



Effects of the extraction techniques on the chemical composition and bioactive properties of lemon balm (*Melissa officinalis* L.) plants grown under different cropping and irrigation regimes

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ARTICLE INFO

Keywords:

Organic cultivation
Deficit irrigation
Bioactive potential
Phenolic compounds
Maceration
Infusion
Ultrasound assisted extraction
Antimicrobial activities

ABSTRACT

The present study aims to determine the combined effect of cropping system and irrigation regime on the chemical composition and bioactive properties of lemon balm aerial parts. For this purpose, lemon balm plants were grown under two farming systems (conventional farming vs organic farming) and two irrigation levels (full irrigation vs deficit irrigation) and harvested twice throughout the growing period. The collected aerial parts were subjected to three different methods of extractions, namely infusion, maceration and ultrasound-assisted extraction and the obtained extracts were evaluated in terms of chemical profile and bioactivities. Five organic acids with varied composition among the tested treatments were identified in all the tested samples for both harvests, namely, citric, malic, oxalic, shikimic and quinic acid. Regarding phenolic compounds composition, the most abundant ones were rosmarinic acid, lithospermic acid A isomer I and hydroxylsalvianolic E, especially for the maceration and infusion extraction methods. Full irrigation resulted in lower EC₅₀ values than deficit irrigation only in the treatments of the second harvest, while variable cytotoxic and anti-inflammatory effects were recorded in both harvests. Finally, in most cases the lemon balm extracts has similar or better activity than the positive controls, while the antifungal activity of lemon balm extracts was stronger than the antibacterial effects. In conclusion, the results of the present study showed that the implemented agronomic practices, as well as the extraction protocol may significantly affect the chemical profile and bioactivities of lemon balm extracts, suggesting that both the farming system and the irrigation schedule may improve the quality of the extracts depending on the implemented extraction protocol.

1. Introduction

Lemon balm (*Melissa officinalis* L.) is a medicinal species commonly cultivated throughout the world (Bonacina et al., 2017). Its above-ground parts are used for flavoring purposes, while its content in

bioactive compounds make it possible to use this matrix for the treatment of various disorders and chronic diseases (Barros et al., 2013; Bukvicki et al., 2020; Guran et al., 2021; Kim et al., 2020). The main compounds associated with the recorded health effects include various phenols, essential oils, monoterpenes and sesquiterpenes (Chrysargyris,

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Petropoulos, & Tzortzakis, 2022; de Assis et al., 2020; Németh-Zámbori et al., 2016).

The current climate change and the excessive use of natural resources have made it necessary to re-evaluate the existing farming systems and agronomic practices aiming to shift to alternative and more sustainable techniques. The commercial cultivation of medicinal plants is very promising, since it allows the exploitation of arable arid and semi-arid lands that are usually not suitable for conventional food crops due to high input requirements, while at the same time it may protect medicinal species from irrational harvest that put them under genetic erosion threat (Ahuja & Mohan, 2016). For this purpose, organic farming is a highly appreciated system due to its low inputs and the rational use of resources compared to conventional and intensified cropping (Malik, Suryapani, & Ahmad, 2011).

Considering that for most of the cultivated medicinal plants there are no officially registered pesticides, chemical pest control and weed management, and it is not always possible to implement alternative non-chemical methods (Malik et al., 2011; Raei & Alami-milani, 2014). Moreover, organic farming is reported to affect several quality parameters of medicinal plants, allowing the production of tailor-made products based on the specific market needs and concerns (Hallmann & Sabała, 2020; Lv et al., 2012). In the case of lemon balm, several studies have reported the potential of organically farming for this particular species where any losses in fresh herb biomass could be compensated by the higher quality of the final product (Ievinsh, Andersone-Ozola, & Zeipiņa, 2020; Mihajlov, Ilieva, Markova, & Zlatkovski, 2013; Seidler-Łożykowska et al., 2015).

On the other hand, the shortage of irrigation water, especially in semi-arid and arid regions of the world, makes it necessary to shift to sustainable irrigation techniques such as deficit irrigation, aiming to achieve the highest water use efficiency without negatively affecting the crop yield and the quality of the marketable product (Feres & Soriano, 2007; Geerts & Raes, 2009; Mushtaq & Moghaddasi, 2011; Ouda, Zohry, & Noreldin, 2020). In this context, medicinal plants are suitable crops to implement such practices due to their low input requirements and their resilience to abiotic stress factors such as water stress (Rajeswara Rao, 2016). However, except for the effects that cultivation practices may have on plant development and crop yield, a high impact is also expected on metabolic and physiological processes that may alter the chemical composition of plants and eventually their bioactive properties (Abbaszadeh, Farahani, & Morteza, 2009; Moradkhani et al., 2010; Petropoulos, Daferera, Akoumianakis, Passam, & Polissiou, 2004; Petropoulos, Daferera, Polissiou, & Passam, 2009). Therefore, the bioactive compounds content in lemon balm is highly affected by stress factors such as water and salinity stress (Bonacina et al., 2017; Said-Al Ahl, Sabra, Gendy, & Astatkie, 2018), while the cropping system (de Assis et al., 2020) and pre- and post-harvesting practices (Antal, Chong, Law, & Sikolya, 2014; Said-Al Ahl et al., 2018) may also alter the chemical profile of the essential oils of the plant. These effects could be crucial for the quality of the final product, since specific compounds such as citronellal, geraniol and nerol may cause oral toxicity when their content exceeds specific amounts (Stojanović et al., 2019). Therefore, further research is needed to fine tune those cultivation practices that may improve the quality of the marketable product.

However, except for the quality of the raw material, the extraction protocol may also affect the chemical profile and the bioactivities of the final product of medicinal plants. So far, various extraction methods have been suggested including traditional processing such as maceration, decoction, cooking and infusion or novel techniques such as the ultrasound- and microwave-assisted extraction among others (Gonçalves, Gomes, Costa, & Romano, 2013; Guran et al., 2021; Hawrylak-Nowak et al., 2021; Pavlović et al., 2021). Moreover, other factors associated with the extraction conditions and the extracted plant part may affect the recovery efficiency of bioactive substances and as a consequence the bioactive properties of the extract (Morsli, Grigorakis, Halahlah, Poulianiti, & Makris, 2021; Sharma et al., 2021). Therefore,

depending on the end use of the extract and the desired final composition various methods have to be tested to optimize and adjust the extraction protocol accordingly (Li, Jiang, & Wong, 2007; Rasul, 2018; Sultana, Anwar, & Ashraf, 2009).

Considering the increasing needs for irrigation water in the Mediterranean basin, the current study was designed to compare two irrigation systems, namely full and deficit irrigation, under two farming systems (conventional and organic farming) aiming to reveal the effects that these agronomic factors may have on the chemical profile and the bioactive properties of lemon balm. Another aim of this study was to suggest environmentally friendly irrigation regimes to the cultivation of MAPs, using lemon balm as a model crop due to its wide cultivation in Cyprus and other Mediterranean countries, without compromising the quality of the final product. Furthermore, the collected aerial parts were subjected to three extraction processes (infusion, maceration and ultrasound assisted extraction protocols) to identify the effect the extraction method may have on the bioactivities of the marketable product, thus increasing the added value of the crop. Considering that most of the studies regarding the lemon balm quality refer to the cropping system and the irrigation regime or the extraction protocol, the novelty of this study is the evaluation of the combined effects of different farming systems under different irrigation regimes and extraction methods.

2. Materials and methods

2.1. Plant material and growing conditions

The experiment was performed during spring-summer of 2018 in Limassol, Cyprus. Seedlings of lemon balm (*Melissa officinalis*) were obtained from the Cypriot National Centre of Aromatic Plants in seed trays, when they reached the stage of 3–4 leaves and a height of 4–5 cm. The experimental conditions and the layout have been previously described in detail by Chrysargyris et al. (2022). The experiment was conducted for approximately four months. The experimental treatments with three replicates of 30 plants (360 plants in total) each included: i) Conventional cropping with full irrigation (CF), ii) Conventional cropping with deficit irrigation (CD), iii) Organic cropping with full irrigation (OF), and iv) Organic cropping with deficit irrigation (OD). The cultivation practices implemented and the growing conditions during the experiment have been described in detail by Chrysargyris et al. (2022).

