Chemia Naissensis, Vol 3, Issue 1, RESEARCH ARTICLE, 121-130

Antioxidant activity of *Micromeria croatica* (Pers.) Schott grown in plant tissue culture *in vitro* versus ones from the natural habitats

Svetlana M. Tošić[1*], Dragana D. Stojičić[1], Bojan K. Zlatković[1], Violeta D. Mitić[2], Marija D. Ilić[2], Marija S. Marković[1], Vesna P. Stankov Jovanović[2]

1- University of Niš, Faculty of Sciences and Mathematics, Department of Biology and Ecology, Višegradska 33, 18000 Niš, Serbia

2-University of Niš, Faculty of Sciences and Mathematics, Department of Chemistry, Višegradska 33, 18000 Niš, Serbia

Svetlana M. Tošić: tosicsvetlana59@yahoo.com
Dragana D. Stojičić: draganadstojicic@gmail.com
Bojan K. Zlatković: bojanzlat@pmf.ni.ac.rs
Violeta D. Mitić: violeta.mitic@pmf.pmf.edu.rs
Marija D. Ilić: marija.fertico@gmail.com
Vesna P. Stankov-Jovanović:sjvesna@yahoo.co.uk

ABSTRACT

Micromeria croatica, like many other species belonging to the Lamiaceae family, is characterized by good antioxidant activity. To avoid the exploitation of natural plant populations, it is recommended to grow them *in vitro* culture. The present study aimed to examine and compare the antioxidant potential of *M. croatica* obtain through nodal culture *in vitro* and collected from natural habitats. Different antioxidant methods were used: DPPH, ABTS, total reducing power, total phenol content, and flavonoid content. The obtained results indicate that the cultivation of plants by the *in vitro* culture technique stimulates the synthesis of secondary metabolites that promote antioxidant activity. It is increased in micropropagated plants primarily due to the increased phenol content by 136%. The possibility to test and then apply in practice the biological activity of the herb *M. croatica* is limited by the fact that the species is a local endemic.

Keywords: antioxidant activity, plant tissue culture in vitro, endemic, Micromeria croatica

[*]Svetlana Tošić
e-mail address: tosicsvetlana59@yahoo.com
address: Višegradska 33, PO Box 224 Niš, 18000 Serbia

Introduction

Rare, endemic or vulnerable species from the Lamiaceae family are really a challenge for various studies, especially in order to preserve the diversity of plant species and their natural population. Plant species of the genus *Micromeria* are relatively widespread. At the same time, many *Micromeria* taxa have a small limited geographical distribution or manifest local endemism. In the flora of Serbia, there are seven species of the genus *Micromeria*, which are classified into two sections, sects. *Micromeria* and sect. *Pseudomelisa*. This study refers to a *Micromeria croatica* belonging to a sect. *Micromeria*. Following the geographical criteria, such as the position and size of the range of the species, *M. croatica* is an endemic taxon limited to the western part of the Balkan Peninsula, including the easternmost enclave of in W. Serbia.

Endemic, endangered, and rare plant species with small populations and habitats near urban centers target negative anthropogenic actions. Many biotechnological methods are developing for biodiversity protection. One of them is a plant tissue cultured *in vitro* that has a prominent role as an *ex-situ* method.

Lamiaceae family representatives are a source of valuable bioactive molecules. Among the natural antioxidants belonging to secondary metabolites are mainly in the focus of interest. Phenols and flavonoids are the most prominent secondary metabolites in plants. Phenol compounds are very present in the Lamiaceae family. They have high antioxidant activity and the potential to catch free radicals and to chelate metals. Flavonoids enable different biological activities.

