measuring zones of inhibition of microbial growth surrounding the plant extracts in the wells. The zones of inhibition were measured in millimeters. Antibacterial activity was assumed in the presence of a growth inhibition zone ≥ 8.0 mm. The tests were performed in triplicate to determine the reproducibility of the results. The complete experiment was carried out under strict aseptic conditions.

Statistical analysis. All analytical assays were carried out in triplicate and expressed as mean values ± standard deviation (SD). Statistical analysis was performed with Statistica 10, StatSoft Inc. (2007).

Results and Discussion

According to the experimental data (Table 2) only leaf, flower and root methanolic extracts of *Betonica bulgarica* exhibited activity against *S. aureus*. The root extracts have the highest antibacterial activity. The zones of inhibition (ZI) at concentration of 16 mg/mL were very large (15.5 – 18.17 mm). Moreover, further dilutions of the root extracts showed concentration-dependent effect against *S. aureus* and some activity even at concentration of 0.25 – 1 mg/mL. Root extracts from the area of Ablanovo exhibited the highest activity against *S. aureus* (ZI 18.17 mm) and were active at concentration of 0.25 mg/mL (at 0.5 mg/mL there was statistically significant difference with the negative control). The leaf and flower extracts demonstrated low and statistically insignificant activity against *S. aureus*. Leaf and flower extracts from the area of Ablanovo again have the highest inhibitory activity against *S. aureus* with ZI of 9.0 mm and 9.33 mm, respectively.

All methanolic extracts of *B. bulgarica* exhibited either low and statistically insignificant activity against *E. coli* or a lack thereof. Ablanovo root extracts showed the highest activity (ZI 9.17 mm) followed by leaf extracts from Karandilska polyana (ZI 9.00 mm).

Only root methanolic extracts showed activity against *B. cereus*, with large ZI (11.17 – 12.5 mm) and statistically significant difference with methanol control. Ablanovo root extracts again demonstrated the highest antimicrobial activity (ZI 12.5 mm).

As a whole, methanolic extracts from *B. bulgarica* of Ablanovo area exhibited the

highest antibacterial activity. This is probably due to the type of soil and climatic conditions of Ablanovo area, which are presumably favourable for the growth of plants rich in antimicrobial constituents.

ZI of the positive control (gentamicin at concentration of 12.5 mg/mL) for *S. aureus*, *E. coli* and *B. cereus* were 21 mm, 17 mm and 23 mm, respectively, which means that all microorganisms were sensitive to positive control.

Our results regarding the antibacterial activity of *B. bulgarica* methanolic extracts are corresponding to the findings of Leblebici et al. (2016) who found that ethanolic extracts of 6 *Stachys* species (*S. annua* ssp. *cilicia*, *S. setifera* ssp. *lycia*, *S. sosnowskyi*, *S. tmolea*, *S. cretica* ssp. *anatolica* and *S. iberica* ssp. *iberica* var. *densipilosa*) were more inhibitory against *S. aureus* and *B. cereus* than *E coli*. Lotfipour et al. (2008) reported higher activity of methanolic extracts of *S. fruticulosa* and *S. schtschegleevii* against *S. aureus* than *B. cereus* and lack of any inhibitory activity against *E. coli.* Saeedi et al. (2008) found that methanolic extracts of four *Stachys* species (*S. byzantina*, *S. inflata*, *S. lavandulifolia* and *S. laxa*) demonstrated higher activity against *S. aureus* than *E. coli* as a whole. Moreover, methanolic extracts of *S. laxa* were not inhibitory against *E. coli*. Mahboubi et al. (2012) reported much higher activity of ethanolic extracts of *S. byzantina* against *S. aureus* than *E. coli*.

