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EFFECT OF THE VEGETATION CYCLE ON TOTAL PHENOLIC AND FLAVONOID COMPOUNDS IN *Hypericum perforatum L.* AND *Melissa officinalis L.* COLLECTED IN MONTENEGRO

SUMMARY

Effect of vegetation cycle on total phenolic content (TPC) and total flavonoid content (TFC) in 80% methanolic and 50% ethanoic extracts in wild growing *Hypericum perforatum* L. and *Melissa officinalis* L. from different habitats in Montenegro was analyzed.

It was found that the type of extraction solvent and the altitude of selected habitats affect the value of the detected TPC and TFC. The highest TPC and TFC values were found in 80% methanolic extract of H1 locality in the month of July (16.66 mgGAE/gDW and 6.91 mgQE/gDW) as well as of M1 locality in the month of August (41.66 mgGAE/gDW and 11.07 mgQE/gDW) while 50% ethanolic extracts showed lower TPC and TFC content.

The TPC and TFC values of *H. perforatum* L. extracts were found to be highly variable in quantity and their dynamics was found irregular, while for the *M. officinalis* L. extracts the TPC and TFC were found to change in quantity in a regular manner during the plants vegetation cycle with the peak in the flowering stage (August), while the lowest concentration of these bioactive substances was found during fruiting stage (September) which is of the great value for the harvesting time recommendation.

Keywords: total phenolic content, total flavonoids content, *Hypericum* L., *Melissa* L.

INTRODUCTION

Plants are producing a multitude of bioactive compounds (Dixon, 2001), among them polyphenols like phenolics and flavonoids, that have been a limitless source of experimental analysis (Sujana *et al*., 2013) and which also have numerous different functions (Halliwell 2006, Albayrak *et al*., 2010, Sarbu *et al*., 2019, Obložinský *et al*., 2006). Phenolics possess antioxidant, antiproliferative, anticancer and anti-inflammatory activities, as well as antibacterial, antiviral and

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antifungal potential (Halliwell, 2006), while flavonoids are considered to have antithrombotic, antineoplastic and anti-hypertensive activities (Benayad *et al.*, 2014, Sarbu *et al.*, 2019) as well as antilipoxygenase activity (Obložinský *et al.*, 2006) among the others.

Phenolics and flavonoids, which are responsible for the biological activities of *Hypericum perforatum* L., can often be found in the leaves and flowers of a plant (Zobayed et al., 2006) and the disposal model of some flavonoids in plants reproductive complex during the flowering stage was also studied in a related *Hypericum* species (Mártonfi, 2006). *H. perforatum* L. is well recognized when it comes to flavonoids content (quercetin and kaempferol at the first place) (Nahrstedt and Butterweck, 2010) which are found in its leaves as glycosides located in special compartments of the cells of epidermis, and which are responsible for UV-protection (Germ, *et al*., 2010).

Leaves from *Melissa officinalis* L. contain flavonoids (quercitrin, rhamnocitrin, luteolin), as well as polyphenolics (rosmarinic acid, caffeic acid and protocatechuic acid) (Sofowora, *et al.*, 2013). The biological action of its extracts is primarily connected, similar to other plants, to the phenolics, flavonoids and terpenoids content (Škrovánková, *et al*., 2012).

In the literature there are several studies about phytochemical content, antimicrobial, antimutagenic and cytotoxic activity of essential oils from plants growing in Montenegro (Šćepanović *et al*., 2019, Damjanović-Vratnica *et al*., 2015, Bošković, *et al*., 2018, Božović *et al*., 2015, 2018; Artini *et la*., 2018, Tadić *et al*., 2017, Stešević *et al*., 2016, Vuković-Gačić *et al.*, 2006), while the effect of vegetation cycle on chemical content and antibacterial activity of essential oil was investigated by Damjanović-Vratnica *et al*., (2011) for the *Satureja montana* L. species.