The main difficulty for the intensive use of species of the Lamiaceae family for pharmaceutical and phytochemical purposes is the tremendous individual variability due to genetic and biochemical heterogeneity. Overcoming this problem involves establishing an *in vitro* plant growing system (Saha et al., 2012). Quantitative and qualitative content of secondary metabolites may vary even among the plants of the same population. It is also under the seasonal influence. To avoid these differences, breeding plants by plant tissue culture *in vitro* has an advantage over other breeding methods. Except for this one, plant tissue culture has many other favorable features. A small amount of starting plant material is required for plant propagation by *in vitro* culture. In a short time, we can get many genetically uniform plantlets at the same ontogenetic stage. They all have the same origin, a little fragment of the parent plant, so-called explant. Just from one explant, several thousand plants can be obtained after a few weeks (Akin-Idowu et al., 2009).

Microcloned plants often grow better and faster than plants that grow *in vivo* because they are free of pathogens, bacterial, and fungal infections, and they often have better yields than conventionally grown plants (Debnath & Teixeira da Silva, 2007). The *in vitro* culture method also has excellent potential for mass production of natural secondary metabolites (Matkowski, 2008; Mulabagal & Tsay, 2004). Plant propagation *in vitro* is faster than *in vivo* propagation. A small space is required for plant propagation *in*

vitro culture. Thanks to controlled conditions, it is possible to predict the production time of individual plants.

A model system of growing *M. croatica in vitro* was established (Tošic et al., 2019; Kereša et al., 2018). To the best of authors' knowledge, there is no information concerning the antioxidant properties of micropropagated *M. croatica.* The *in vitro* culture method is widely used in the propagation of endemic and rare plants in danger of extinction to preserve the diversity of flora. Recent studies show that *M. croatica* exhibits promisingly suitable biological activities (antioxidant, antimicrobial, antiphytoviral, hepatoprotective). Despite functional biological activities, *M. croatica,* as endemic species, can not be exploited from natural habitats for commercial purposes. In line with that, the goal of the research is to evaluate the antioxidant potential of methanolic extracts of micropropagated *M. croatica* and compare it with the potential of naturally grown plants.

Experimental

Plant material

Above ground parts of *M. croatica* individuals at the vegetative stage of development were harvested from a natural population in May 2012. The location of the population was Beli Rzav gorge, W. Serbia (latitude 43° 46'26 "N and longitude 19° 27'44 "E) and the voucher specimen (Nº 6913) was deposited in the Herbarium collection of the Faculty of Science and Mathematics, University of Niš (HMN). *M. croatica* was already introduced into plant tissue culture *in vitro* (Tošic et al., 2019). For the analysis of the antioxidant activity of the extracts, micropropagated shoots that were growing on Murashige and Skoog (MS) media (1962) supplemented with 3% sucrose and 7% agar (Torlak, Belgrade) without the presence of phytohormones were used. Plant tissue cultures were growing at 25 ± 2 °C and under 16-h photoperiod in four weeks. After that, cultures were maintained by passages, which means replacing one-node stem segments on a new MS medium. The passages were done four times until we did not reach the desired amount of plant material.

Extracts preparation protocol

Both samples of plant material (natural and micropropagated) were dry and ground to powder. 1.0 g of dry weight was extracted with 10 ml methanol (Tosic at al. 2015). Obtained extracts were kept on cold (255 K) until analysis.

Antioxidant assays

All prepared extracts for antioxidant activity evaluation were of the initial concentration of 1 mg/mL. All measurements were done in triplicate, and results expressed as mean value ±standard deviation. Five different methods were used to assess the antioxidant activity of the extracts.

Determination of total reducing power using Fe(III)/Fe(II) redox pair and determination of Radical Scavenging Activity by DPPH radical was done according to the procedure described by Mitic (Mitic et al., 2011). BHT standard (butylated hydroxytoluene) solution of the same concentration as *M. croatica* extracts (1 mg/mL) was used as a reference. For the ABTS scavenging activity, we used the Trolox solution (final concentration 0–15 M) as a reference standard. The obtained results were expressed as μmol Trolox/g dry weight of extract (Li et al., 2007). Determination of Total Phenolic Content was quantified by Folin-Ciocalteu spectrophotometric method, and we used gallic acid as a reference standard while the absorbance values of the samples were read at 760 nm against a reagent blank (Mitic et al., 2011). Quantification of total flavonoid content was based on the calibration routine and is expressed as mg routine/g of dry extract (mg/g DE) (Mitic et al., 2011).