On the other hand, our findings are somewhat dissimilar to the data of Jassbi et al. (2014), who evaluated antibacterial activity of methanolic extracts from 9 *Stachys* species (*S. acerosa*, *S. benthamiana*, *S. byzantina*, *S. obtusicrena*, *S. lavandulifolia S. pilifera*, *S. pubescens*, *S. spectabilis* and *S. persica*) through nutrient broth microdilution assay. These authors found that the extracts from *Stachys* species showed similar activity against *S. aureus*, *E. coli* and *B. cereus* as a whole, with small differences. The extracts were the most active against *B. cereus*, followed by *E. coli* and *S. aureus*. Dulger & Aki (2009) reported much higher activity of ethanolic extract of *S. pseudopinardii* against *B. cereus* (ZI 25 mm) than *S. aureus* (ZI 13 mm). The extract was not inhibitory against *E. coli*. However, Abichandani et al. (2010) found that methanolic extract of *S. schtschegleevii* exhibited similar activity against *S. aureus* and *E. coli* (ZI 10 mm), but did not inhibit *B. cereus*. According to Yavuz et al. (2017) methanolic extract of *S. annua* inhibited *E. coli* (ZI 17 mm) but not *S. aureus* (ZI 7 mm).

According to the experimental data leaf, flower, stem and root extracts of *B. bulgarica* showed either a lack of antifungal activity or low and statistically insignificant one (Table 3).

These results are similar to the data of Saeedi et al. (2008), who found that methanolic extracts of four *Stachys* species (*S. byzantina*, *S. inflata*, *S. lavandulifolia* and *S. laxa*) did not show any antifungal activity against *Aspergillus niger*. Skaltsa et al. (2003) studied antimicrobial activity of essential oils from eight *Stachys* species (*S. alopecuros, S. scardica, S. cretica* ssp. *cretica*, *S. germanica* ssp. *heldreichii, S. recta, S. spinulosa, S. euborica, S. menthifolia*) and reported better activity against bacterial species (*P. aeruginosa, E. coli, B. subtilis, B. cereus, Micrococcus flavus* and *Staphylococcus epidermidis*) than fungi (*Aspergillus niger*, *Penicillium ochrochloron*, *Epidermophyton floccosum*, *Candida albicans* and *Trichophyton menthagrophytes*), which is similar to our findings. On the other hand, the essential oils of *Stachys pubescens* exhibited noticeable antifungal activity against *Fusarium oxysporum*, *Aspergillus flavus* and *Alternaria alternata* (Mohammadi et al., 2014). According to Lazarević et al. (2010) the antifungal activity of *Stachys* spp. extracts was very dependent on the type of solvent. Diethyl ether extracts of *S. germanica*, *S. plumosa* and *S. scardica* exhibited very high activity against *Aspergillus niger* (with ZI at least 24 mm), but ethyl acetate extracts were not inhibitory against this mould. As a whole, there is a lack of enough literature data about the antifungal activity of *Betonica* (*Stachys*) species which hinders the comparison of our data with similar findings.

Table 2. Diameter of inhibition zones (mm) of methanolic extracts from *B. bulgarica* (n=3, mean± SD). Legend: * – no activity. Different letters in the table denote significant differences between zones of inhibition of plant extracts and negative control (methanol) values according to LSD test ($p \leq 0.05$).

***B. bulgarica* Population/Organs**	**Methanol extract (mg/mL)**	***S. aureus***	***E. coli***	***B. cereus***
Pashova livada				
Leaves	16	8.33 ± 0.58^{a}	-	-
Flowers	16	8.0 ± 0.0^{a}	-	-
Seeds	16	-	8.17 ± 0.29^{a}	-
Stems	16	-	8.83 ± 0.29^{a}	-
Roots	16	16.0 ± 0.5^{b}	8.5 ± 0.5^{a}	11.33 ± 0.29^{ab}
	8	14.83 ± 0.86^{ab}		
	4	13.83 ± 1.04^{ab}		
	2	11.33 ± 0.58^{ab}		
	1	8.5 ± 0.5^{a}		
	0.5	-		
Above Avdjiiski trap				
Leaves	16	7.67 ± 0.58^{a}	-	-
Flowers	16	7.33 ± 0.58^{a}	-	-
Seeds	16	-	-	-
Stems	16	-	-	-
Roots	16	15.83 ± 0.29^{ab}	8.0 ± 0.5^{a}	11.5 ± 0.5^{ab}
	8	14.33 ± 0.58^{ab}		
	4	13.33 ± 0.29^{ab}		
	2	11.33 ± 0.58^{ab}		
	1	8.5 ± 0.5^{a}		
	0.5	-		
Karandilska polyana				
Leaves	16	-	9.0 ± 0.0^{a}	-
Flowers	16	8.67 ± 0.58^{a}	-	-
Seeds	16	-	7.17 ± 0.29^{a}	-
Stems	16	-	-	-
Roots	16	15.5 ± 0.5^{ab}	8.17 ± 0.29^{a}	11.17 ± 0.29^{ab}
	8	14.33 ± 0.58^{ab}		
	4	13.5 ± 0.5^{ab}		
	2	11.0 ± 0.0^{ab}		
	1	8.33 ± 0.29^{a}		
	0.5	-		
Ablanovo				
Leaves	16	9.0 ± 0.0^{a}	-	-
Flowers	16	9.33 ± 0.58^{a}	-	-
Seeds	16	-	7.5 ± 0.87^{a}	-
Stems	16	-	8.17 ± 0.29^{a}	-
Roots	16	18.17 ± 0.29^{ab}	9.17 ± 0.76^{a}	12.5 ± 0.5^{ab}
	8	16.17 ± 0.29^{ab}		

