ORIGINAL ARTICLE

PHYTOSOCIOLOGICAL STUDY AND ITS INFLUENCE ON THE BIOSYNTHESIS OF ACTIVE COMPOUNDS OF TWO MEDICINAL PLANTS *MENTHA PIPERITA* L. AND *MELISSA OFFICINALIS* L.

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Abstract

The aim of the paper was the phytosociological study and its influence upon the biosynthesis of active compounds of two medicinal plants (*Mentha piperita* L. - peppermint and *Melissa officinalis* L. - lemon balm), which are widely used in phytotherapy. Our research was performed on dried leaves and the active substances content was determined by means of spectrophotometric (for evaluation of flavones, phenolcarboxylic acids and total phenolic contents) and volumetric methods (for evaluation of the essential oil content). Our results have shown that in the common (phytosociological) crops the active compounds are synthesized in higher amounts compared to the control crops. Moreover, for the common crops, horizontal and vertical development was much more obvious. In addition, our results regarding peppermint leaves, have shown that the active substances content have a different distribution throughout the day, with an upwards course during the morning and midday, whilst a descending direction was seen in the evening.

Rezumat

Studiul fitosociologic a urmărit influența în dezvoltarea și în biosinteza principiilor active între două specii medicinale frecvent asociate în fitoterapie, *Mentha piperita* L. și *Melissa officinalis* L. Cercetările au fost efectuate pe produsele vegetale de tip *folium*, iar conținutul în principii active a fost evaluat prin metode spectrofotometrice (acizi fenolcarboxilici, flavone, polifenoli totali) și volumetrice (ulei volatil). Rezultatele au demonstrat că în loturile fitosociologice se sintetizează o cantitate mai mare de principii active, iar dezvoltarea pe orizontală și verticală este mult mai evidentă comparativ cu loturile martor.

Keywords: phytosociology, *Mentha piperita* L*., Melissa officinalis* L.

Introduction

Phytosociology, a branch of botany, represents a new field with growing interest, in scientific research. Phytosociology deals with plant communities and it is based on the ability of certain species to coexist in heterogeneous groups [2], to influence themselves during plant development [21] and to establish inter-relationships within an area delimited by vegetation [11]. Taking into consideration the scientific data [1, 4, 18], in the current phytosociological context, this paper presents the research carried out on two medicinal plants belonging to the *Lamiaceae* family, that are frequently associated in phytotherapy. Taking into account the premise of our study, we have observed the development of peppermint and lemon balm medicinal plants, associated in phytosociological groups. Furthermore, we have also studied the influence of their association upon the active compounds content of leaves. It is well known that peppermint leaves (*Menthae folium*) are commonly used for symptomatic relief of gastro-intestinal disorders and skin conditions [23, 24]. *Peppermint leaves* have tonic and stomachic

properties (due to the essential oil and bitter substances content); choleretic-cholagogue and spasmolytic effects (due to flavones, polymethoxylated flavones, essential oil esters, caffeic acid and chlorogenic acid content) [23, 24]. Moreover, peppermint leaves are commonly used for their antidiarrheal and antibacterial effects (due to tannins and essential oil content). Menthol, the main active compound in peppermint essential oil, has antiemetic (due to a slight anaesthesia of the gastric mucosa) and anti-itching properties [23, 24]. Peppermint leaves active substances content is variable and greatly influenced by pedoclimatic conditions, thus *Menthae folium* contain up to 3.02 - 6.32% flavones, 2.70 - 5.52% phenolcarboxylic acids and 5.72 - 11.51% polyphenols [5, 16]. Lemon-balm leaves are widely used for emotional disorders treatment, since the main constituents of *Melissae folium* essential oil (aldehydes and esters) are known for their sedative and spasmolytic effects. On addition, lemon balm leaves have a strong choleretic-cholagogue effect, due to phenolcarboxylic acids (caffeic and chlorogenic acids) and bitter substances content [23, 24]. HPLC studies regarding lemon balm

leaves reported a variable content of active compounds (caffeic acid 1.28 - 1.95 g/100 g leaves, total phenolic content 2.25 g/100 g leaves, rutin 1.81%) depending on plants habitat and harvest area [14, 17, 20].