Results and Discussion

Numerous phenol compounds with antioxidant activities are identified in many plant species of the Lamiaceae family. Among the species of the genus, *Micromeria* has a noticed role. We analyzed the whole antioxidant activity of extract instead of particular compounds, because compounds may have additive, synergistic or antagonistic properties.

Whereas during *in vitro* cultivation, phytohormones added to the nutrient medium may inhibit certain enzymes involved in the biosynthetic pathways of secondary metabolites (Rothe et al., 2003). To assess the antioxidant activity of *Micromeria* shoots grown *in vitro*, only shoots cultivated in the absence of phytohormones were used.

All used methods for measuring antioxidant activities show that there are differences in obtained values among methanol extracts that originated from micropropagated and natural growing plants (Table 1).

Table 1. Antioxidant properties of *M. croatica* methanol extracts estimated by DPPH, ABTS, total reducing power, total phenolics, and total flavonoids assays

Extracts	**DPPH**%	**ABTS** (Trolox equivalents, μg/ml)	**TRP** (Ascorbate equivalentsμg/mg of dry extract)	**Total phenols** (gallic acid equivalents μg/mg of dry extract)	**Flavonoids** (rutin equivalents μg/mg of dry extract)
M. croatica nature	3.30±0.34	0.57±0.02	137.22±8.28	10.61±0.31	33.06±1.18
M. croatica in vitro	2.88±0.03	1.05±0.01	256.38±15	25.04±0.66	18.70±0.72

BHT 3.18±0.02% (RSD=0.65%) at concentration of 1 mg/ml

Due to the simple procedure and high reproducibility, the DPPH method is often used to measure antioxidant activity. There are small differences between plant samples from natural habitats and *in vitro* culture. Plantlets of *M. croatica* regenerated on the MS medium possess lower or closely similar DPPH antiradical scavenging capacities as naturally grown plants. The difference is the possibility to catch free DPPH radicals is the smallest, 12.73%.

The ABTS method is widely used to assess antioxidant activity. With this method, a large number of samples can be analyzed quickly, and a smaller number of interfering compounds occur during the measurement (Surveswaran et al., 2007). The value obtained by the ABTS method is 84.21% greater for the extract of shoots from plant tissue culture than value for the extract of nature originated plants.

The content of flavonoids in the plant extract decreases during cultivation *in vitro* culture. The content of flavonoids is 42.44% greater in shoots from natural habitats than in micropropagated plants.

The most apparent difference we got for the total phenols content. Shoots that were breeding in plant tissue culture *in vitro* have 136% higher phenol content compared with shoots of plants from natural habitat (Figure 1).

The total reducing power determined for shoots that have been developed and grown *in vitro* has a higher value than plants from natural habitats. This fact could be explained by the increased content of total polyphenols (136%) in the buds of *in vitro* grown plants. The total reducing power evaluated for *in vitro* cultivated shoots is bigger for 86.84% than for shoots collected from the natural population (Figure 1).