	4	14.17 ± 0.29 [ab]		
	2	13.67 ± 0.58 [ab]		
	1	13.0 ± 0.0 [ab]		
	0.5	10.83 ± 0.29 [ab]		
	0.25	8.0 ± 0.0 [a]		
	0.12	-		
Methanol	0	7.0 ± 0.0 [a]	7.0 ± 0.0 [a]	6.0 ± 0.0 [a]
Gentamicin	12.5	21 ± 0.0	17 ± 0.0	23 ± 0.0

Table 3. Diameter of inhibition zones in mm of methanolic extracts (32 mg/mL) from *B. bulgarica* (mean± SD). Legend: * – no activity. According to LSD test ($p \leq 0.05$) the results denote no significant differences between zones of inhibition of plant extracts and negative control (methanol) values.

Betonica bulgarica **Population/Organs**	***A. ochraceus***	***F. moniliforme***	***F. graminearum***	***P. verrucosum***
Pashova livada				
Leaves	7.17 ± 0.29 [a]	7.0 ± 0.0 [a]	-	-
Flowers	-	7.17 ± 0.29 [a]	-	-
Stems	-	-	-	-
Roots	-	7.17 ± 0.29 [a]	7.0 ± 0.0 [a]	-
Above Avdjiiski trap				
Leaves	-	-	-	-
Flowers	8.67 ± 0.58 [a]	-	-	-
Stems	-	-	-	-
Roots	-	7.17 ± 0.29 [a]	7.0 ± 0.0 [a]	--
Karandilska polyana				
Leaves	-	-	-	-
Flowers	-	7.0 ± 0.0 [a]	-	7.0 ± 0.0 [a]
Stems	-	-	-	6.83 ± 0.29 [a]
Roots	-	7.0 ± 0.0 [a]	7.0 ± 0.5 [a]	-
Ablanovo				
Leaves	-	-	-	8.17 ± 0.76 [a]
Flowers	-	-	-	-
Stems	-	-	7.0 ± 0.0 [a]	8.0 ± 0.0 [a]
Roots	-	8.0 ± 0.0 [a]	8.17 ± 0.29 [a]	7.83 ± 0.29 [a]
Methanol	6.0 ± 0.0 [a]	6.0 ± 0.0 [a]	6.0 ± 0.0 [a]	6.0 ± 0.0 [a]

We see some experimental data concerning the antibacterial and antifungal activity of *Betonica* (*Stachys*) species that sometimes differ greatly from our results. The distinctions could be primarily attributed to factors such as plant species, bacterial strains and method of extract preparation, but also to geographic conditions, collection time and vegetation phase of the plants, etc. (Dimitrova-Dyulgerova et al., 2015; Igwaran et al., 2017). Climatic differences and geographical area may change the amount and types of secondary metabolites of plant species. Moreover, the plants of the same genus differ in chemical composition and content of antimicrobial substances such as monoterpenes, sesquiterpenes, diterpenes, triterpenes, flavonoids, biflavonoids, glycosides, phenolic acids, etc. (Leblebici et al., 2016). These factors could strongly influence antimicrobial activity of the plants

examined which could explain the differences found.