Materials and Methods

Peppermint and lemon balm were planted in experimental crops with a dimension of 50 cm x 300 cm, 400 cm distance between batches, 30 cm distance between seedlings and 5 seedlings/group. The crop was generated in the suburban area of Turnu Măgurele city, Teleorman County (43°44′44.16″ Northern latitude, 24°52′53.40″ Eastern longitude, with a temperate-continental climate specific to the Romanian Plain), in 2018. Crops were observed between 2018 and 2019. A series of techno-agricultural works were carried out (obtaining seedlings, preparing the land, planting) before obtaining the experimental crops. All batches were monitored through-out their development (between 2018 and 2019), by the horticulturist Ciocăneală Ștefan, and were subjected to the same operations like watering, weeding, loosening of the soil, etc. It is worth mentioning that during the whole experiment, we have not used pesticides or fertilizers. Quantitative analysis was performed on peppermint and lemon balm leaves, harvested during the flowering period (June, 2019). Leaves were dried in the laboratory of Pharmacognosy, Phytochemistry and Phytotherapy department and stored in paper bags. The batches for the analysed medicinal plants and the corresponding leaves were encoded as follows: MM – peppermint control crop, MF – peppermint phytosociological (common) crop, MLM – lemon balm control crop and MLF – lemon balm phytosociological (common) crop.

Our phytosociological study continued in 2020. For peppermint, we have also determined the distribution of active substances throughout the day. With that end in view, quantitative assays were performed on fresh leaves harvested in the month of June 2020 (control and common crops), using the following schedule (8.30 a.m., 12.30 p.m., 16.30 p.m. – 1st day, 20.30 p.m. – 2nd day, 8.30 a.m. and 12.30 p.m. – 3rd day). Besides, leaves were collected (following the above mentioned schedule) from the base, middle and top parts of peppermint plants.

Reagents and solvents. All chemicals (aluminium chloride, chlorogenic acid, ethanol, hydrochloric acid, rutin, sodium hydroxide, sodium nitrite, tannic acid) were purchased from Sigma-Aldrich (Germany).

Obtaining the extractive solutions

Preparation of samples for the spectrophotometric assays: 1 g of dried leaves (from each batch) were heated twice with 25 mL 70% ethanol (v/v) and 50% ethanol (v/v) on a reflux condenser for 30 min. After cooling, the solutions were filtered in a 50 mL volumetric flask and filled to mark with the same solvent.

Spectrophotometric assays. The flavonoids content (FL) was determined based on the chelating reaction with aluminium chloride [8]. Phenolic acids (PAC) were assessed based on the formation of oxymes in the presence of sodium nitrite/hydrochloric acid and sodium hydroxide [9]. The total phenolic (TPC) content was evaluated based on the capacity to reduce molybdenic compounds (VI) [3, 6, 10, 13, 15, 22]. For all spectrophotometric determinations, a Jasco V-530 spectrophotometer (Jasco, Japan) was used. The following calibration curves were used to determine the active compounds content: rutin (linearity range: 5.0 - 35.0 µg/mL, r = 0.9998, n = 11), chlorogenic acid (linearity range: 11.3 - 52.7 µg/mL, r = 0.9998, n = 6) and tannic acid (linearity range: 2.0 - 12.0 µg/mL, r = 0.9990, n = 10). The essential oil content was determined based on a volumetric method, using the Neo Clevenger apparatus.

