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PRODUCTION OF POLYPHENOLIC COMPOUNDS WITH HIGH ANTIOXIDANT ACTIVITY IN *MELISSA OFFICINALIS* L. CALLUS LINE

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Abstract

The importance of antioxidant components for disease prevention and health quality improvement has attracted much research attention over the last decades. Aromatic herbs have been recognized as important sources of antioxidants, Melissa officinalis L. being one of the species with many therapeutic actions. Plant extracts represent mixtures of numerous compounds and their biological actions are often attributed to polyphenol components, such as flavonoids and phenolic acids, which possess antioxidant activities. The aim of our research was to obtain and characterize a highly metabolite-producing callus line of lemon balm, with antioxidant activity. Our callus' performances were represented by a very high antioxidant capacity (18.74 mM Trolox equivalents/g DW), a high content of total polyphenols (2.57 mg gallic acid equivalents/g DW) and an especially rich content of flavonoids (19.21 mg rutin equivalents/g DW). This lemon balm callus line was compared to plant tissues grown in ex vitro conditions and proved to have clearly superior productive qualities. Also, our results showed values 4 to 18 times higher than those reported in other studies which investigated different types of lemon balm extracts.

Key words*: callus line, Melissa officinalis L., antioxidant activity, polyphenols.*

INTRODUCTION

Polyphenols are secondary metabolites specific to higher plants and, currently, over 8000 such compounds are known (Koleva et al., 2021). Half of them belong to the flavonoid group and are found as aglycones, glycosylated forms or methylated derivatives with strong antioxidant activity (Kumar & Pandey, 2013), most of them being of interest mainly for the food, medicine, cosmetics and pharmaceutical industries.

Due to the growing economic importance of polyphenols, it was vital to reconsider genetic resources, but also to direct research towards finding new methods of producing and extracting bioactive compounds from plants (Radomir et al., 2019).

In many medicinal plants which are part of the spontaneous flora, the risk of overexploitation by excessive and unsustainable harvesting causes the destruction of natural habitats and the decline of species (Coskun & Kapdan, 2024). Also, the changing climatic conditions significantly affect the biological potential of medicinal plants in terms of population density, but also have effects on harvesting periods. The spontaneous flora is subject to different types of stress which can lead to genetic changes that can compromise the biosynthetic value of the plant material. All this highlights the need for new approaches to improve the production of secondary metabolites.

The introduction of *in vitro* cultures as biological model has led to obtaining important scientific data on valorisation through unconventional methods. *In vitro* cultures have proven to be efficient and reproducible alternatives for the production of plant material in increased quantities, but also with improved qualities. The use of *in vitro* culture techniques for the production of aromatic plants has made it possible to regulate the production in accordance with the requirements, independent of season, phenomena of dormancy, with a high content of active principles, which can be supplied in fresh condition (Radomir et al., 2019).

Mellissa officinalis L., known as lemon balm, is one of the aromatic plants with many medicinal and economical values. It is a perennial herb belonging to the mint family, characteristic to

the Mediterranean region, distributed from Central and Southern Europe to Central Asia, Russia, Caucasus and Iran (Petrova et al., 2021). This Mediterranean species grows spontaneously in southern and western Romania (Hanganu et al., 2008). It is cultivated in Europe, Asia and the United States as a culinary plant with a particular taste, but also for medicinal purposes, due to its chemical composition rich in essential oils with health benefits (Doğan et al., 2021).

M. officinalis plants are rich sources of various secondary metabolites such as phenolic acids, flavonoids, terpenes, volatile oils or tannins (Petrova et al., 2021). It is used in traditional medicine for the multitude of proven pharmacological effects, such as: anti-inflammatory, antispasmodic, sedative, anxiolytic, cardioprotective, antipsychotic, antitumor, antiviral, antimicrobial or antioxidant (Shakeri et al., 2016; Ieri et al., 2017).

Cultivation of lemon balm by conventional methods is not difficult, however to obtain a homozygous population producing valuable compounds in constant quantity and quality is an endeavour (Meftahizade et al., 2010). Through *in vitro* culture it is possible to obtain a stable quantity, but also to stimulate the production of certain metabolites by using growth regulators or elicitors. However, the performances are dependent on the genotype, the endogenous hormones' concentrations and the initial explant response, i.e. the interaction between the exogenous hormone concentrations and the endogenous hormones receptors (Phillips & Garda, 2019).