The main goal of this study is to present dynamics of total phenolic and total flavonoids content during vegetation cycle in two different plant species – *H. perforatum* L. and *M. officinalis* L. wild-growing in Montenegro.

MATERIAL AND METHODS

Plant material

Wild-growing plants of *H. perforatum* L. and *M. officinalis* L. were collected in Montenegro in localities H1 (Ljubišnja), H2 (Vijenac) (Table 1.a) and M1 (Zenica), M2 (Stari Bar) (Table 1.b) respectively. The plant material was collected by hand from July to September 2014. The plant material was determined in the laboratory of the Study program for Biology, Faculty of Natural Sciences and Mathematics, University of Montenegro, and genus and species of the plant were identified and confirmed.

a)Aparatus

For the purpose of extraction water bath from the manufacturer Vims elektrik was used, and after the extraction, sand bath and automated rotor-vacuum evaporator IKA rv 10 were used for removing dissolvent from the extract.

Table 1. Localities description for *H. perforatum* L. (a) and *M. officinalis* L. (b).

a.

No.	Locality	Coordinates	Altitude
1.	Ljubišnja / H1	43°17'48"N 19°05'58"E	1509m
2.	Vijenac / H2	43°21'03"N 19°27'21"E	1373m

b.

No.	Locality	Coordinates	Altitude
1.	Zenica / M1	43°17'29"N 19°21'03"E	846m
2.	Stari Bar / M2	42°05'46"N 19°08'09"E	178m

All measurements were performed on a UV-VIS spectrophotometer CECIL CE 2021 in quartz cuvettes which were 10mm wide and with 83% light transmitting ability at 200nm.

b)Chemicals and reagents

Folin-Ciocalteu reagent was obtained from a producor named ALDRICH Chemistry. Solutions of 7,5% $NaHCO_3$, 2,5% $AlCl_3xH_2O$, and 10% ($Na(CH_3COO)*3H_2O$) were freshly prepared. Gallic acid and quercetin were used as reference substances.

Preparation of extracts

Hydroalcoholic extraction was done according to a modified method of Fialová *et al*., (2008). Namely, to the 0.10g of powdered drug (dried herba of *H. pefroratum L.* or dried folium of *M. officinalis* L.) 15ml of a) 50% ethanol and b) 80% methanol was added in a glass flask, and it was left to boil in a water bath for 30 min. Filtrate was poured into a measuring flask where the dilution was done. For the purpose of total phenolics determination filtrate was diluted 5 times, and for the purpose of total flavonoids determination, it was diluted 10 times.

Total phenolic content (TPC)

The TPC were determined according to the Habila et al., (2010) by using Folin-Ciocalteau assay. Namely, in 1.5 ml of Folin-Ciocalteau working solution was added 1.5 ml of $NaHCO_3$ and 200 µl of examinating samples. The samples were incubated at room temperature for 30 min. Then, absorbance was measured spectrophotometrically at 765 nm. The results for TPC concentration were expressed as milligrams of gallic acid equivalents per gram of dry weight (mgGAE/gDW). All the analyses were repeated 6 times.

Total flavonoids content (TFC)

The TFC were determined spectrophotometrically by $AlCl_3$ method, according to modified assay described in Zou, *et al*., (2004). 1ml 2.5% $AlCl_3x6H_2O$ was mixed with 1ml of NaOH and left for the reaction for 5 min. Following this, 2ml of 10% ($Na(CH_3COO)*3H_2O$) and 6ml of 70% ethanol and 1ml of examinating sample were added. After the incubation of 30 min on the room temperature, the absorbance was measured at 420 nm. TFC was expressed