Statistical analysis. For each extract, three samples were analysed and all assays were carried out in tri-plicate (n = 3). The results are expressed as mean ± standard deviation. Standard deviation was determined using Microsoft Office programme (Excel®, 2010). The distribution of active substances for peppermint leaves was performed using the open source software R [19]. The main goal was to evaluate the behaviour of a continuous outcome variable, denoted with Concentration, for each analysed active substance (flavones, phenolcarboxylic acids and total phenolic content). The factors that affected the numerical variable concentration were a focal variable denoted with Type (with 2 levels: Control and Phytosociological) and other two moderator variables: Time (with 6 levels) and Position (with 3 levels: base, middle and top of the leaf). We evaluated the statistical significant differences between the two study groups for the 5% standard level of significance (or p value < 0.05). Because the data set contained relatively small samples, acceptance of the conditions of normality, homoscedasticity and sphericity could be questioned, therefore the choice of more robust estimators such as trimmed mean and computational tests based on the bootstrap method were the most appropriate choice [7]. The determination of the effects of the three factors involved on the concentration of each natural compound was performed with the help of the robust statistical analysis multi-way between-subjects ANOVA from the WRS2 package [12].

Results and Discussion

The differences between the control and common groups (for the 2018 - 2019 crops) are obvious in terms of vertical and horizontal development for the analysed medicinal plants (Table I).

Table I

Differences regarding the evolution of analysed medicinal plants

Month/year	Batches			
	MM	MF	MLM	MLF
May 2018	h = 22 cm	h = 24 cm	h = 25 cm	h = 31 cm
June 2018	h = 23 cm	h = 39 cm	h = 33 cm	h = 42 cm
May 2019	h = 19 cm	h = 24 cm	h = 48 cm	h = 66 cm
June 2019	h = 26 cm	h = 37 cm	h = 60 cm	h = 89 cm

MM – peppermint control crop; MF – peppermint phytosociological (common) crop; MLM – lemon balm control crop and MLF – lemon balm phytosociological (common) crop

If initially, the plants did not exceed 10 cm, in May 2018, several differences were observed. Comparing the crops development between the months of May and June 2018, one can note an abundance of the two medicinal species grown in the same crop. Comparing the crops of the months May and June 2018 with those of 2019, there is an obvious evolution for lemon balm compared to peppermint. Moreover, major differences regarding both plants evolution (both vertically and horizontally) were observed between phytosociological and control groups. So, peppermint control group (MM) (measured during the month of June 2019), contained plants with a height of 26 cm, with spread horizontal development, while for the phytosociological group (MF), the height of the plants was 37 cm, with a much lower recurrence horizontally, compared to lemon balm crop. For lemon balm phytosociological crop (MLF) the difference was much obvious. According to our results, the association of lemon balm with peppermint conducted to a much better development both vertically and horizontally, with a plant height of 60 cm in the control group (MLM), respectively 89 cm in the phytosociological group (MLF).

Moreover, for the same crop, we have also noticed a uniform growth along two consecutive months. For instance, peppermint crops (for May - June 2018 period) had a uniform development, the plants height varied between 22 - 23 cm for the control group and 24 - 39 cm for the phytosociological group.

From a macroscopic point of view, several differences (in term of size) were observed for peppermint and lemon balm leaves, harvested in June 2019. Leaves, belonging to the phytosociological crops (MLF, MF) were larger. Analysing leaves, using a stereomicroscope (stereomicroscope with Axiocam ERc5s camera, Zeiss, Germany), we have observed several glandular trichomes (for peppermint leaves), that were mainly found on the lower epidermis. It is important to mention, that there is a positive correlation between the existence of glandular trichomes and the essential oil content. Regarding the amount of dried leaves, we have found significantly higher quantities for the phytosociological groups, compared to the control ones (Table II). Besides, the leaves size was higher for the common crops. We consider that our remarks may be used by indigenous manufactures for the purpose of increasing the production yield of analysed medicinal plants.