Since lemon balm is one of the plant species intensively exploited for volatile oils with significant commercial value, in recent years a series of studies have been carried out specifically on the production of secondary metabolites in *in vitro* cultures (Barros et al., 2013; Rahman et al., 2015; Mokhtarzadeh et al., 2017; Mousavi & Shabani, 2019; Petrova et al., 2021; Kim et al., 2020; Kianersi et al., 2022; Coskun & Kapdan, 2024). In light of the above, this study aims to present a simple, reproducible and optimized protocol for obtaining callus in *M. officinalis* with a metabolic profile rich in polyphenols and flavonoids with increased antioxidant activity and monitoring, on long term, the production of secondary metabolites in this callus culture.

MATERIALS AND METHODS

Plant material

The plant material used for our experiment was represented by *M. officinalis* callus culture initiated from *in vitro* plantlets obtained by aseptically germination of seeds. Seeds were purchased from commercial site (www.semintelegumeflori.ro).

Seeds of lemon balm were sterilized in 70 % ethanol for 30 sec, washed with sterilized distilled water, transferred to 0.1% $HgCl_2$ for 15 min and finally washed three times with sterilized distilled water. Sterilized seeds were inoculated on MS basal medium (Murashige & Skoog, 1962) with 0.7% agar and incubated at 24°C, with 16-h photoperiod, in a growth chamber - Fitotron Gallenkamp SCG 120 (Weiss Technik, Loughborough, UK).

Production of callus cultures

One-month old plantlets were used as a source of explants. Leaf explants were excised and used for callus cultures establishment. Callus cultures were initiated on MS basal medium supplemented with B5 vitamins (Gamborg et al., 1968) and 0.3 mg/l 2,4-D, 1 mg/l NAA (1-naphthalen acetic acid), 0.5 mg/l Kinetin, 0.2 mg casein hydrolysate, 30 g/l sucrose and 7 g/l agar. Cultures were incubated in a growth room at 24±2°C under dark condition. Primary calli were initiated after incubation for one month and callus fragments were subcultured on fresh media every three weeks.

The optimized callus culture was obtained by successive passages for two years on adjusted formula of culture medium with increased levels of 2,4-D at 1mg/l and casein hydrolysate at 0.3 mg/l.

The callus stock from this culture was maintained for twelve years as *M. officinalis* callus long-term culture of in the *in vitro* culture collection of the Institute of Biology Bucharest, Romanian Academy.

The gravimetrical parameters of this callus culture were registered as:

- callus growth rate was calculated as W_f/W_i, where W_f and W_i are the fresh weights (FW, g) at the beginning and the end of the growth period (three weeks);
- dry weight (DW, g) was obtained by drying of 1g from each sample was at 80ºC, until

constant weight, in an Ecocell oven (MMM Group, Germany);

- callus moisture content was calculated as percentage using the formula: M.C (%) = (FW-GW)/FW × 100.

Extracts Preparation

For extract preparation, 1 gram of fresh weight callus was crushed using a mortar and pestle, then extracted with different types of solvents (water, ethanol, methanol) in different proportions (1:1; 1:3; 1:5 m/v). After vortexing vigorously for 1 minute, extracts were placed on an orbital shaker (Heidolph Instruments GmbH, Germany) for 2 days under continuous shaking, in the dark, at room temperature (RT). After this maceration, samples were centrifuged two times (15 min, 12000 rpm, 4°C) using an Eppendorf centrifuge (Germany). The supernatant was transferred into a clean tube and stored at 4 °C.

Evaluation of Total Phenolic Content

The classical Folin-Ciocalteu method was used for the total phenolic content estimation (Stankovic et al., 2012). A calibration curve with gallic acid between 10-100 µg/mL ($R^2 = 0.9968$) was made and the results were calculated as mg of gallic acid equivalents (GAE)/g of callus DW.

Evaluation of Flavonoid Content

A protocol with aluminium chloride adapted from Cai et al. (2010) was used for flavonoid concentrations' estimation. The results were calculated by extrapolation on a standard curve with rutin between 100-1000 µg/ml ($R^2 = 0.9996$) and expressed as mg of rutin equivalents (RE)/g of callus DW.

Evaluation of Antioxidant Activity Through DPPH Assays

The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) method was applied according to Marxen et al. (2007). Results were calculated as mM of Trolox equivalents (TE)/g of callus DW, according to a standard curve between 50-150 µg Trolox/ml (R2 = 0.9873).

For all colorimetric determinations, a Spectronic Helios Gamma UV-Vis spectrophotometer (Thermo Fisher Scientific) was used and biochemical determinations were performed in triplicate.