Irrigation schedule was programmed according to the methodology of Chrysargyris et al. (2022). In brief, irrigation was scheduled approximately every 4–5 days. All plants received full irrigation for two months and then deficit irrigation treatments were applied for three further weeks and until the 1st harvest (May 2018). The same irrigation schedule was implemented for three more weeks and up to the 2nd harvest (June 2018), applying full irrigation after the 1st harvest for plant regrowth and then subjecting the corresponding plants to the same deficit irrigation conditions. Plants of full irrigation treatments received full irrigation throughout the experimental period.

Harvests took place by removing the aboveground parts of the plants. The yield was determined by measuring the fresh weight of each plant and then extrapolating to the number of plants per hectare, while the dry matter content was recorded after drying fresh samples at 72 °C in a forced-air oven until weight was constant. Chemical analyses were performed in triplicate in batch samples of fresh samples of leaves from each treatment.

2.2. Extracts preparation

To prepare the extracts, three different methods of extractions, namely infusion (I), maceration (M) and ultrasound assisted (U). In particular, the following conditions were applied for each extraction method:

2.2.1. Infusion (I)

In this technique, the water was heated to boiling temperature (100 °C) and mixed with the ground plant material in the proportion of 1 g/200 mL (sample/water) and left for 5 min (Guimarães, Barros, Dueñas, et al., 2013). Afterwards, the obtained solution was filtered using a filter paper (Whatman n°4), and further freeze-dried (-47 °C, 0.045 bar, FreeZone 4.5, Labconco, Kansas City, MO, USA).

2.2.2. Maceration (M)

Maceration was performed according to Lopes et al. (2018), using an ethanol/water solution as solvent (80:20 v/v) which was added to the sample at a ratio of 1 g/30 mL and placed under continuous stirring for 1 h, followed by filtration using a filter paper (Whatman n° 4). Then, the residue was re-extracted with 30 mL of the same solution. The hydro-ethanolic fractions were subjected to evaporation using a rotary vacuum evaporator (Büchi, R-210, Flawil, Switzerland) and the remaining aqueous fractions were freeze-dried.

2.2.3. Ultrasound assisted extraction (U)

The grounded samples were extracted using standard conditions, namely 33.0 ± 3.2 min, 371.7 ± 19.3 W and $39.9 \pm 1.4\%$ ethanol, using 1.5 g of sample for 50 mL of solution. After the extraction, the solutions were filtered through a Whatman No. 4 paper and subsequently the ethanol was removed through a rotary evaporator, and the obtained extracts were freeze-dried (Roriz, Barros, Carvalho, Santos-buelga, & Ferreira, 2014).

2.3. Chemical characterization

2.3.1. Organic acids

Organic acids composition was evaluated by ultra-fast liquid chromatography (UFLC) coupled to a photodiode array detector (PDA) with prior extraction using 4.5% (w/v) metaphosphoric acid in 1.5 g of freeze-dried sample (Pereira, Barros, Carvalho, & Ferreira, 2013). For the quantification of the detected compounds, calibration curves were prepared from commercial standards (e.g. ascorbic, citric, oxalic, quinic malic, and fumaric acid), while the results were presented in g/100 g dw.

2.3.2. Phenolic compounds

The implemented protocol and equipment were previously described by Bessada, Barreira, Barros, Ferreira, & Oliveira (2016), where the dried extracts (~10 mg) from the different extraction conditions were dissolved in 2 mL of 20:80 (v/v) ethanol/water solution and filtered through 0.22 µm disposable filter discs. To quantify the phenolic compounds, the following calibration curves were used: A - Rosmarinic acid (280 nm) $y = 191291x - 652903$, $R^2 = 0.999$, Linearity = [2.5–80], LOD (Limit of Detection) = 2.51, LOQ (Limit of quantification) = 7.59 µg/mL; and B - Rosmarinic acid (370 nm) $y = 325364x - 1E + 06$, $R^2 = 0.9986$, Linearity = [2.5–80], LOD = 4.42, LOQ = 13.39. The results are expressed as mg/g extract.

2.4. Bioactive properties

2.4.1. Antioxidant activity

2.4.1.1. Thiobarbituric acid reactive substances (TBARS). The TBARS assay was carried out according to procedure described by Lockowandt et al. (2019). Briefly, a solution of porcine brain cells was incubated with the sample plus FeSO₄ and ascorbic acid at 37 °C for 1 h. Trichloroacetic acid and thiobarbituric acid were added and further heated at 80 °C for 20 min. Afterwards, a centrifugation step was applied and the color intensity of the malondialdehyde (MDA)-TBA complexes that were created in the system was recorded at 532 nm. The results were presented as IC₅₀ values (µg/mL) (Lockowandt et al., 2019).

2.4.1.2. Cellular antioxidant activity (CAA). The cellular antioxidant activity (CAA) was assessed in a murine macrophage cell line (RAW 246.7) through a methodology previously described by Albuquerque et al., (2023). The assay aimed to evaluate the extracts' ability to prevent the oxidation of intracellular 7'-dichlorodihydrofluorescein (DCFH). Quercetin served as the positive control, while 7'-dichlorodihydrofluorescein and Dulbecco's modified Eagle's medium (DMEM) were utilized as negative controls. The results were reported as the percentage of inhibition of oxidation at the highest concentration tested (2000 µg mL⁻¹).

2.5. Cytotoxic and anti-inflammatory activities

2.5.1. Cytotoxicity

The obtained extracts were analyzed against four tumor cell lines obtained from the Leibniz-Institut DSMZ: AGS, Caco-2, MCF-7, and NCI-H460 (Guimarães, Barros, Calhella, et al., 2013). Regarding non-tumor cells, a primary non-tumor cell line from porcine liver (PLP2) was studied. The cells were washed with Hanks' Balanced Salt Solution (HBSS) with 100 U/mL penicillin and 100 µg/mL streptomycin, to obtain 1x1 mm³ explants. Cells were sub-cultured and plated in a 96-well microplate at a density of 1.0×10^4 cells/well and the evaluation was possible using the Sulforhodamine B method (Sigma- Aldrich, St. Louis, MO, USA). Absorbance reading was achieved at 540 nm and the positive control used was ellipticin. The results were relevant when it was possible to obtain extract concentrations capable to inhibit 50% of cell growth (GI₅₀: µg/mL).

2.5.2. Anti-inflammatory activity

Macrophage cell line RAW 264.7 obtained from mouse was cultured in Dulbecco's modified eagle's medium (DMEM) fortified according to the protocol previously described by Souza et al. (2015). The results were presented as EC₅₀ values (µg/mL), which correspond to the concentration of the sample that provides 50% inhibition of nitric oxide (NO) production. Dexamethasone (50 µM) was the applied positive control.

2.6. Antimicrobial activity

2.6.1. Antibacterial potential

The antibacterial analyses were performed against several pathogenic bacterial strains including Gram-negative strains: *Escherichia coli* (ATCC 25922), *Salmonella Typhimurium* (ATCC 13311), *Enterobacter cloacae* (ATCC 35030); and Gram-positive strains: *Staphylococcus aureus* (ATCC 11632), *Bacillus cereus* (clinical isolate), and *Listeria monocytogenes* (NCTC 7973), using the microdilution method, according to Reis et al. (2014). The microorganisms were provided by the Laboratory of Mycology, Department of Plant Physiology, Institute of Biological Research "Siniša Stanković", University of Belgrade, Serbia. The MIC (minimum inhibitory concentration), and MBC (minimum bactericidal concentration) were determined for each sample in comparison with commercial food additives considered as positive controls (sodium benzoate (E211) and potassium metabisulfite (E224)); Dimethyl sulfoxide (DMSO; 5%) was used as the negative control. The results are presented in mg/mL.

2.6.2. Antifungal potential

Antifungal activity was evaluated through the microdilution method according to Heleno et al. (2013). For the antifungal bioassays, six mycomycetes were used: *Aspergillus fumigatus* (ATCC 9197), *Aspergillus versicolor* (ATCC 11730), *Aspergillus niger* (ATCC 6275), *Aspergillus ochraceus* (ATCC 12066), *Penicillium funiculosum* (ATCC 36839) and *Penicillium verrucosum* var. *ciclopium* (food isolate). The organisms were obtained from the Laboratory of Mycology, Department of Plant Physiology, Institute of Biological Research "Siniša Stanković", Belgrade, Serbia. The MIC were determined as also the MFC (minimum fungicidal

concentration) that corresponds to the lowest concentration with no visible growth, indicating 99.5% killing of the original inoculum, expressed in mg/mL.