Table II

The size and amount of leaves for analysed crops

Batches	Leaves size (cm)	Leaves amount (g)
MM	L = 3 cm; width base = 1.5 cm; width top = 1.5 cm	498 g
MF	L = 6.5 cm; width base = 3.5 cm; width top = 2 cm	754 g
MLM	L = 5 cm; width base = 3.5 cm; width top = 2 cm	803 g
MMF	L = 6 cm; width base = 4 cm; width top = 2.5 cm	1279 g

MM – peppermint control crop; MF – peppermint phytosociological (common) crop; MLM –lemon balm control crop and MLF – lemon balm phytosociological (common) crop

Regarding the quantitative analysis, we have found significant differences between analysed batches (Table III). Besides, the solvent represents a key factor, which greatly influences the content of active substances. According to our results, leaves harvested from phytosociological crops (MF, MLF) have a higher content of active substances compared to control groups (Table III). For peppermint, we have found a content of 1.85 g % flavones (for the common crop), compared to 1.12 g % for the control crop (for 70% alcoholic

solutions); while lemon balm leaves contain 22.22 g % PCAs (for the common crop), compared to 17.36 g % (for the control group) (for 50% alcoholic solutions). TPC is increased for both herbal products in the phyto-sociological crops, with an unequal distribution regarding the type of solvent. For peppermint leaves a higher amount was determined for 70% alcoholic (10.1874 ± 0.4661) solutions, while for lemon balm 50% alcohol led to a higher phenolic content (7.1192 ± 0.0999).

Table III
Results for spectrophotometric and volumetric assays

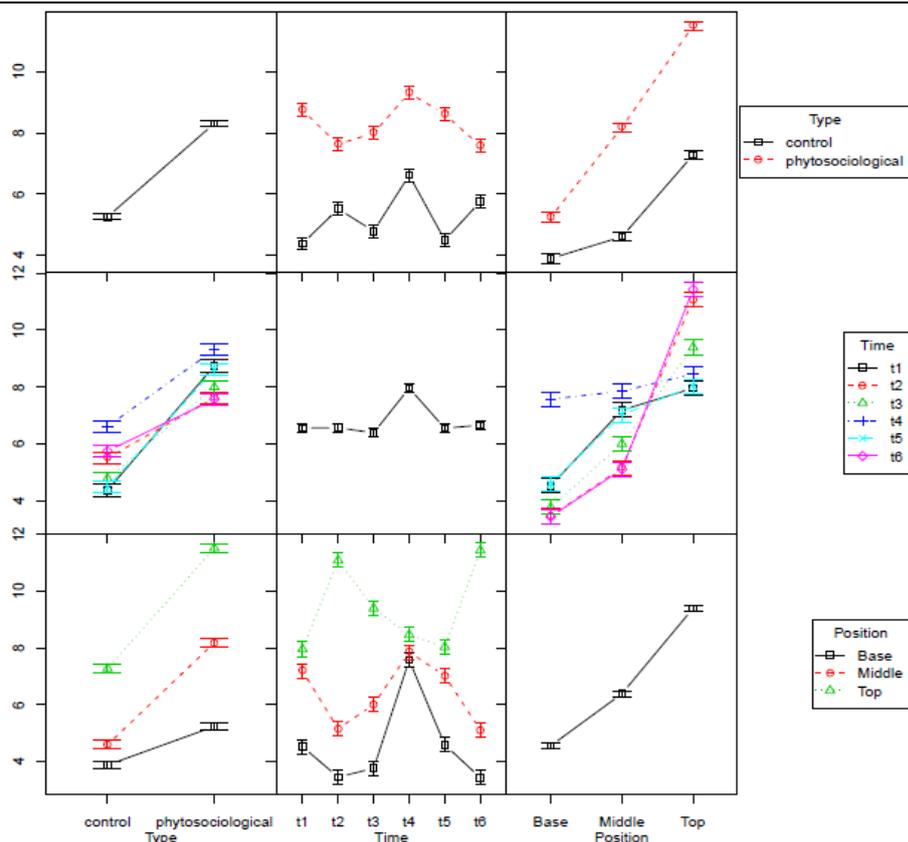| Solvent	MM	MF	MLM	MLF
g FL expressed in rutin/100 g dried leaves				
70% Alcohol	1.1223 ± 0.2576	1.8507 ± 0.2992	1.8538 ± 0.5767	2.4158 ± 0.8011
50%Alcohol	0.9748 ± 0.2038	1.8173 ± 0.1849	1.7444 ± 0.07164	2.1718 ± 0.7634
g PCAs expressed in chlorogenic acid/100 g dried leaves				
70%Alcohol	12.2345 ± 1.0167	13.8455 ± 2.0607	12.1843 ± 0.6242	20.2219 ± 2.6006
50%Alcohol	8.8808 ± 1.6324	10.8075 ± 0.4515	17.3679 ± 0.6865	22.2269 ± 0.9863
g TPC expressed in tannic acid//100 g dried leaves				
70%Alcohol	8.4538 ± 0.9820	10.1874 ± 0.4661	3.8451 ± 0.3310	6.6502 ± 0.7380
50%Alcohol	8.9389 ± 1.4589	9.6069 ± 1.1012	5.5494 ± 0.3895	7.1192 ± 0.0999
mL essential oil/100 g fresh herbal product				
	2.3	2.6	0.1	0.2