Evaluation of flavonoids and phenolic acids production by HP-TLC

The methanolic extracts were separated using HP-TLC system (CAMAG, Muttenz, Switzerland) on glass TLC silica gel 60 F_{254} plates. Two mobile phases were used: one for phenolic acids (A) ethyl acetate: acetic acid: formic acid: water (100:10:10:18, v/v) and one for flavonoids (B) chloroform: ethyl acetate: acetone: formic acid (40:30:20:10, v/v), according to Jesionek et al. (2015). The plates were visualized under UV light (254 nm and 365 nm). The standards caffeic acid, gallic acid, chlorogenic acid, salicylic acids, cinnamic acid, maslinic acid, ursolic acid, catechin, proanthocyanin B1, resveratrol, rutin, quercetin, myricetin 3-O-glucoside and kaempferol 3-O-glucoside were purchased from Sigma and Extrasynthesis. The flavonoids' separation was highlighted with 1% NP reagent combined with 5% PEG 4000 solution. For the assessment of antioxidant activity, a 0.2% methanolic DPPH solution was sprayed onto the plate.

Statistical analysis

All the experiments consisted of 3 to 9 replicates for biochemical and gravimetrical parameters. The experimental data were subjected to normality and homogeneity of variance tests, after which a one-way analysis of variance (ANOVA) was performed, to assess the statistical significance. To determine the differences between means, we used the Tukey HSD test. All analyses were performed using MS Excel (Microsoft) and Origin, Version 2025 (OriginLab Corporation, Northampton, MA, USA). The results were reported as means ± standard deviation (SD) and *p*-values $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) were considered statistically significant.

RESULTS AND DISCUSSIONS

In the last decades, plant cell and tissue culture techniques, specifically biotechnologies applied for the elicitation of biologically active compounds biosynthesis, have proven to be valuable tools for the study of secondary metabolite production in plants.

In our callus induction experiment from leaf fragments, primary calli were obtained and multiplied by successive subcultivations on the same type of medium. Subsequently, a callus line was selected (Figure 1), which showed high metabolic performances, especially flavonoids, in concentration of 19.21±2.75mg RE/g FW, a rich content of total polyphenols of 2.57±0.05

mg GAE/g FW and a high antioxidant activity of 18.47±1.96 mM TE/g FW.

Figure 1. Callus initiated from leaf explants of *M. officinalis*

Our callus tissues had a high content of water over 95% and by expressing the metabolite concentrations as g dry weight, our ethanolic extracts of lemon balm callus presented increased values. The flavonoid concentration was 626±89.84 mg RE/g DW, clearly superior to those presented in literature: 25.8±6.26 mg RE/g in extracts from plants from the Southeast region of Romania (Spiridon et al., 2011); 12.5± 2.11 mg RE/g DW in commercial plants from Iran (Moradi et al., 2016); 57.76 mg RE/g in *M. officinalis* leaves from Bulgaria (Koleva et al., 2021). Using other standards, other authors reported flavonoid concentrations of 72.28 mg quercetin equivalents (QE)/g DW for plants yield in farms from Egypt (Hassan et al., 2019); 12.65±0.66 mg QE/g DW for methanolic extracts of *M. officinalis* leaves from southern Bulgaria (Dogan et al., 2021) or 45.71 ± 0.40 mg catechin equivalents (CE)/g DW for plants harvested from the Macedonian area (Tusevski et al., 2014). Our callus ethanolic extracts displayed an antioxidant activity of 610.43±22.70 mM TE/g DW, which can be compared with the value of 406.03 ± 13.57 µmol TE/g DW obtained in methanolic plants extracts by Tusevski et al. (2014).

The increased concentration of flavonoids and enhanced antioxidant activity negatively impacted the growth of the callus culture, therefore optimization of culture conditions was necessary. The administered hormonal balance was modified by increasing the auxin concentration from 0.3 to 1 mg/l 2,4-D to promote cell proliferation, but also the casein hydrolysate concentration from 0.2 to 0.3 mg/l.

Subsequently, the methanol extracts, obtained from the optimized callus line, were compared with the leaf, stem or root extracts coming from potted plants grown in greenhouse conditions. The concentrations of polyphenols and flavonoids and the antioxidant activity of the callus extracts were significantly superior to potted plants' extracts (Figure 2).