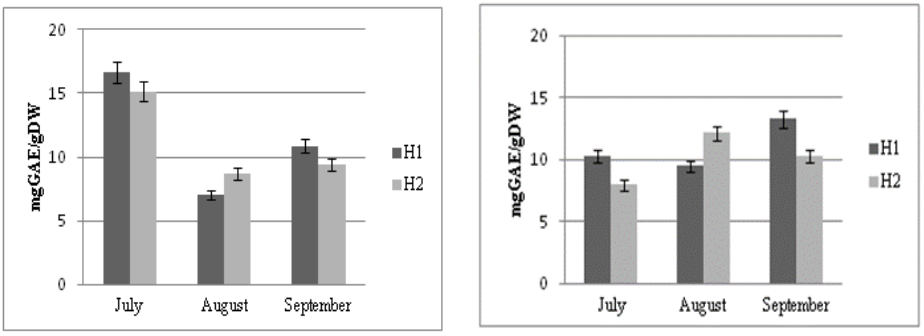
as mg quercetin per gram of dry weight (mgQE/gDW). All the analyses were repeated 6 times.

RESULTS AND DISCUSSION

Phenolics and flavonoids are bearers of plants biological activities (Olech, *et al*., 2012, Božin, *et al*., 2013). In this paper the variation in TPC and TFC of the wild-growing *H. perforatum* L. and *M. officinalis* L. from several different localities and different habitats in Montenegro, during their phenological cycle was investigated.

During the observation and analyses of TPC and TFC, in the dried herb (*herbae*) of wild-growing *H. perforatum* L., which were obtained during three consecutive months (July, August, September) for 80% methanolic and 50% ethanolic extracts **(Figure 1. and 2.),** a stable regularity in the dynamics of these bioactive substances during the vegetation period was not found.

In terms of 80% methanolic extracts **(Figure1.a)** the peak of TPC was found in July (16.66 mgGAE/gDW - for the H1 locality), while 50% ethanolic extract showed the peak of TPC in September (13.5 mgGAE/gDW - for the H1 locality) **(Figure 1.b).**



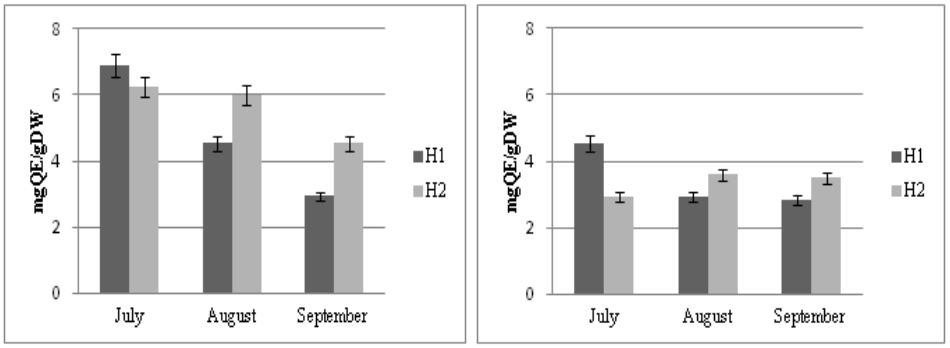
a) **b)**

Figure 1.a) Dynamics of total phenolic content in 80% methanolic hydroalcoholic extract of the wild-growing *H. perforatum* L. *herba* from the locality of H1 (Ljubišnja) i H2 (Vijenac). **b)**Dynamics of total phenolic content in 50% ethanolic hydroalcoholic extract of the wild-growing *H. perforatum* L. *herba* from the locality of H1 (Ljubišnja) i H2 (Vijenac).

When it comes to TFC in 80% methanolic extracts of *H. perforatum* L. **(Figure 2.a)** their peak was found in July (6.91 mgQE/gDW - for the H1 locality) and the 50% ethanolic **(Figure 2.b)** extract also showed the peak of TFC in July (4.54 mgQE/gDW - for the H1 locality).