2.7. Statistical tools

Chemical profile and bioactivities were determined in three replicates for each treatment and each analysis was performed three times. Data were analyzed with Statgraphics 5.1. plus (Statpoint Technologies, Inc., Warrenton, VA, USA) and a four-way ANOVA was applied to determine the effect of Cropping system (C), Irrigation regime (I), Extraction method (E), and Harvesting (H). In the case of organic acids, a three-way ANOVA was performed (Cropping system (C), Irrigation regime (I), and Harvesting (H)), since only one extract was analyzed due to protocol restrictions. The tested factors showed significant interactions, while the comparison of means was performed with the Tukey's honestly significant difference (HSD) test ($p = 0.05$).

3. Results and discussion

The multifactor analysis showed a significant interaction among the tested factors for all the determined parameters, except for the case of cytotoxic effects against NCI-H640 cells (see [Supplementary Tables S1-S4 in the supplementary material](#)).

3.1. Organic acids composition

The organic acids composition in relation to the cropping system, irrigation method, extraction protocol and harvesting time of lemon balm aerial parts is presented in [Table 1](#). Five organic acids were detected in all the studied samples for both harvests, namely, citric, malic, oxalic, shikimic and quinic acid, while fumaric acid was identified in traces. Moreover, a varied composition was reported depending on the growing condition (e.g. cropping system and irrigation regime).

In particular, quinic acid was the most abundant one, especially in the first harvest for the conventional cropping system (C) under deficit irrigation (16.4 g/100 g dw). Oxalic and malic acids were also detected in the highest amounts in plants grown under conventional cultivation (O) in both harvests. However, deficit irrigation was the most beneficial for oxalic acid under deficit irrigation, regardless of the harvest (2.62 g/100 g dw and 2.94 g/100 g dw in the first and second harvest, respectively), whereas malic acid was the highest under deficit and full irrigation in the first and second harvest (2.3 g/100 g dw and 2.52 g/100 g dw, respectively). On the other hand, shikimic and quinic acids were benefited by organic cultivation in both harvests either under full or deficit irrigation. In particular, the highest amounts of shikimic acid

were recorded for the organic farming under deficit irrigation in the first harvest (12.3 g/100 g dw) or for the organic farming (regardless of the irrigation regime) in the second harvest (11.07–11.38 g/100 g dw).

Citric acid was the richest in the case of OF treatment in the first harvest (2.3 g/100 g dw), and in OD treatment in the second harvest (1.55 g/100 g dw). Finally, total organic acids content was the highest for the OD treatment in both harvests (22.9 g/100 g dw and 23.1 g/100 g dw, for the first and second harvest respectively), while organic farming resulted in higher amounts of total organic acids for both irrigation regimes, especially in the second harvest.

Similarly to our study, [Ertas & Yener \(2020\)](#) suggested that quinic acid was the richest non-phenolic organic acid in herbal teas of lemon balm obtained after maceration and extraction using various solvents (acetone, methanol, petroleum ether and water), especially in methanol extraction. A similar finding was also reported by [Yilmaz \(2020\)](#) who also identified quinic acid as the prevalent organic acid, while he suggested a variable content depending on the extraction solvent (acetone, methanol and water) with methanol giving the best results.

In contrast, [Villalva et al. \(2021\)](#) that performed a detailed analysis of bioactive compounds in lemon balm using an ultra-high resolution Quadrupole Time-of-Flight (QTOF) equipment suggested the presence of various organic acids including gluconic, quinic, tartaric, malic and succinic acids. In the current study, the effects of limited irrigation were profound for the conventional farming and the first harvest, while in organic farming water deficit resulted in slightly increased total organic acids content in both harvests. It could be assumed that organic farming improved soil physicochemical conditions in the long term, especially water holding capacity, which may alleviate the adverse effects of water restriction due to better availability of irrigation water ([Kannan, Saravanan, Krishnakumar, & Natarajan, 2018](#); [Liebig & Doran, 1999](#)). On the other hand, the increased content of organic acids under deficit irrigation of conventional farming in the first harvest could be attributed to the osmoprotective role of organic acids under stress condition ([Petropoulos et al., 2020](#); [Venekamp, Lampe, & Koot, 1989](#)). In contrast, the prolonged stress in the second harvest could induce other plant protection mechanisms against oxidation stress apart from osmoprotection ([Chaves et al., 2002](#); [Van Oosten & Maggio, 2015](#)), hence no significant differences were recorded in total organic acids amounts between the irrigation regimes in organic farming in our study.

3.2. Phenolic compounds profile

The identification parameters and the quantification of the detected phenolic compounds are presented in [Table 2](#) and [Table 3](#), respectively. Eight phenolic acids were identified in the studied samples, all being previously detected in *Melissa officinalis* L. infusions by [Barros et al.](#)

Table 1

Composition of organic acids (g/100 g dw) of lemon balm aerial parts in relation to the cropping system (Conventional: C and Organic: O) and the irrigation regime (Full irrigation: F and Deficit irrigation: D) in both harvests.

Organic Acid							
Samples	Oxalic acid	Quinic acid	Malic acid	Shikimic acid	Citric acid	Fumaric acid	Σ Organic Acids
1st harvest							
CF	1.26 ± 0.02d	8.31 ± 0.03d	0.95 ± 0.02c	0.222 ± 0.002e	0.89 ± 0.03f	tr	10.74 ± 0.01e
CD	2.62 ± 0.02b	16.4 ± 0.1a	2.3 ± 0.1b	0.22 ± 0.01e	1.04 ± 0.02e	tr	21.3 ± 0.2b
OF	0.93 ± 0.03f	10.0 ± 0.2c	0.060 ± 0.001d	10.4 ± 0.3c	2.3 ± 0.1a	tr	21 ± 1b
OD	0.65 ± 0.02 g	9.84 ± 0.31c	0.06 ± 0.01d	12.3 ± 0.1a	1.97 ± 0.04b	tr	22.9 ± 0.5a
2nd harvest							
CF	1.65 ± 0.05c	9.5 ± 0.4c	2.52 ± 0.02a	0.19 ± 0.01e	0.81 ± 0.03g	tr	13.7 ± 0.5d
CD	2.94 ± 0.02a	9.9 ± 0.1c	0.038 ± 0.001d	9.9 ± 0.3d	1.10 ± 0.04e	tr	12.9 ± 0.1d
OF	0.87 ± 0.03f	8.3 ± 0.3d	0.060 ± 0.002d	11.07 ± 0.03b	1.4 ± 0.1d	tr	20.3 ± 0.3c
OD	1.01 ± 0.04e	10.6 ± 0.3b	0.67 ± 0.01d	11.38 ± 0.35b	1.55 ± 0.03c	tr	23.1 ± 0.6a

* Equations used in the calculation of organic acids: Oxalic acid, $y = 1E + 07x + 231891$; Quinic acid $y = 671557x + 14583$; Malic acid, $y = 950041x + 6255,6$; Shikimic acid, $y = 5E + 07x + 109778$; Citric acid, $y = 1E + 06x - 10277$; Fumaric acid, $y = 1E + 08x + 614399$. Means in the same column followed by different letters are significantly different according to Tukey's HSD test at $p < 0.05$.

Table 2

Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, and tentative identification of the phenolic compounds present in the hydroethanolic extracts of lemon balm aerial parts.