Results are mean $\pm$ SD (n = 3); MM – peppermint, control crop; MF – peppermint phytosociological (common) crop; MLM – lemon balm control crop and MLF – lemon balm phytosociological (common) crop; FL – flavones; PCAs – phenolcarboxylic acids; TPC – total phenolic content

Regarding the distribution of active substances, for peppermint leaves, our results pointed out that they follow an upwards course during the morning and midday, whilst a descending direction was seen in the evening. For flavones, there was a statistically significant three way interaction, p = 0.008 (Figure 1a). There was a statistically significant simple two-way interaction between the crop type (control or phytosociological) and Position (p = 0.001) but not for Time factor (p = 0.86).
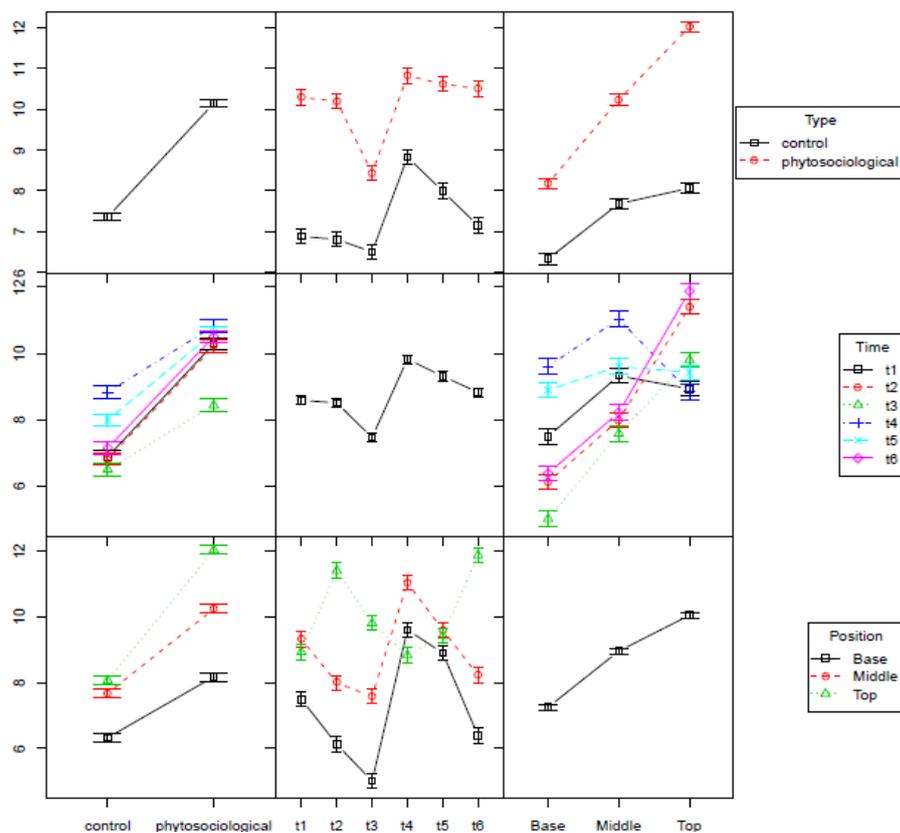
There was a statistically significant simple main effect between the crop type (p = 0.002) for every kind of Position analysed, but there was no evidence related to factor Time (p = 0.07) (Figure 1a). Regarding the phenolcarboxylic acids and the total phenolic contents, we