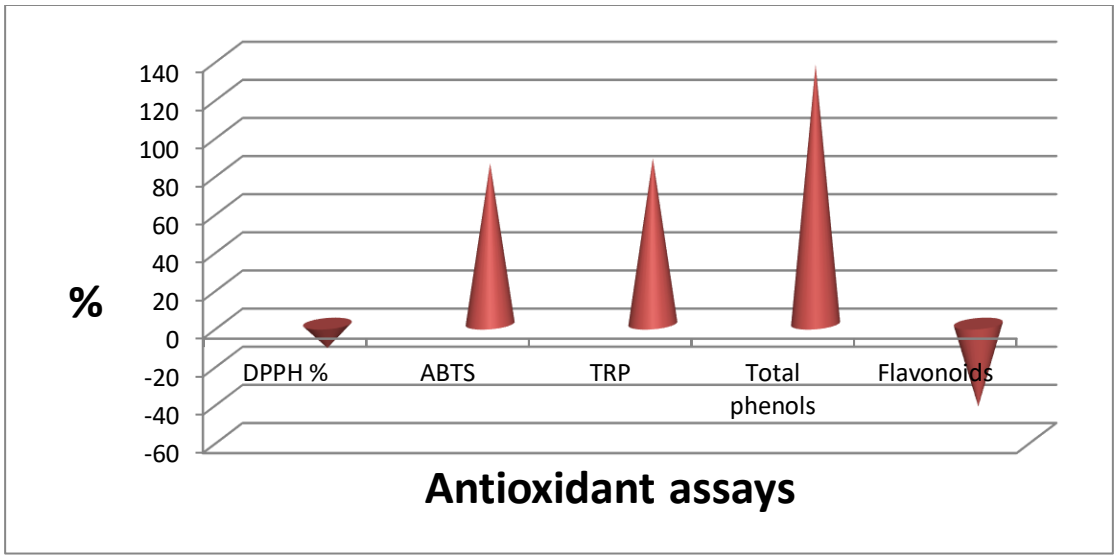

Figure 1. Degree of deviation (%) of antioxidant activities of *M. croatica* plants grown in plant tissue culture *in vitro* from the antioxidant activities obtained for plants growing in open field conditions

For different species of *Micromeria*, it has been confirmed that the total polyphenol content contributes to antioxidant activity (Vladimir-Knežević et al., 2011). The correlation between the antioxidant activity of the extracts and the polyphenol content was also confirmed in other Lamiaceae species (Tosun et al., 2009; Lopez, 2007).

We chose to use methanol for preparing extract as it is a useful solvent for phenol compounds extraction. It turned out that the most species belonging to genus *Micromeria* show better antioxidant activity in polar extracts. The methanol extract of *M. croatica* has antioxidant and pro-oxidant activity. It protects DNA and lipid molecules from damage. In low concentrations, it has an antioxidant effect on proteins, and, in high concentrations, it has a prooxidative effect. In high concentrations, methanol extract has a cytotoxic effect on HEp2 cells and leads to the formation of ROS species (Šamec et al., 2015).

Antioxidant properties are also characteristic of other species of *Micromeria*. Vladimir-Knežević et al. (2011) compared the antioxidant activity of ethanolic extracts of *M. croatica*, *M. juliana*, and *M. thymifolia*. Among them, *M. croatica* had the best antioxidant activity. The acetone extract of *M. cilicica* exhibits good antioxidant activity (Oeztuerk et al., 2011). The methanol extract of *M. fruticosa ssp. serpyllifolia* originating from Turkey has significant antioxidant activity measured by the DPPH method (Güllüce et al., 2004). The methanol extract of *M. myrtifolia* has good DPPH activity as well as reducing power, while essential oils and hexane extract do not show activity in free radical scavenging but have reducing potential. Also, the methanolic extract of *M. myrtifolia*, unlike chloroform and hexane, has a high content of polyphenols (Formisano et al., 2014).

The change in antioxidant activity of *M. croatica* shoots from the culture concerning shoots of plants from natural habitats follows the data obtained for *M pulegium*. We notice the similar data in earlier research, by comparing antioxidant activities of extracts prepared from micropropagated and nature growing plant of *M. pulegium* under the same experimental conditions (Tosic et al. 2015). Good antioxidant activity of micropropagated plant's extracts was recorded for different plant species. Extracts obtained from cultures of different organs of *Salvia officinalis* have good or even better antioxidant activity compared to extracts of the same plant organs from natural habitats (Grzegorczyk et al., 2007). Phenolic compounds with good antioxidant activity are present in the culture of *S. officinalis* shoots (Santos-Gomes et al., 2002). In experiments with different varieties of basil, it was observed that *in vitro* culture of tissues or cells accumulates more secondary metabolites than donor plants (Kiferle et al., 2011).