Peak	Rt (min)	λ_{max} (nm)	[M-H] ⁻ (m/z)	MS ² (m/z)	Tentative identification
1	11.39	276	537	493(67), 359(17), 313(31), 295(100), 269(26), 97(20), 179(77)	Lithospermic acid A isomer I
2	15.56	322	473	311(21), 293(15), 267(<5), 179(82), 149(100), 135(30)	Caftaric acid hexoside
3	16.72	272	735	537(100), 519(21), 493(7), 357(3), 321(12), 297(12), 295(6)	Hydroxylsalvianolic E
4	19.74	325	719	539(12), 521(9), 359(100), 197(9), 179(12), 161(51), 135(5)	Sagerinic acid
5	21.47	328	359	197(98), 179(89), 161(100), 135(42)	Rosmarinic acid
6	24.15	325	537	493(100), 359(21), 197(19), 179(13), 161(35)	Lithospermic acid A isomer II
7	27.09	327	537	493(100), 359(23), 197(25), 179(12), 161(45)	Lithospermic acid A isomer III
8	28.89	324	829	667(89), 535(100), 491(15), 311(34), 293(6), 197(5), 179(11)	Salvianolic acid C derivative

(2013), while Villalva et al. (2021) reported the presence of several hydroxybenzoic and hydroxycinnamic derivatives such as rosmarinic, lithospermic, salvianolic and sagerinic acids, as well as luteolin derivatives. Varied amounts of individual compounds were recorded depending on the tested factors. In the first harvest, the richest compounds were rosmarinic acid, lithospermic acid A isomer I, and hydroxylsalvianolic E (peaks 5, 1, and 3, respectively) in all the studied samples.

The highest amounts for these compounds were recorded in samples obtained after maceration (peak 5) or after infusion (peaks 1 and 3) from plants grown under conventional farming and full irrigation in both cases. Moreover, the highest total phenolic compounds content was measured for the samples obtained after maceration due to the highest amounts of peak 4. Similar results were recorded in the second harvest regarding peak 5 and 3, while peak 1 was the highest in samples obtained after infusion from plants grown under conventional farming and deficit irrigation or under organic farming, regardless of the irrigation schedule. Similarly to the first harvest, the highest total phenolic compounds content was measured for the samples obtained after maceration due to highest amounts of peak 5. Finally, plants subjected to prolonged stress, as in the case of the second harvest, showed a higher total phenolic compounds content when grown under full irrigation regime, regardless of the farming system and the extraction method. In contrast, the samples of the first harvest and deficit irrigation, resulted in lower amounts of total phenols in plants grown under conventional farming, regardless of the extraction method, whereas contrasting trends were observed in organic farming, except for the case of ultrasound extraction

where deficit irrigation increased total phenols content.

According to Németh-Zámbori et al. (2016), the severity of water deficit was associated with increasing content of phenolic compounds in lemon balm shoots, whereas opposing trends were recorded for roots. Moreover, in the same line Chrysargyris et al. (2022) who evaluated lemon balm plants cultivated under identical conditions with the present study suggested that water stress increased total phenolic compounds, especially in the second harvest. In addition, de Assis et al. (2020) suggested that organic cultivation of lemon balm resulted in higher total phenols content which is not in agreement with the present study. Chrysargyris et al. (2019) reported significant differences in total phenols content in *Sideritis perfoliata* L. subsp. *perfoliata* among two harvestings, which was also recorded in the case of organic cultivation in the present results. These contradictions could be associated with the different methods implemented for the assessment of phenolic compounds profile, since the colorimetric assays used for total phenols assessment are less sensitive than analytical protocols (liquid chromatography) used in the present study due to interference with other antioxidant compounds (Blainsk, Lopes, & de Mello, 2013; Ferioli, Giambanelli, & Antuono, 2020). The variable results reported in the literature could also indicate the incorporation of different antioxidant systems (enzymatic or non-enzymatic) which could induce the biosynthesis of protective molecules that help plants to subsist oxidative stress (Safari, Akramian, Salehi, & Mansour, 2022). Moreover, the extraction conditions and processing of samples prior to extraction may also have an effect on bioactive compounds content as reported by Palamutoglu, Kasnak, Dursun, & Ünalı (2022) who evaluated total phenols content in ground or non-ground lemon balm tissues subjected to different infusion times and temperatures. The results reported by Ziagova, Mavromatidou, Samiotis, & Amanatidou (2022) were in the same line, since a significant impact of the extraction process on total phenols content was also suggested.

3.3. Antioxidant activity

The antioxidant activity of the obtained extracts is reported in Table 4. The obtained results showed a variation in the antioxidant effects of lemon balm in both harvests and implemented assays. In the case of the TBARS assay, the highest EC₅₀ values that indicate lower antioxidant activity were observed in the extracts after maceration of plant material obtained from OD treatment, regardless of the harvest. On the other hand, several extracts showed high antioxidant activity with EC₅₀ values that did not differ significantly with each other, with the exception of ODM sample in which all the extracts recorded lower antioxidant activity (higher EC₅₀ values) compared to the positive control. Moreover, full irrigation resulted in lower EC₅₀ values in the corresponding treatments of extraction and cropping system of the second harvest, whereas no specific trends were recorded in the first harvest. In the case of cellular antioxidant activity, the results showed a varied response for the tested extracts with higher inhibition percentages being observed in the case of ODM treatment in the first harvest and the treatments CFU, OFU and ODU in the second harvest. According to Wojciechowska, Dugosz-Grochowska, Koton, & Zupnik (2015), antioxidant activity is correlated to bioactive compounds content, especially in phenolic compounds such as rosmarinic acid which was the richest phenolic compound in the present study. However, the detected amounts of rosmarinic acid and total phenolic compounds in the tested extracts are not in accordance with the recorded antioxidant activity. Therefore, despite the well-evidenced association of phenolic compounds with antioxidant activity, the differences between the tested assays could be associated with the contribution of other bioactive compounds to the overall antioxidant capacity of the extracts, as well as with differences in the extraction protocols (Kolli, Markaki, Zoumpoulakis, & Proestos, 2016; Stagos et al., 2012). Moreover, the decrease of the TBARS antioxidant activity under organic cultivation recorded in the second harvest is in accordance with the lowest total phenolics content recorded for

Table 3

Phenolic compounds composition (mg/g extract) of lemon balm aerial parts in relation to the extraction method (Infusion: I; Maceration: M and Ultrasound-assisted: U), the cropping system (Conventional: C and Organic: O) and the irrigation regime (Full irrigation: F and Deficit irrigation: D) in both harvests.