Conclusions

In the present study root extracts of *Betonica bulgarica* exhibited the highest antibacterial activity against *S. aureus* and *B. cereus* with large zones of inhibition. All extracts demonstrated either low and statistically insignificant activity against *E. coli* or a lack thereof. Seed and stem extracts did not have any activity against *S. aureus*. Leaf, flower, seed and stem extracts were not inhibitory against *B. cereus*. As a whole, methanolic extracts from *B. bulgarica* of Ablanovo area showed the highest activity against *S. aureus, B. cereus* and *E. coli*. Plant extracts of *B. bulgarica* exhibited either a lack of antifungal activity or low and statistically insignificant one.

Acknowledgements

This work was supported by the Bulgarian Ministry of Education and Science under the National Research Programme "Healthy Foods for a Strong Bio-Economy and Quality of Life" approved by DCM #577/17.08.2018.

References

Abichandani, M., Nahar, L., Singh, P., Chitnis, R., Nazemiyeh, H., Delazar, A. & Sarker, S. D. (2010). Antibacterial and free-radical-scavenging properties of *Stachys schtschegleevii* (Lamiaceae). *Archives of Biological Sciences, 62*(4), 941-945. doi: 10.2298/ABS1004941A.

Amirghofran, Z., Bahmani, M., Azadmehr, A. & Javidnia, K. (2007). Immunomodulatory and apoptotic effects of *Stachys obtusicrena* on proliferative lymphocytes. *Medical Science Monitor, 13*(6), BR 145-150. PMID: 17534229

Bankova, V., Koeva-Todorovska, J., Stambolijska, T., Ignatova-Groceva, M.-D., Todorova, D. & Popov, S. (1999). Polyphenols in *Stachys* and *Betonica* species (Lamiaceae). *Z. Naturforsch., 54c,* 876-880. doi: 10.1515/znc-1999-1104.

Das, K., Dang, R. & Gupta, N. (2009). Comparative antimicrobial potential of different extracts of leaves of *Stevia rebaudiana* Bert. *International Journal of Natural and Engeneering Sciences. 3*(1), 65-68.

Dimitrova-Dyulgerova, I., Merdzhanov, P., Todorov, K., Seymenska, D., Stoyanov, P., Mladenov, R. & Stoyanova, A. (2015). Essential oils composition of *Betonica officinalis* L. and *Stachys sylvatica* L. (Lamiaceae) from Bulgaria. *Comtes rendus de l'Academie bulgare des Sciences, 68*(8), 991-998.

Dulger, G. & Aki, C. (2009). Antimicrobial activity of the leaves of endemic *Stachys pseudopinardii* in Turkey. *Tropical Journal of Pharmaceutical Research, 8*(4), 371-375. doi: 10.4314/tjpr.v8i4.45231.

Ebrahimabadi, A. H., Ebrahimabadi, E. H., Djafari-Bidgoli, Z., Kashi, F. J., Mazoochi, A. & Batooli, H. (2010). Composition and antioxidant and antimicrobial activity of the essential oil and extracts of *Stachys inflata* Benth from Iran. *Food Chemistry, 119*(2), 452-458. doi: 10.1016/j.foodchem.2009.06.037.

Genova, E. (2011). Betonica bulgarica. In: *Red Data Book of the Republic of Bulgaria, vol. 1 Plants and Fungi*. Joint edition of the Bulgarian Academy of Science and Ministry of Environment and Water, Sofia, Bulgaria. Retrieved from: e-ecodb.bas.bg.

Gerdzhikova, M., Grozeva, N., Pavlov, D., Panayotova, G. & Todorova M. (2015). Leaves area characteristics of *Betonica bulgarica* Degen et Neič. during vegetation. *Agricultural Science and Technology, 7*(4), 486-493.

Grozeva, N., Gerdzhikova, M., Pavlov, D., Panayotova, G., & Todorova, M. (2016). Morphological variability of the Bulgarian endemic *Betonica bulgarica* Deg. et Neič. (Lamiaceae) from Sinite Kamani Natural Park, Eastern Balkan range. *Acta Botanica Croatica, 75*(1), 81-88. doi: 10.1515/botcro-2016-0020.