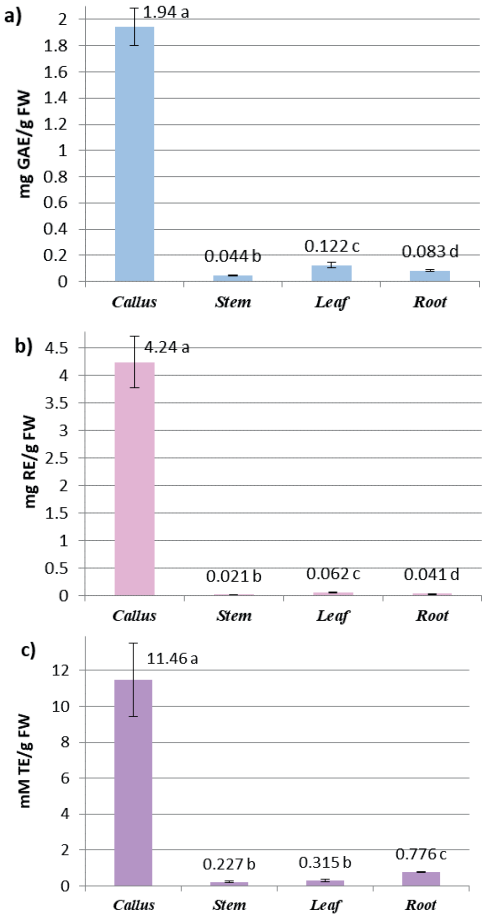

Figure 2. The total polyphenolic content (a), flavonoid concentration (b) and antioxidant activity (c) in methanolic extracts of *M. officinalis*. The data are presented as mean ± SD. Values with different letters are significantly different ($p < 0.001$).

Two years post-initiation, the optimized callus line (Figure 3) was subjected to a new extraction with different solvents (water, methanol and ethanol) to determine the levels of polyphenols and flavonoids and the antioxidant activity.

Figure 3. Optimized callus culture of *M. officinalis*

For the extraction of phenolic compounds, the best results were obtained in methanol, while for flavonoids, the ethanol extraction proved to be more advantageous (Figure 4).

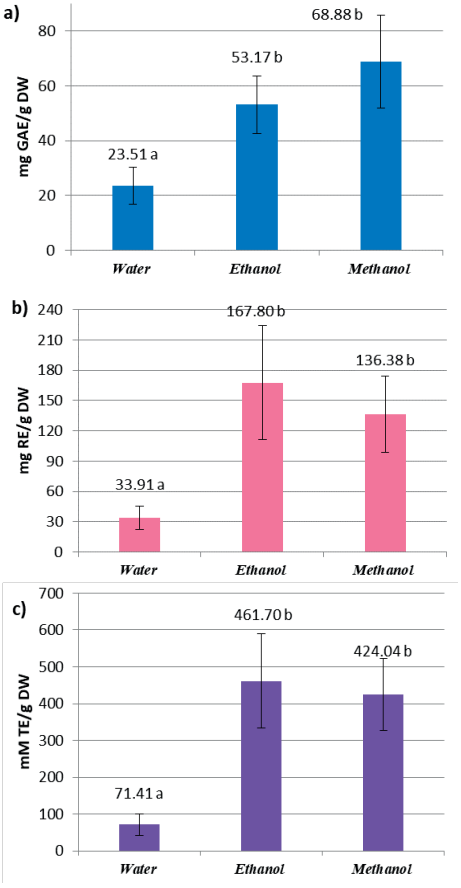

Figure 4. The total polyphenolic content (a), flavonoid concentration (b) and antioxidant activity (c) in different types of extracts from *M. officinalis* calli. The data were recorded at the end of the subcultivation period (3 weeks) and are presented as mean ± SD. Values with different letters are significantly different ($p < 0.01$)

The aqueous extract registered the weakest values. Moreover, the literature specifies that due to the high content of constituents with phenolic and flavonoid origin, extraction in ethanol, methanol and water would be recommended for *M. officinalis* extracts, as compared to acetone or hexane (Mabrouki et al., 2018; Stini et al., 2024). Hydroalcoholic solutions of methanol and ethanol proved to be much more efficient in the extraction of phenolic acids, in particular for rosmarinic acid, the main compound of *M. officinalis* extracts, compared to water (Angelov & Condoret, 2007; Wang et al., 2004; Duda et al., 2015).