Hydroethanolic Extrates	Phenolic Compounds								Σ Total phenolics
	1 ^A	2 ^B	3 ^A	4 ^B	5 ^B	6 ^B	7 ^B	8 ^B	
	1st harvest								
CFI	27.4 ± 0.8d	0.78 ± 0.01m	20.7 ± 0.2a	1.57 ± 0.02k	107.6 ± 1.3l	2.38 ± 0.04kl	4.2 ± 0.1l	1.63 ± 0.03ij	166.3 ± 2.5f
CDI	24.9 ± 0.3e	9.95 ± 0.04a	13.8 ± 0.1f	2.27 ± 0.05def	94.2 ± 0.4p	2.37 ± 0.03kl	4.87 ± 0.06ij	1.64 ± 0.03ij	154.02 ± 1.09hij
OFI	19.4 ± 0.1i	1.36 ± 0.01ghi	11.25 ± 0.02i	2.36 ± 0.05cd	92.6 ± 0.7p	2.60 ± 0.02j	5.68 ± 0.06e	1.07 ± 0.02l	136.3 ± 0.7m
ODI	22.15 ± 0.01gh	1.54 ± 0.01cd	9.90 ± 0.01k	2.21 ± 0.02fgh	99.8 ± 0.1n	3.12 ± 0.03ef	6.76 ± 0.05a	1.88 ± 0.04fg	147.4 ± 0.1k
CFM	11.5 ± 0.2l	1.27 ± 0.03j	10.41 ± 0.08j	2.16 ± 0.01fgh	155.7 ± 0.5b	5.28 ± 0.03a	4.72 ± 0.02j	2.21 ± 0.04c	193.3 ± 0.6a
CDM	10.8 ± 0.1mn	1.31 ± 0.05ij	6.17 ± 0.05p	2.23 ± 0.03efg	126.4 ± 0.1f	2.28 ± 0.08l	4.4 ± 0.1k	2.19 ± 0.08c	155.7 ± 0.1hi
OFM	11.1 ± 0.3lmn	1.60 ± 0.04c	8.1 ± 0.1m	2.65 ± 0.02a	135.1 ± 0.7e	2.33 ± 0.01kl	3.34 ± 0.01o	1.97 ± 0.02ef	166.2 ± 1.1f
ODM	12.75 ± 0.06k	1.18 ± 0.02k	7.6 ± 0.3n	2.50 ± 0.05b	140.9 ± 0.4d	2.94 ± 0.06gh	2.3 ± 0.1q	1.53 ± 0.09jk	171.75 ± 0.07d
CFU	22.8 ± 0.2fg	1.28 ± 0.02j	16.2 ± 0.3d	1.76 ± 0.04j	118.8 ± 0.8h	2.9 ± 0.2hi	5.67 ± 0.06e	2.48 ± 0.02b	171.9 ± 0.4d
CDU	22.2 ± 0.2g	1.32 ± 0.04hij	11.9 ± 0.1h	1.88 ± 0.01i	106.01 ± 0.01l	3.1 ± 0.1efg	5.1 ± 0.1gh	2.11 ± 0.02cd	153.52 ± 0.06ij
OFU	18.1 ± 0.3j	1.28 ± 0.05j	13.2 ± 0.1g	2.25 ± 0.07defg	124.1 ± 0.2g	2.90 ± 0.04h	4.96 ± 0.04hi	1.73 ± 0.07hi	168.82 ± 0.01
ODU	19.18 ± 0.01i	1.42 ± 0.03efg	11.4 ± 0.1i	2.22 ± 0.09fgh	40.7 ± 0.1q	3.21 ± 0.01e	3.51 ± 0.01n	1.81 ± 0.01gh	83.4 ± 0.3o
	2nd harvest								
CFI	28.8 ± 0.3c	1.44 ± 0.04ef	18.61 ± 0.01b	2.14 ± 0.07gh	109.3 ± 0.4k	2.76 ± 0.01ij	5.18 ± 0.06fg	2.54 ± 0.02b	170.8 ± 0.6de
CDI	30.2 ± 0.4a	1.52 ± 0.04cd	14.70 ± 0.02e	1.95 ± 0.08i	97.3 ± 0.3o	3.05 ± 0.05fgh	5.3 ± 0.1f	2.09 ± 0.06cde	156.1 ± 0.6h
OFI	29.91 ± 0.01ab	1.48 ± 0.01de	17.2 ± 0.5c	2.7 ± 0.1a	124.6 ± 1.3g	4.4 ± 0.1b	6.4 ± 0.01b	2.6 ± 0.1b	189.2 ± 1.7b
ODI	30.1 ± 0.1a	1.58 ± 0.05c	11.3 ± 0.3i	2.35 ± 0.04cde	36.0 ± 0.2r	3.41 ± 0.03d	4.46 ± 0.07k	2.04 ± 0.06de	91.27 ± 0.04n
CFM	11.42 ± 0.01lm	1.24 ± 0.02jk	8.87 ± 0.09l	2.11 ± 0.03h	163.7 ± 0.4a	2.35 ± 0.06kl	3.70 ± 0.03m	1.62 ± 0.03ijk	195.0 ± 0.1a
CDM	11.5 ± 0.2l	1.25 ± 0.04jk	6.8 ± 0.2o	1.90 ± 0.03i	124.70 ± 0.04g	1.95 ± 0.02m	3.48 ± 0.07no	1.51 ± 0.07jk	153.1 ± 0.1j
OFM	10.69 ± 0.08n	1.25 ± 0.03jk	8.3 ± 0.1m	2.42 ± 0.02bc	153.84 ± 0.01c	1.57 ± 0.05n	2.20 ± 0.05q	1.49 ± 0.01k	181.8 ± 0.3c
ODM	11.10 ± 0.05lmn	1.05 ± 0.01l	5.17 ± 0.09q	1.85 ± 0.01ij	116.5 ± 0.2i	1.38 ± 0.02o	1.67 ± 0.04r	1.00 ± 0.01l	139.7 ± 0.4l
CFU	25.25 ± 0.06e	1.38 ± 0.03fgh	16.06 ± 0.03d	2.23 ± 0.06fgh	111.7 ± 2.2j	3.2 ± 0.1ef	5.86 ± 0.03d	2.4 ± 0.1b	168.1 ± 1.9ef
CDU	29.3 ± 0.6bc	1.86 ± 0.05b	14.42 ± 0.01ee	2.24 ± 0.06fgh	102.4 ± 0.7m	3.6 ± 0.1c	6.1 ± 0.1c	2.73 ± 0.08a	162.6 ± 1.7g
OFU	22.9 ± 0.3f	1.47 ± 0.07de	13.38 ± 0.04fg	2.41 ± 0.08bc	116.09 ± 1.03i	2.44 ± 0.02k	3.11 ± 0.04p	2.01 ± 0.04def	163.8 ± 1.6g
ODU	21.5 ± 0.6h	1.85 ± 0.03b	9.6 ± 0.3k	2.19 ± 0.04fgh	101.3 ± 1.0mn	1.44 ± 0.02no	1.17 ± 0.03s	1.98 ± 0.08def	141.0 ± 0.9l

Calibration curves used for quantification: A - Rosmarinic acid (280 nm) $y = 191291x - 652903$, $R^2 = 0.999$, Linearity = [2.5–80], LOD (Limit of Detection) = 2.51, LOQ (Limit of quantification) = 7.59 µg/mL and B - Rosmarinic acid (370 nm) $y = 325364x - 1E + 06$, $R^2 = 0.9986$, Linearity = [2.5–80], LOD = 4.42, LOQ = 13.39. In each column means followed by different Latin letters are significantly different according to Tukey's HSD test ($p < 0.05$).

the organic cropping system. This result is in agreement with the report of de Assis et al. (2020), whereas contrasting results were reported by Hallmann & Sabała (2020) and Kazimierczak, Hallmann, & Rembialkowska (2015) who suggested an increase of antioxidant compounds under organic farming. Considering that the study of Hallmann & Sabała (2020) refers to other species, while Kazimierczak et al. (2015) did not evaluate the antioxidant activity but only the presence of antioxidant compounds could justify the contradictory results compared to the results of this study. Another possible explanation for these differences could be associated with the harvesting time, since according to Slađana & Tijana (2021) and Luță, Ghica, Costea, & Elena (2020) a diurnal and seasonal variation in the antioxidant compounds content of lemon balm should be expected.

3.4. Cytotoxic and anti-inflammatory effects

The cytotoxic and anti-inflammatory properties are presented in Table 5. Different cytotoxic effects against tumor cell lines, as well as anti-inflammatory activities were recorded in both harvests. In particular, in the first harvest the lower EC₅₀ values against AGS, Caco and MFC-7 tumor cell lines were observed for the U extracts and for the organic cultivation, either for fully irrigated (31 µg/mL; AGS cell line) or deficit irrigated plants (Caco (47 µg/mL) and MFC-7 lines (107 µg/mL)). Regarding NCI-H460 tumor cell line, the lowest GI₅₀ values (the concentration that inhibits in 50% the cells growth) were recorded for maceration of plant material obtained from conventional farming and full irrigation (185 µg/mL), while the same extract was the most

Table 4

Antioxidant activity (TBARS and Cellular antioxidant activity) of lemon balm aerial parts in relation to the extraction method (Infusion: I; Maceration: M and Ultrasound-assisted: U), the cropping system (Conventional: C and Organic: O) and the irrigation regime (Full irrigation: F and Deficit irrigation: D) in both harvests.

Hydroethanolic Extracts	Antioxidant activity TBARS EC ₅₀ Values (µg/mL)		Cellular antioxidant activity (CAA) (% of oxidation inhibition) Maximum tested concentration: 2000 µg/mL	
	1st harvest	2nd harvest	1st harvest	2nd harvest
	CFI	41.6 ± 1.5cd	4.7 ± 0.1h	0
CDI	6.0 ± 0.2h	5.6 ± 0.2h	0	0
OFI	5.8 ± 0.2h	5.5 ± 0.1h	29g	0
ODI	11.4 ± 0.5gh	8.5 ± 0.4h	21h	0
CFM	47 ± 1c	23 ± 1ef	70e	80c
CDM	9.1 ± 0.3h	44 ± 2cd	71e	83b
OFM	37 ± 2d	42 ± 2cd	65f	83b
ODM	952 ± 39a	187 ± 7b	76d	81c
CFU	6.1 ± 0.2h	6.5 ± 0.3h	0	89a
CDU	5.8 ± 0.3h	11.1 ± 0.2gh	0	84b
OFU	26.7 ± 0.3e	17.5 ± 0.4fg	0	87a
ODU	5.8 ± 0.1h	22.1 ± 0.2ef	0	88a

Positive control TBARS: Trolox = 139 ± 5 µg/mL; positive control CAA: quercetin % oxidation inhibition: 0.3 µg/mL inhibits 95%. Means of the same antioxidant activity assay for both harvests followed by different Latin letters are significantly different according to Tukey's HSD test (*p* < 0.05).