Good antioxidant activity due to polyphenol content has also been reported in endemic species of other families such as Rubiaceae and Myrtaceae (Angaji et al., 2012). Since endemic species cannot be exploited from natural habitats, it is recommended to grow them *in vitro*.

Conclusion

Plants are exposed to different and the altering environmental factors during their growth and development. The environmental factors may have impact on antioxidant activity of plants. Culture conditions appear to be stressful for plant tissues leading to increased antioxidant potential. The change of antioxidant potential represents an adaptation of plants on these conditions. During cultivation using *in vitro* culture, the antioxidant potential of *M. croatica* shoots is preserved or even better. Methanolic extracts of *M. croatica* shoots grown *in vitro* show higher antioxidant activity than extracts of plants from natural habitats. Higher antioxidant activity is attributed to the higher content of phenolic compounds in shoots grown *in vitro*. Plants grown *in vitro* do not lose their antioxidant potential and can serve as an alternative for production of active metabolites. According to the antioxidant activity, *Micromeria* species could be used to prevent or treat diseases caused by free radicals and oxidants. They can also be used as a source of additives in the food industry.

Acknowledgment

This research was financially supported by the Ministry of Education, Science and Technological Development ofthe Republic of Serbia, Grant Nos.
The Contract number is 451-03-68/2020-14.

Conflict-of-Interest Statement

The authors declare that they have no conflict of interest. All authors took part in experimental research, and they agree with publishing data.

References

Akin-Idowu, P. E., Ibitoye, D. O., & Ademoyegun, O. T. (2009). Tissue culture as a plant production technique for horticultural crops. African Journal of Biotechnology, 8(16), 3782-3788.

Angaji, S. A., Mousavi, S. F., & Babapour, E. (2012). Antioxidants: A few key points. Annals of Biological Research, 3 (8), 3968-3977.

Formisano, C., Oliviero, F., Rigano, D., Saabb, A. M., & Senatore, F. (2014). Chemical composition of essential oils and *in vitro* antioxidantproperties of extracts and essential oils of *Calamintha origanifolia* and *Micromeria myrtifolia*, two Lamiaceae from the Lebanon flora. Industrial Crops and Products, 62, 405–411.

Grzegorczyk, I., Matkowski, A., & Wysokin'ska, H. (2007). Antioxidant activity of extracts from *in vitro* cultures of *Salvia officinalis* L. Food Chemistry, 104, 536–541.

Güllüce, M., Sökmen, M., Şahin, F., Sökmen, A., Adigüzel, A., & Özer, H. (2004). Biological activities of the essential oil and methanolic extract of *Micromeria fruticosa* (L) Druce ssp *serpyllifolia* (Bieb) PH Davis plants from the eastern Anatolia region of Turkey. Journal of the Science of Food and Agriculture, 84(7), 735–741.

Kereša, S., Andrijanić, Z., Kremer, D., Habuš- Jerčić, I., Barić, M., Batelja- Lodeta, K., Bolarić, S., & Bošnjak- Mihovilović, A. (2018). Efficient micropropagation and rooting of *Micromeria croatica* (Pers.) Schott (Lamiaceae). Poljoprivreda, 24 (2), 27-33.

Kiferle, C., Lucchesini, M., Mensuali-Sodi, A., Maggini, R., Raffaelli, A., & Pardossi, A. (2011). Rosmarinic acid content in basil plants grown *in vitro* and in hydroponic. Central European Journal of Biology, 6, 946-957.

Li, H. B., Cheng, K. W., Wong, C. C., Fan, K. W., Chen, F., & Jiang, Y. (2007). Evaluation of antioxidant capacity and total phenolic content of different fractions of selected microalgae. Food Chemistry 102, 771–776.

Lopez, V., Akerreta, S., Casanova, E., Garcia-Mina, J. M., Cavero, R. Y., & Calvo, M. I. (2007). *In vitro* antioxidant antirhizopus activities of Lamiaceae herbal extracts. Plant Foods and Human Nutrition, 62, 151-155.