Grozeva, N., Todorova, M., Gerdzhikova, M., Panayotova, G., Getova, N. & Donchev, D. (2014). New data for Bulgarian endemic *Betonica bulgarica* Deg. et Neič. of Sinite Kamani Natural Park Sliven. *Journal of Bioscience and Biotechnology, SE/Online*, 205-210.

Grujic-Jovanovic, S., Skaltsa, H., Marin, P. & Sokovic, M. (2004). Composition and antibacterial activity of the essential oil of six *Stachys* species from Serbia. *Flavour and Fragrance Journal, 19*(2), 139-144. doi: 10.1002/ffj.1275.

Hajdari, A., Mustafa, B., Franz, C. & Novak, J. (2010). Total flavonoids, total phenolics and antioxidant activity of *Betonica officinalis* L. from Kosovo. *ISHS Acta Horticulturae, 860*, 75. doi:10.17660/ActaHortic.2010.860.8.

Hajdari, A., Mustafa, B., Franz, C. & Novak, J. (2011). Variability of essential oils of *Betonica officinalis* (Lamiaceae) from different wild populations in Kosovo. *Natural Product Communications, 6*(9), 1343-1346. doi: 10.1177/1934578X1100600931.

Hossain, M. A. & Rahman, S. M. M. (2015). Isolation and characterisation of flavonoids from the leaves of medicinal plant *Orthosiphon stamineus*. *Arabian Journal of Chemistry*, *8*, 218–221.

Igwaran, A., Iweriebor, B., Okoh, S., Nwodo, U., Obi, L. & Okoh, A. (2017). Chemical constituents, antibacterial and antioxidant properties of the essential oil flower of *Tagetes minuta* grown in Cala community Eastern Cape, South Africa. *BMC Complementary and Alternative Medicine, 17,* 351. doi: 10.1186/s12906-017-1861-6.

Jassbi, A., Miri, R., Asadollahi, M., Javanmardi, N. & Firuzi, O. (2014). Cytotoxic, antioxidant and antimicrobial effects of nine species of woundwort (*Stachys*) plants. *Pharmaceutical Biology, 52*(1), 62-67. doi: 10.3109/13880209.2013.810650.

Khavani, M., Sharifzadeh, M., Hadjiakhoondi, A. & Shafiee, A. (2005). Phytochemical investigation and anti-inflammatory activity of aerial parts of *Stachys byzantina* C. Koch. *Journal of Ethnopharmacology, 97*(3), 463-468. doi: 10.1016/j.jep.2004.11.037.

Lazarević, J., Palić, R., Radulović, N., Ristić, N. & Stojanović, G. (2010). Chemical composition and screening of the antimicrobial and antioxidative activity of extracts of *Stachys* species. *Journal of the Serbian Chemical Society, 75*(10), 1347-1359. doi: 10.2298/JSC100601117L.

Leblebici, S., Kaygusuz, Ö., Korkmaz, T. & Darcan, C. (2016). The antimicrobial activities of the leaves of some endemic *Stachys* species spreading in West Anatolia, Turkey. *Mitteilungen Klosterneuburg, 66*(6), 18-28.

Lotfipour, F., Nazemiyeh, H., Fathi-Azad, F., Garaei, N., Arami, S., Talat, S., Sadegpour, F. & Hasanpour, R. (2008). Evaluation of antibacterial activities of some medicinal plants from North-West Iran. *Iranian Journal of Basic Medical Sciences, 11*(2), 80-85. doi: 10.22038/ijbms.2008.5200.

Mahboubi, A., Mojab, F., Kamalinejad, M. & Ziabbary, S. M. (2012). Evaluation of antimicrobial effect of ethanol and chloroform extracts of *Stachys byzantina*. *Research in Pharmaceutical Sciences, 7*(5), S750.

Mohammadi, A., Nazari, H., Imani, S. & Amrollahi, H. (2014). Antifungal activities and chemical composition of some medicinal plants. *Journal de Mycologie Medicale, 24*(2), e1-8. doi: 10.1016/j.mycmed.2014.02.006.