Table 5

Cytotoxic and anti-inflammatory activity (GI₅₀; µg/mL) of lemon balm aerial parts in relation to the extraction method (Infusion: I; Maceration: M and Ultrasound-assisted: U), the cropping system (Conventional: C and Organic: O) and the irrigation regime (Full irrigation: F and Deficit irrigation: D) in both harvests.

Hydroethanolic Extracts	Cytotoxicity Activity GI ₅₀ (µg/mL)					Anti-inflammatory Activity GI ₅₀ (µg/mL) RAW
	AGS	Caco	MFC-7	NCI-H460	PLP2	
Irrigation	1st harvest					
CFI	51 ± 3bcd	48 ± 2gh	290 ± 18a	224 ± 20abcde	>400	293 ± 8c
CDI	45 ± 2efgh	51 ± 5fgh	292 ± 14a	245 ± 22ab	>400	276 ± 6d
OFI	40.6 ± 0.1ghij	55 ± 4defg	287 ± 3a	216 ± 2abcde	>400	319 ± 14ab
ODI	37 ± 2jk	58 ± 2cdef	278 ± 28ab	197 ± 10cde	>400	257 ± 8.0e
CFM	40 ± 3hij	54 ± 1efgh	238 ± 11def	185 ± 13e	>400	116 ± 8h
CDM	64 ± 6.0a	57 ± 1cdef	226 ± 4efg	234 ± 14abc	>400	>400
OFM	68 ± 7.0a	64 ± 2c	238 ± 10def	223 ± 20abcde	>400	>400
ODM	54.3 ± 0.4b	54 ± 1efgh	256 ± 23bcd	224 ± 16abcd	>400	>400
CFU	39 ± 1ijk	55 ± 1defg	243 ± 2cde	211 ± 18bcde	>400	318 ± 11ab
CDU	41 ± 1ghij	54 ± 2efgh	213 ± 11fg	217 ± 21abcde	>400	>400
OFU	31 ± 2l	53 ± 5efgh	136 ± 3k	228 ± 1abcd	>400	323 ± 6.0a
ODU	44.1 ± 0.5fghi	47 ± 1gh	107 ± 5l	244 ± 14ab	>400	152 ± 10g
	2nd harvest					
CFI	34 ± 1kl	46 ± 4h	139 ± 12jk	226 ± 21abcd	>400	244 ± 11e
CDI	22.0 ± 0.3m	55 ± 4defg	266 ± 11abc	254 ± 16a	>400	192 ± 3f
OFI	22 ± 1m	58 ± 1cdef	55 ± 2m	236 ± 21ab	>400	>400
ODI	20.3 ± 0.5 m	60 ± 6cde	235 ± 6def	245 ± 24ab	>400	>400
CFM	46 ± 1defg	63 ± 4cd	169 ± 10i	190 ± 7de	>400	>400
CDM	50 ± 1bcde	78 ± 1b	199 ± 6gh	243 ± 18ab	>400	>400
OFM	48 ± 3cdef	20 ± 1i	159 ± 15ijk	212 ± 17bcde	>400	>400
ODM	45 ± 1efgh	17 ± 2i	166 ± 5j	255 ± 15a	>400	273 ± 7d
CFU	24 ± 1m	157 ± 10a	175 ± 9hi	248 ± 26ab	>400	305 ± 9bc
CDU	20 ± 2m	63 ± 4cd	183 ± 18hi	236 ± 22ab	>400	148 ± 7g
OFU	42 ± 3ghij	58 ± 3cdef	216 ± 8efg	222 ± 18abcde	>400	13 ± 2i
ODU	52 ± 1bc	54 ± 5efgh	278 ± 15ab	245 ± 21ab	>400	13 ± 2i

* Positive control ellipticine: AGS: 1.23 ± 0.03 µg/mL; Caco2: 1.21 ± 0.02 µg/mL; MFC-7: 1.02 ± 0.02 µg/mL; NCI-H460: 1.01 ± 0.01 µg/mL; PLP2: 1.4 ± 0.1 µg/mL; Anti-inflammatory Activity RAW 6.3 ± 0.4 µg/mL. In each column, means followed by different Latin letters are significantly different according to Tukey's HSD test (*p* < 0.05).

effective extract against inflammation (116 µg/mL).

In the second harvest, extraction via infusions and ultrasounds resulted in higher cytotoxicity against AGS (CDI (22.0 µg/mL), OFI (22 µg/mL), ODI (20.3 µg/mL), and CDU (20 µg/mL) treatments). The highest activity against Caco and NCI-H460 cell lines was recorded for maceration and organic farming, regardless of the irrigation regime (20 and 17 µg/mL in full and deficit irrigation, respectively; Caco cell line), or conventional farming × full irrigation treatment (CFM (190 µg/mL); NCI-H460 cell line). On the other hand, infusion × organic farming × full irrigation treatment resulted in the highest activity against MCF-7 cell line (55 µg/mL). Finally, none of the analyzed extracts presented toxicity for the non-tumor tested cell line PLP2 at the maximum tested concentration.

Based on the literature reports, methanolic extracts obtained from lemon balm have been associated with cytotoxic and antitumor effects against MRC-5 and HCT-116 cell lines (Pavlović et al., 2021), while macerated ethanolic extracts showed activity against squamous (FaDu and SCC-4) and colorectal (Caco-2 and HT-29) cancer cell lines (Guran et al., 2021). Moreover, the ultrasound-assisted ethanolic extracts of lemon balm showed significant anti-inflammatory activity by inhibiting the secretion of proinflammatory cytokines by LPS-induced THP-1 macrophages (Villalva et al., 2021). Choi et al. (2020), suggested the combined ethanolic extracts of lemon balm and dandelion had significant anti-inflammatory activity against RAW264.7 cell line and anti-tumor activity against HepG2 hepatocellular carcinoma cell line. Ethanolic extracts of lemon balm were also more effective against MCF-7 and MDA-MB-231 breast cancer cells and less effective against MCF-10A normal cell lines than methanolic extracts, despite the highest content of the latter in phytochemicals and particularly in rosmarinic and ursolic acids (Ghiulai et al., 2020). The same authors suggested that the activity of extracts is associated with a multi-targeted mechanism of action regulated by the content of multiple compounds in the extracts and not

only the main compounds (Ghiulai et al., 2020). This finding is in agreement with our study, where the treatments that resulted in the highest rosmarinic acid and total phenolic compounds content (maceration × conventional farming × full irrigation in both harvests) were not associated with the highest activity against breast cancer cell lines, although they were the most effective against NCI-H460 (both harvests) and RAW (only in the first harvest) cell lines. Moreover, Weidner et al. (2015) reported apoptotic and antiproliferative effects of ethanolic

lemon balm extracts against HT-29 and T84 colon cancer cells, while a lemon balm fermented beverage (also known as Kombucha) was effective against HeLa, MCF7, and HT-29 cancer cell lines (Četojević-Simin et al., 2012). Therefore, based on the scarce literature reports regarding the cytotoxic and anti-inflammatory effects of lemon balm extracts, it could be assumed that the effects of the extraction protocols and cultivation practices in our study could regulate the tested bioactive properties through the changes in the overall composition of extracts rather

Table 6

Antibacterial effects of lemon balm aerial parts in relation to the extraction method (Infusion: I; Maceration: M and Ultrasound-assisted: U), the cropping system (Conventional: C and Organic: O) and the irrigation regime (Full irrigation: F and Deficit irrigation: D) in both harvests.