Present data for TPC values were lower than in Sarikurkcu *et al*., (2020) and it seems that this is the case due to the different extraction type. This

investigation also showed significantly lower TPC and TFC data than Tausevski *et al*., (2019) found in flowering stage samples collected in Northern Macedonia.



a) **b)**

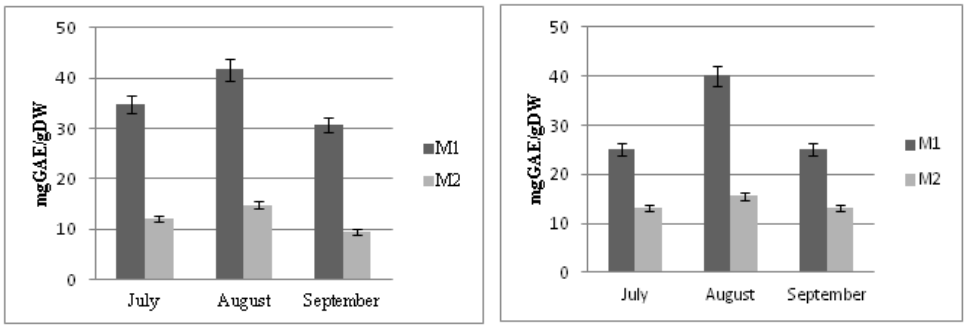
Figure 2.a) Dynamics of total flavonoid content in 80% methanolic hydroalcoholic extract of the wild-growing *H. perforatum* L. herba from the locality of H1 (Ljubišnja) i H2 (Vijenac).**b)** Dynamics of total flavonoid content in 50% ethanolic hydroalcoholic extract of the wild-growing *H. perforatum* L. herbae from the locality of H1 (Ljubišnja) i H2 (Vijenac).

The TPC and TFC values were found to be highly variable in quantity and their dynamics was found irregular unlike in Kazlauskas and Bagdonaite (2004) and Couceiro *et al.,* (2006). We also take into consideration data obtained by Toker, (2009) and Ciraki, *et al*. (2013) for *Hypericum triquetrifolium* Turra species, which is known for its higher content of hypericin when compared with *H. perforatum* L. (Al-Snafi, 2018). The research of Toker, (2009) encompassed three (vegetative, full flowering and mature fruiting stage) and of Ciraki,*et al.* (2013) five developmental stages (vegetative stage, floral budding stage, full flowering stage, fresh fruiting stage and mature fruiting stage) and both researchers concluded that a peak of bioactive substances concentration was in full flowering stage, which is not the case in this research.

During the monitoring of the content of TPC and TFC, in the dried leaf (*folium*) of wild-growing *M. officinalis* L. during three consecutive months (July, August, September) for 80% methanolic and 50% ethanolic extracts **(Figure 3. and 4.),** the maximum value of these bioactive substances was detected in the month of August during the flowering period (for 80% methanolic extract on the locality M1 TPC = 41,66 mgGAE/gDW and TFC = 11,07 mgQE/gDW **(Figure 3.a and Figure 4.a)** and for 50% ethanolic extracts on the locality M1 TPC = 40.15 mgGAE/gDW and TFC= 8,42 mgQE/gDW **(Figure 3.b and Figure 4.b),** while the lowest values were found in the month of September

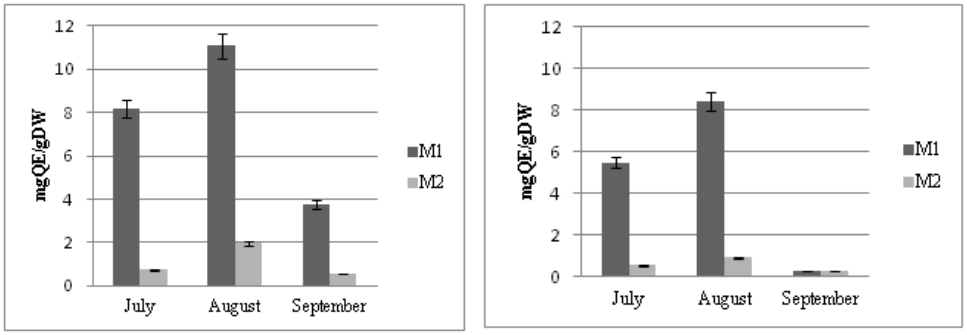
This range fits the value from Moacă *et al*., (2018) who found that TPC in the *M. officinalis* L. leaves extract was 32.76 mg GAE/g dry material, as well as Rehan *et al*., (2014). A higher TPC equal to 227.6 mg GAE/gDW was calculated