The methanolic extracts presented a high concentration of polyphenols (68.88±16.98 mg GAE/ g DW), which is comparable to the values reported in other studies: 54.9±2.14 mg GAE/ g (Spiridon et al., 2011); 71. 02 mg GAE/ g DW (Hassan et al., 2019); 70.86 mg GAE/ g DW (Tusevski et al., 2014); 51±0.5 mg GAE/ g (Alizadeh Behbahani & Shahidi, 2019); 63 mg GAE/g dry ethanolic extract (Mambrouki et al., 2018) or with the results obtained by Koleva et al. (2021) in *M. officinalis* leaf extracts (65.17 mg GAE/g).

Depending on the extraction method used, other authors (Manolescu et al., 2022) reported, in a recent study, concentrations between 24.20-70.07 mg GAE/g, the highest values being obtained by using the microwave-assisted extraction technique, while by maceration, as in our study, a total polyphenol content of maximum 32.26±0.26 mg GAE/g was obtained. A polyphenol content ranging from 18.17±0.04 to 64.17±052 mg GAE/g was also reported by Petkova et al. (2017), using the infusion technique from lemon balm plants cultivated in Bulgaria.

Lower polyphenol concentrations were registered by other authors using water decoction and infusion: 43.51±1.50, respectively 27.17±0.51 mg GAE/g of DW (Popova et al., 2016) or between 32.86- 53.99 mg GAE/g DW by using ultrasound-assisted extraction in ethanol-water solvent (Manolescu et al., 2022).

However, higher concentrations were estimated in the literature for *M. officinalis* extracts, reaching to 184.33±0.5 mg GAE/g (Dogan et al., 2021) or over 200 mg GAE/g in the case of methanolic extracts reported by Moradi et al. (2016) or Papoti et al. (2019). Possible explanations for the large differences in the polyphenol

contents shown previously are: the techniques and extraction parameters;the harvesting period of the plant material; the drying protocol of the plants; the geographical area of cultivation, but also the characteristics of the plant material (Farhat et al., 2014; Sellami et al., 2012; Dent et al., 2017). Moreover, a recent study showed the difference in the production of polyphenols in two ecotypes of *M. officinalis* from Iran, Esfahan and Ilam, which had different biosynthetic capacities and the different responses to elicitation with methyl jasmonate (Kianeresi et al., 2022).
Although the metabolite concentrations of were lower in the optimized culture than in initial phases (Table 1), the advantage of producing plant biomass with a high concentration of metabolites supports a high production yield through *in vitro* callus culture of *M. officinalis* and stimulation with different elicitors would be inadvisable for this, already performing culture.

Table 1. Comparison of metabolites' accumulation in *M. officinalis* callus culture over time. The data were recorded at the end of subcultivation period (3 weeks) and are presented as mean ± SD. Values from the same column are significantly different ($p < 0.001$)

Callus type	Total phenol content (mg GAE/ g callus DW)	Flavonoid content (mg RE/ g callus DW)	Antioxidant activity (mM TE/g callus DW)
Initial	83.83±5.85[a]	626.06±89.84[a]	610.43±22.70[a]
Optimized	68.88±16.98[b]	167.80±56.76[b]	461.70±128.55[b]
Long term	11.14±1.28[c]	16.09±6.07[c]	19.57±7.19[c]

This culture, efficient in the synthesis of polyphenols and flavonoids, was maintained in the IBB *in vitro* cultures collection for a period of 12 years, but recent determinations in the metabolite's contents have demonstrated the loss of biosynthetic performances. Moreover, the culture, also, showed a different morphological aspect (Figure 5), being white with dehydration zones, clearly differing from the white-brownish appearance of the optimized culture.
Through multiple and repeated subcultivations, the callus lost their biosynthetic performances, maintaining the proliferative capacity, the growth rate varying between 17.5-24.78 after 3 weeks of cultivation.
HP-TLC analyses of *M. officinalis* callus extracts highlighted its capacity to synthesize compounds belonging to the phenolic acid class, as well as flavonoids (Figure 6).