have found a similar behaviour regarding the interaction related to all factors involved in our statistical analysis. There was a statistically significant three way interaction (p < 0.0001) and simple two-way interactions between all factors (p < 0.01) (Figures 1b and 1c).
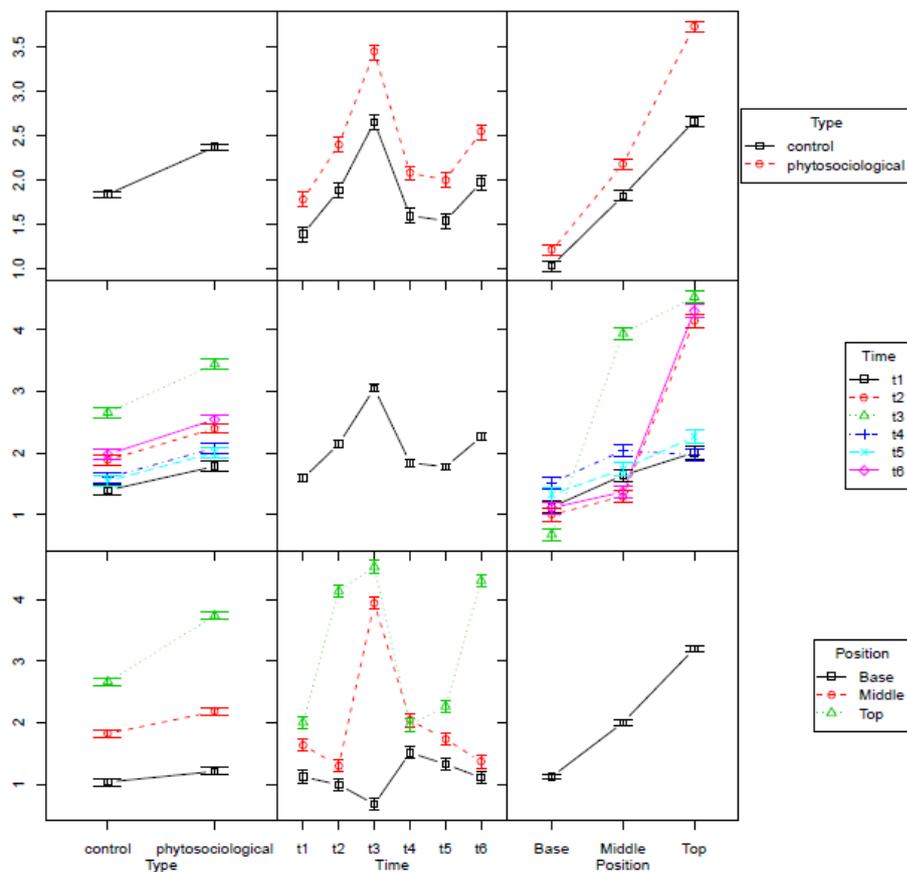
1a. Three factors interaction plot for FL

Figure 1.
Distribution of active substances content for *Mentha officinalis* L. dependent on leaves batch, time of leaves harvest and part of the medicinal plant (base, middle or top)
FL – flavones; PCAs – phenolcarboxylic acids; TPC – total phenolic content

Conclusions

Our research has shown a positive interrelationship between analysed medicinal plants, *Mentha piperita* L. and *Melissa officinalis* L., in terms of development and active substances content. The aim of our future research will be the extension of phytosociological studies on other medicinal plants.

Acknowledgement

Conflict of interest

The authors declare no conflict of interest.

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