for the hydroalcoholic extract from *M. officinalis* L. leaves (Moradi, *et al*., 2016) while the TFC of 12.5±2.11 mg/g as reported by Moradi *et al*., (2016), was in accordance with present data.



a) **b)**

Figure 3.a) Dynamics of total phenolic in 80% methanolic hydroalchoholic extract of dried wild-growing *M. officinalis* L. *folium* from the localities M1 (Zenica) and M2 (Stari Bar). **b)** Dynamics of total phenolic in 50% ethanolic hydroalchoholic extract of dried wild-growing *M. officinalis* L. *folium* from the localities M1 (Zenica) and M2 (Stari Bar).



a) **b)**

Figure 4.a Dynamics of total flavonoids in 80% methanolic hydroalchoholic extract of dried wild-growing *M. officinalis* L. *folium* from the localities M1 (Zenica) and M2 (Stari Bar).**b** Dynamics of total flavonoids in 50% ethanolic hydroalchoholic extract of dried wild-growing *M. officinalis* L. *folium* from the localities M1 (Zenica) and M2 (Stari Bar).

Total polyphenols in spray-dried extract before and after hydrodistillation was found the highest in *M. officinalis* L. among *Lamiaceae* family species, along with *Origanum vulgare* (Grigore *et al*. 2019).

For the *M. officinalis* L. species, the TPC and TFC were found to change in quantity in a regular manner during the plants vegetation cycle with the peak in

the flowering stage (August), while the lowest concentration of these bioactive substances was found during fruiting stage (September), which is in a good agreement with the results of Saeb *et al*., (2011) who did the research on the plant material collected in India during three different plants development stages (vegetative growth, flowering stage and after flowering). Similarly, the amount of rosmarinic acid (%) was studied in samples of *M. officinalis* L. leaves in dependence of the plant ontogenetic phase at harvest time (Tóth *et al*., 2003). Statistically non-significant variability of this phenolics acid was found. Nevertheless, maximum content was found in the full flowering stage (3.91 %) and minimum content in the stage just before flowering (3.50 %).

For the both of afore mentioned species, 80% methanolic extracts had higher concentrations of investigated bioactive substances than the 50% ethanolic extracts, which suggests 80% methanol being a better solvent for this purpose. The variation of TPC values according to the solvent used for the extraction is also described in the literature (Moacă *et al*., 2018). We should also take into account that content of bioactive substances in plants is changed according to climate, environmental conditions, different habitat and locality (Stefanović, *et al*., 2018). For instance, the methanolic extract gained from the aerial parts of *M. officinalis* L. wild-growing in Romania had a TPC of 22 mg GAE/g extract (Armatu *et al*., 2010) and those from Bulgaria *(herbae*) a TPC of 48.86 mg GAE/100 g dry weight (Atanassova *et al*., 2011). These findings reinforce present results on TPC in *M. officinalis* L. leaf extracts which highly differs depending on a habitat, especially altitude of the selected localities, for example TPC values for the M1 locality (846m) (August) was 41.66 mgGAE/gDW while for the M2 locality (178m) was (August) 14.77 mgGAE/gDW.

CONCLUSIONS

In this study variation of TPC and TFC during three consecutive months of vegetation cycle was presented for the two wild-growing plant species: *H. perforatum* L. and *M. officinalis* L. The results of this investigation point towards the recommendation of collecting plant material of *M. officinalis* L. in Montenegro during the flowering phase (month of August) in which there is the peak of TPC and TFC, which is not the case with *H. perforatum* L.

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