Figure 5. Long-term callus culture of *M. officinalis*

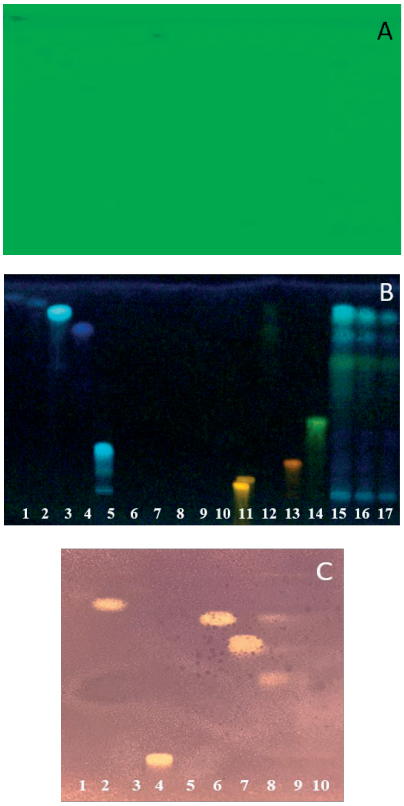

Figure 6. HP-TLC plates of *M. officinalis* callus methanolic extracts migrated in elution system B and visualized under UV light 254 nm **(a)**, derivatized with NP reagent under 366 nm fluorescent lamp (**b**) with lanes (1 – vanillin; 2 – resveratrol; 3- caffeic acid; 4 – gallic acid; 5 – chlorogenic acid; 6 – salicylic acid; 7 – trans cinnamic acid; 8- maslinic acid; 9 – ursolic acid; 10 – catechin; 11 – rutin; 12 – quercetin; 13 – myricetin-3-O-glucoside; 14 – kaempferol 3-O-glucoside; 15-20 – callus extracts) and stained with DPPH reagent for antioxidant activity testing **(c)** with lanes (1– vanillin; 2 – resveratrol; 3 – salicylic acid; 4 – chlorogenic acid; 5 – trans cinnamic acid; 6 – caffeic acid; 7 – gallic acid ; 8-10 – callus extracts)

Among the phenolic acids, the presence of caffeic acid was detected both at 254 nm and 366 nm and post exposure to the NP reagent it displayed a turquoise fluorescence. On TLC plates stained with DPPH solution, its antioxidant activity was also highlighted. Chlorogenic acid was identified on fluorescent TLC plates at 254 and 366 nm, but the staining with the NP and DPPH reagents was weak, this compound probably being present in small quantities. Gallic acid was highlighted at 254 nm and on plates stained with DPPH, revealing a weak antioxidant activity. Among flavonoids, kaempferol 3-O-glucoside was identified both at 254 nm and by staining with the NP reagent and, also, traces of rutin and myricetin 3-O-glucoside. For the flavonoids separated in TLC, no antioxidant activity was determined. The other flavonoid compounds with antioxidant activity remained unidentified due to the lack of standards.

As shown in literature, the main components of *M. officinalis* extracts are phenolic and cinnamic acids (Stini et al., 2024). The most commonly identified phenolic acids were protocatechuic acid, gallic acid and its dimer ellagic acid. Among the cinnamic acids, cinnamic, p-coumaric, caffeic, caftaric and ferulic acids were described. In fact, a significant category in lemon balm is represented by caffeic acid derivatives such as caffeoylquinic esters or caffeoyl-3,4-dihydroxyphenyllactic acid, which form chlorogenic and respectively, rosmarinic acids. Rosmarinic acid is considered the main major compound of lemon balm extracts (Ieri et al., 2017; Kianeresi et al., 2022). Among the flavonoids, quercetin, luteolin, apigenin, hesperin, hesperetin, naringenin, rutin, myricetin (Stini et al., 2024), as well as several glycosylated derivatives including kaempferol 3-O-glucoside (Kuo et al., 2020) were identified. In lemon balm from France, the major flavonoid found was luteolin 3-O-glucoside (Heitz et al., 2000).

Among the compounds identified by in our study, caffeic acid possesses anti-inflammatory, antioxidant and immunomodulatory effects (Anwar et al., 2012), while chlorogenic acid can be an efficient antioxidant, anti-inflammatory, hepatoprotective, cardioprotective, antidiabetic, and antihypertensive agent (Santana-Galvez et al., 2017; Naveed et al., 2018).

CONCLUSIONS

Our results showed a special capacity for the synthesis of secondary metabolites in the first phases of callus culture initiation. The biosynthesis performances were comparable or superior to the results reported in the literature and significantly increased compared to plants grown in pots.

After optimizing the culture growth conditions, the synthesis performances decreased, being in balance with the proliferation and production of callus biomass. The long-term culture lost its biosynthesizing performances, while it maintained the proliferation capacity.

The best solvent proved to be methanol, for the extraction of polyphenols and ethanol, for the extraction of flavonoids.

The present study demonstrates the efficiency of *in vitro* culture techniques, as alternative methods for the production of useful compounds in *M. officinalis*.

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