Hydroethanolic Extracts		<i>Staphylococcus aureus</i> (ATCC 11632)	<i>Bacillus cereus</i> (clinical isolate)	<i>Listeria monocytogenes</i> (NCTC 7973)	<i>Enterobacter cloacae</i> (ATCC 35030)	<i>Salmonella</i> Typhimurium (ATCC 13311)	<i>Escherichia coli</i> (ATCC 25922)
1st harvest							
CFI	MIC	1.02	0.51	1.02	1.02	0.51	1.02
	MBC	2.04	1.02	2.04	2.04	1.02	2.04
CDI	MIC	0.50	0.25	0.50	0.50	0.50	0.50
	MBC	1.00	0.50	1.00	1.00	1.00	1.00
OFI	MIC	1.01	0.51	0.51	0.51	0.51	0.51
	MBC	2.02	1.01	1.01	1.01	1.01	1.01
ODI	MIC	1.01	0.51	0.51	0.51	0.51	0.51
	MBC	2.02	1.01	1.01	1.01	1.01	1.01
CFM	MIC	1.02	0.51	1.02	1.02	1.02	1.02
	MBC	2.04	1.02	2.04	2.04	2.04	2.04
CDM	MIC	1.00	0.50	2.01	2.01	1.00	1.00
	MBC	2.01	1.00	4.02	4.02	2.01	2.01
OFM	MIC	2.00	0.50	2.00	2.00	1.00	1.00
	MBC	4.00	1.00	4.00	4.00	2.00	2.00
ODM	MIC	1.03	0.52	1.03	1.03	1.03	1.03
	MBC	2.06	1.03	2.06	2.06	2.06	2.06
CFU	MIC	1.02	0.51	1.02	1.02	1.02	1.02
	MBC	2.04	1.02	2.04	2.04	2.04	2.04
CDU	MIC	2.03	0.51	1.02	1.02	1.02	1.02
	MBC	4.06	1.02	2.04	2.04	2.04	2.04
OFU	MIC	1.00	0.50	1.00	1.00	0.50	0.50
	MBC	2.01	1.00	2.01	2.01	1.00	1.00
ODU	MIC	1.00	0.50	1.00	1.00	1.00	1.00
	MBC	2.01	1.00	2.01	2.01	2.01	2.01
E211	MIC	4.00	0.50	1.00	2.00	1.00	1.00
	MBC	4.00	0.50	2.00	4.00	2.00	2.00
E224	MIC	1.00	2.00	0.50	0.50	1.00	0.50
	MBC	1.00	4.00	1.00	0.50	1.00	1.00
2nd harvest							
CFI	MIC	0.25	0.25	1.00	0.50	1.00	0.50
	MBC	0.50	0.50	2.00	1.00	2.00	1.00
CDI	MIC	1.00	0.50	0.50	0.50	0.50	0.50
	MBC	2.01	1.00	1.00	1.00	1.00	1.00
OFI	MIC	0.51	1.02	1.02	0.51	0.51	1.02
	MBC	1.02	2.03	2.03	1.02	1.02	2.03
ODI	MIC	1.02	0.51	1.02	1.02	1.02	0.51
	MBC	2.03	1.02	2.03	2.03	2.03	1.02
CFM	MIC	1.03	0.51	2.05	2.05	1.03	1.03
	MBC	2.05	1.02	4.10	4.10	2.05	2.05
CDM	MIC	1.02	1.02	1.02	1.02	1.02	0.51
	MBC	2.04	2.04	2.04	2.04	2.04	1.02
OFM	MIC	1.04	0.52	1.04	1.04	1.04	1.04
	MBC	2.08	1.04	2.08	2.08	2.08	2.08
ODM	MIC	1.01	0.51	2.02	2.02	1.01	1.01
	MBC	2.02	1.01	4.04	4.04	2.02	2.02
CFU	MIC	1.02	0.51	1.02	1.02	1.02	0.51
	MBC	2.04	1.02	2.04	2.04	2.04	1.02
CDU	MIC	1.00	0.50	1.00	1.00	0.50	1.00
	MBC	2.00	1.00	2.00	2.00	1.00	2.00
OFU	MIC	1.005	0.50	1.005	0.50	1.005	0.50
	MBC	2.01	1.005	2.01	1.005	2.01	1.005
ODU	MIC	1.02	1.02	1.02	0.51	1.02	0.51
	MBC	2.04	2.04	2.04	1.02	2.04	1.02
E211	MIC	4.00	0.50	1.00	2.00	1.00	1.00
	MBC	4.00	0.50	2.00	4.00	2.00	2.00
E224	MIC	1.00	2.00	0.50	0.50	1.00	0.50
	MBC	1.00	4.00	1.00	0.50	1.00	1.00

MIC- minimal inhibitory concentration; MBC – minimal bactericidal concentration. E211 - Sodium benzoate; E224 - Potassium Metabisulfite.

than the changes in the most abundant compounds such as rosmarinic acid.

3.5. Antimicrobial properties

Antimicrobial effects of the tested extracts are recorded in Table 6 (antibacterial) and Table 7 (antifungal). The antibacterial activity of the obtained extracts varied against the studied bacteria, depending on the

evaluated factors (e.g. extraction protocol, cropping system, irrigation regime, and harvesting time). The results herein indicate that in most cases the lemon balm extracts have similar or better activity than the positive controls. In particular, the extract of the first harvest obtained from the infusion of aboveground parts of plants grown with conventional cropping and deficit irrigation (CDI) were more efficient than positive control against *Bacillus cereus* (lower MIC and similar MBC values compared to E211), as well as against *Staphylococcus aureus*

Table 7

Antifungal effects of lemon balm aerial parts in relation to the extraction method (Infusion: I; Maceration: M and Ultrasound-assisted: U), the cropping system (Conventional: C and Organic: O) and the irrigation regime (Full irrigation: F and Deficit irrigation: D) in both harvests.

Hydroethanolic Extracts		<i>Aspergillus fumigatus</i> (ATCC 9197)	<i>Aspergillus versicolor</i> (ATCC 11730)	<i>Aspergillus niger</i> (ATCC 6275)	<i>Aspergillus ochraceus</i> (ATCC 12066)	<i>Penicillium funiculosum</i> (ATCC 36839)	<i>Penicillium verrucosum</i> var. <i>cyclopium</i> (food isolate)
1st harvest							
CFI	MIC	0.51	0.51	0.51	1.02	1.02	1.02
	MFC	1.02	1.02	1.02	2.03	2.03	2.03
CDI	MIC	0.50	0.25	0.25	0.50	0.50	1.00
	MFC	1.00	0.50	0.50	1.00	1.00	2.00
OFI	MIC	4.04	1.01	4.04	0.51	1.01	1.01
	MFC	8.08	2.02	8.08	1.02	2.02	2.02
ODI	MIC	1.01	1.01	0.51	1.01	0.51	0.51
	MFC	2.02	2.02	1.01	2.02	1.01	1.01
CFM	MIC	1.02	1.02	0.51	1.02	0.51	2.03
	MFC	2.03	2.03	1.02	2.03	1.02	4.06
CDM	MIC	1.00	1.00	1.00	0.50	1.00	0.50
	MFC	2.01	2.01	2.01	1.00	2.01	1.00
OFM	MIC	1.00	1.00	1.00	0.50	1.00	2.00
	MFC	2.00	2.00	2.00	1.00	2.00	4.00
ODM	MIC	1.03	1.03	0.52	1.03	0.52	1.03
	MFC	2.06	2.06	1.03	2.06	1.03	2.06
CFU	MIC	1.02	0.51	0.51	1.02	1.02	1.02
	MFC	2.03	1.02	1.02	2.03	2.03	2.03
CDU	MIC	1.02	0.51	0.51	1.02	1.02	1.02
	MFC	2.03	1.02	1.02	2.03	2.03	2.03
OFU	MIC	1.00	1.00	1.00	0.50	0.50	0.50
	MFC	2.01	2.01	2.01	1.00	1.005	1.00
ODU	MIC	1.00	1.00	1.00	0.50	1.00	1.00
	MFC	2.01	2.01	2.01	1.00	2.01	2.01
E211	MIC	1.00	2.00	1.00	1.00	1.00	2.00
	MFC	2.00	2.00	2.00	2.00	2.00	4.00
E224	MIC	1.00	1.00	1.00	1.00	0.50	1.00
	MFC	1.00	1.00	1.00	2.00	0.50	1.00
2nd harvest							
CFI	MIC	1.00	1.00	1.00	0.50	1.00	0.50
	MFC	2.00	2.00	2.00	1.00	2.00	1.00
CDI	MIC	1.00	0.50	0.50	0.50	0.50	0.50
	MFC	2.01	1.00	1.00	1.00	1.00	1.00
OFI	MIC	1.02	0.51	0.26	0.51	0.51	1.02
	MFC	2.03	1.02	0.51	1.02	1.02	2.03
ODI	MIC	1.02	1.02	1.02	0.51	1.02	1.02
	MFC	2.03	2.03	2.03	1.02	2.03	2.03
CFM	MIC	2.05	2.05	2.05	1.03	1.03	2.05
	MFC	4.10	4.10	4.10	2.05	2.05	4.10
CDB	MIC	1.02	1.02	1.02	0.51	1.02	1.02
	MFC	2.04	2.04	2.04	1.02	2.04	2.04
OFM	MIC	2.08	1.04	0.52	0.52	0.52	0.52
	MFC	4.16	2.08	1.04	1.04	1.04	1.04
ODM	MIC	1.01	0.51	0.51	0.51	0.51	0.51
	MFC	2.02	1.01	1.01	1.01	1.01	1.01
CFU	MIC	2.06	2.06	0.52	1.03	1.03	1.03
	MFC	4.12	4.12	1.03	2.06	2.06	2.06
CDU	MIC	1.00	0.50	0.25	0.50	0.50	0.50
	MFC	2.00	1.00	0.50	1.00	1.00	1.00
OFU	MIC	1.00	1.00	1.00	0.50	0.50	0.50
	MFC	2.01	2.01	2.01	1.00	1.00	1.00
ODU	MIC	1.02	1.02	1.02	0.51	1.02	0.51
	MFC	2.04	2.04	2.04	1.02	2.04	1.02
E211	MIC	1.00	2.00	1.00	1.00	1.00	2.00
	MFC	2.00	2.00	2.00	2.00	2.00	4.00
E224	MIC	1.00	1.00	1.00	1.00	0.50	1.00
	MFC	1.00	1.00	1.00	2.00	0.50	1.00