Morteza-Semnani, K. & Saeedi, M. (2009). Chemical composition and antimicrobial activity of essential oil of *Stachys persica* Gmel. *Journal of Essential Oil Research, 21,* 279-282. doi: 10.1080/10412905.2009.9700170.

Mostafa, A., Al-Askar, A., Almaary, K., Dawoud, T., Sholkamy, E. & Bakri, M. (2018). Antimicrobial activity of some plant extracts against bacterial strains causing food poisoning diseases. *Saudi*

Journal of Biological Sciences, 25, 361-366. doi: 10.1016/j.sjbs.2017.02.004.

Rusenova, N. & Parvanov, P. (2009). Antimicrobial activities of twelve essential oils against microorganisms of veterinary importance. *Trakia Journal of Sciences, 7*(1), 37-43.

Saeedi, M., Morteza-Semnani, K., Mahdavi, M. & Rahmi, F. (2008). Antimicrobial studies on extracts of four species of *Stachys*. *Indian Journal of Pharmaceutical Sciences, 70*(3), 403-406. doi: 10.4103/0250-474X.43021.

Salehi, P., Sonboli, A. & Asghari, B. (2007). Chemical composition of the essential oil of Stachys acerosa and its antibacterial and antioxidant activities. *Chemistry of Natural Compounds, 43*(3), 339-341. doi: 10.1007/s10600-007-0126-x.

Serbetci, T., Demirci, B., Güzel, C., Kültür, S., Ergüven, M. & Baser, K. (2010). Essential oil composition, antimicrobial and cytotoxic activities of two endemic *Stachys cretica* subspecies (Lamiaceae) from Turkey. *Natural Product Communications, 5*(9), 1369-1374. PMID: 20922993.

Skaltsa, H., Demetzos, C., Lazari, D. & Sokovic, M. (2003). Essential oil analysis and antimicrobial activity of eight *Stachys* species from Greece. *Phytochemistry, 64*(3), 743-752. doi: 10.1016/s0031-9422(03)00386-8.

Šliumpaitė, I., Venskutonis, P., Murkovic, M. & Ragažinskienė, O. (2013). Antioxidant properties and phenolic composition of wood betony (*Betonica officinalis* L., syn. *Stachys officinalis* L.). *Industrial Crops and Products, 50,* 715-722. doi: 10.1016/j.indcrop.2013.08.024.

StatSoft Inc. (2007). STATISTICA (Data analysis software system), Vers. 10. Retrieved from statsoft.com.

Tzanova, M., Grozeva, N., Gerdzhikova, M., Argirova, M., Pavlov, D. & Terzieva, S. (2018). Flavonoid content and antioxidant activity *of Betonica bulgarica* Degen et Neič. *Bulgarian Chemical Communications, 50* (Special issue C), 90-97.

Velichkova, K., Sirakov, I., Rusenova, N., Beev, G., Denev, S., Valcheva, N. & Dinev, T. (2018). *In vitro* antimicrobial activity on *Lemna minuta*, *Chlorella vulgaris* and *Spirulina* sp. extracts. *Fresenius Environmental Bulletin, 27*(8), 5736-5741.

Vundac, V., Pfeifhofer, H., Brantner, A., Males, Z. & Plazibat, M. (2006). Essential oils of seven *Stachis* taxa from Croatia. *Biochemical Systematics and Ecology, 34,* 875-881. doi: 10.1016/j.bse.2006.04.010.

Yakoub, A., Abdehedi, O., Jridi, M., Elfalleh, W., Nasri, M. & Ferchichi, A. (2018). Flavonoids, phenols, antioxidant, and antimicrobial activities in various extracts from Tossa jute leave (*Corchorus olitorus* L.). *Industrial Crops and Products, 118,* 206-213. doi: 10.1016/j.indcrop.2018.03.047.

Yavuz, C., Kiliç, D., Ayar, A. & Yildirim, T. (2017). Antibacterial effects of methanol extracts of some plant species belonging to Lamiaceae family. *International Journal of Secondary Metabolite, 4*(3), 429-433. doi: 10.21448/ijsm.376691.

Received: 16.10.2020
Accepted: 14.12.2020