Matkowski, A. (2008). Plant *in vitro* culture for the production of antioxidants. A review, Biotechnology Advances, 26, 548–560.

Mitić, V., Stankov-Jovanović, V., Jovanović, O., Palić, I., Djordjević, A., & Stojanović G. (2011). Composition and antioxidant activity of hydrodistilled essential oil of Serbian *Ajuga chamaepitys* (L.) Schreber ssp chia (Schreber) Arcangeli. Journal of Essential Oil Research, 23(6), 70-74.

Mulabagal, V., & Tsay, H. S. (2004). Plant Cell Cultures - An alternative and efficient source for the production of biologically important secondary metabolites. International Journal of Applied Science and Engineering, 2 (1), 29-48.

Murashige, T., & Skoog F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiology, 15, 473-497.

Oeztuerk, M., Kolak, U., Topcu, G., Oeksuez, S., & Choudhary, M. I. (2011). Antioxidant and anticholinesterase active constituents from *Micromeria cilicica* by radical-scavenging activity-guided fractionation. Food Chemistry, 126, 31–38.

Rothe, G., Hachiya, A., Yamada, Y., Hashimoto, T., & Dräger B. (2003). Alkaloids in plants and roots cultures of *Atropa belladonna* overexpressing putrescine *N*-methyltransferase. Journal of Experimental Botany, 54, 2065-2070.

Saha, S., Kader, A., Sengupta, C., & Ghosh, P. (2012). *In Vitro* propagation of *Ocimum gratissimum* L. (Lamiaceae) and its evaluation of genetic fidelity using RAPD marker. American Journal of Plant Sciences, 3, 64-74.

Debnath, C. S., & Teixeira da Silva, A. J. (2007). Strawberry culture *in vitro*: applications in genetic transformation and biotechnology. Fruit, Vegetable and Cereal Science and Biotechnology, 1(1), 1-12.

Santos-Gomes, P. C., Seabra, R. M., Andrade, P. B., & Fernandes-Ferreira, M. (2002). Phenolic antioxidant compounds produced by *in vitro* shoots of sage (*Salvia officinalis* L). Plant Science, 62, 981-987.

Surveswaran, S., Cai, Y. Z., Corke, H., & Sun, M. (2007). Systematic evaluation of natural phenolic antioxidants from 133 Indian medicinal plants. Food Chemistry, 102, 938–953.

Tosun, M., Ercisli, S., Sengul, M., Ozer, H., Pola, T., & Ozturk, E. (2009). Antioxidant properties and total phenolic content of eight *Salvia* species from Turkey. Biological Research, 42, 175-181.

Tošić, S., Stojičić, D., Stankov-Jovanović, V., Mitić, V., Mihajilov-Krstev, T., & Zlatković, B. (2015). Chemical composition, antioxidant and antimicrobial activities of micropropagated and native *Micromeria pulegium* (Lamiaceae) extracts. Oxidation Communications, 38, 55–66.

Tosic, S. M., Stojicic, D. D., Slavkovska, V. N., Mihajilov-Krstev, T. M., Zlatkovic, B. K., Budimir, S. M., & Uzelac, B. B. (2019). Phytochemical composition and biological activities of native and in vitro-propagated *Micromeria croatica* (Pers.) Schott (Lamiaceae). Planta, 249(5), 1365-1377.

Vladimir-Knežević, S., Blažeković, B., Bival -Štefan, M., Alegro, A., Köszegy, T., & Petrik, J. (2011). Antioxidant activities and polyphenolic contents of three selected *Micromeria* species from Croatia. Molecules, 16, 1454-1470.

Šamec, D., Gruz, J., Durgo, K., Kremer, D., Kosalec, I., Valek-Žulj, L., Martinez, S., Salopek-Sondi, B., & Piljac-Žegarac, J. (2015). Molecular and cellular approach in the study of antioxidant/pro-oxidant properties of *Micromeria croatica* (Pers.) Schott, Natural Product Research: Formerly Natural Product Letters, 29(18), 1770-1774.