MIC - minimal inhibitory concentration; MFC – minimal fungicidal concentration. E211 - Sodium benzoate; E224 - Potassium Metabisulfite.

(lower MIC and similar to E211 MBC values). The treatments CDI, OFI and ODI had the same efficacy against *Listeria monocytogenes* compared to E224 (both MIC and MBC values were the same), whereas they recorded similar MIC and lower MBC values to E224 against *Enterobacter cloacae*. The same treatments and OFU treatment had similar MIC and MBC values to E224 against *Escherichia coli*, while all the extracts obtained after infusion were similarly effective against *Salmonella Typhimurium* as E224 positive control. In general, the extracts obtained with infusion showed better overall efficacy against the tested bacteria, especially against *L. monocytogenes*, *S. Typhimurium* and *E. coli* were the extracts had lower or similar MIC and/or MBC values compared to positive controls. In the case of the second harvest, CFI treatment was the most effective against *S. aureus* and *B. cereus*, while CDI treatment recorded the same MIC and MBC value as E224 against *L. monocytogenes*. On the other hand, various treatments (e.g. CFI, CDI, OFI, OFU and ODU) had the same MIC values as E224 against *E. cloacae*, whereas CDI, OFI and CDU treatments had lower MIC and similar MBC values to E224 against *S. Typhimurium*. Finally, most of the extracts (CFI, CDI, ODI, CDM, CFU, OFU and ODU) were equally effective against *E. coli* as E224.

Most of the studies refer to the antimicrobial effects of the essential oils of lemon balm against bacteria and fungi. However, there are several studies where the antimicrobial effects of various lemon balm extracts are evaluated, as reported in detail in the recent review report of Carvalho, Duarte, Ferreira, & Melissa (2021). In a recent study the ethanolic extracts of macerated aerial tissues of lemon balm showed a significant antimicrobial activity against several Gram-positive and Gram-negative bacteria in a dose- and bacterial type depended manner (Guran et al., 2021). Similarly to our study, Abdel-Naime, Fahim, Fouad, & Kamel (2019) suggested that the ethanolic extracts of lemon balm were efficient against *S. aureus* and *P. aeruginosa*, whereas there was no activity against *E. coli* and *Klebsiella pneumoniae*. On the other hand, Zazharskyi, Davydenko, Kulishenko, Borovik, & Brygadyrenko (2019) reported a significant inhibitory activity of lemon balm ethanolic extracts against *S. Typhimurium*, *E. coli* and *K. pneumoniae*. Moreover, Jafarzadeh, Mehdipour Moghaddam, & Bakhshi (2020) suggested a higher activity against *E. coli* for the aqueous compared to the ethanolic extracts of lemon balm, while different activities were also observed by Ertas & Yener (2020) between acetone and methanol extracts of lemon balm against Gram-positive (*S. aureus* and *S. pyogenes*) and Gram negative (*E. coli* and *Pseudomonas aeruginosa*) bacteria. In the study of Albayrak, Aksoy, Albayrak, & Sagdic (2013) who evaluated different extraction methods (methanolic extracts, infusions, decoctions and hydrosols) only the methanolic extracts recorded activity against *B. cereus* and *P. aeruginosa*, while Keskin & Guvensen (2018) also reported a variable antibacterial efficacy for the extracts of lemon balm leaves obtained with different solvents. Moreover, Carocho et al. (2015) and Caleja et al. (2019) suggested the use of lemon balm beverages and infusions, respectively due to their considerable antimicrobial effects.

Regarding the antifungal effects, all the tested extracts had the same or higher MFC values than E224 against *Aspergillus fumigatus*, while CFI and CDI treatments had the lowest MIC values against the same pathogen. Moreover, CDI treatment showed the highest effectiveness against *A. versicolor* and *A. niger* by recording the lowest MIC and MFC values. In contrast, CDI, OFI, CDM, OFM, OFU and ODU treatments were more effective (lower MIC and MFC values) against *A. ochraceus* compared to the rest of the extracts and the positive controls. In the case of *Penicillium funiculosum*, CDI, ODI, CFM, ODM and OFU recorded similar MIC values to E224, while the latter had the lowest overall MFC value. Finally, ODI, CDM and OFU recorded the lowest MIC values against *Penicillium verucosum* var. *cyclopium*. Ertas & Yener (2020) suggested significant differences in the activity of acetone and methanolic extracts of lemon balm against *Candida albicans*, while Zazharskyi et al. (2019) who tested the antimicrobial effects of ethanolic extracts of lemon balm did not observe any effects against *L. monocytogenes* and *C. albicans*. According to Araújo et al. (2019), fresh herb of lemon balm macerated with ethanol and further extracted with various solvents showed a variable

effectiveness against human pathogenic fungi due to differences in phytochemical content of the various extracts. In contrast to our study, Guran et al. (2021) suggested that the antibacterial activity of lemon balm extracts was stronger than the antifungal effects, although they reported a dose depended response. The variable antimicrobial effects reported in the literature could be attributed to differences in phytochemicals profile and content which can be affected by several pre-harvest factors such as the growing location, the harvesting time, the growth stage of the plant, the cultivar and the growing period among others, while post-harvest parameters such as the extraction protocol and processing may also result in variable bioactive compounds content (Carvalho et al., 2021). Cocan et al. (2018) suggested that the antimicrobial effects could be correlated to the presence of various hydroxycinnamic acids; Abdel-Naime, Fahim, Abdelmohsen, et al. (2019) reported triterpene glycosides as the main responsible compounds for these effects, while Şener, Gür, Verep, Güneş, & Altuner (2017) and Tantry et al. (2014) attributed the antimicrobial effects of lemon balm ethanolic extracts to the presence of flavonoids such as naringenin, luteolin, myricetin, rutin and naringin or phenolic acids such as rosmarinic acid.

4. Conclusions

The current climate change and the irrational use of natural resources urges the adoption of sustainable agronomic practices, particularly in the arid and semi-arid region of the world. In the present study, the deficit irrigation combined with organic cropping system were tested in the cultivation of lemon balm plants aiming to identify those conditions that may enhance the quality of the final product and thus compensate for any yield losses compared to conventional and high input cropping systems. The results of this work indicate that both practices may affect the chemical profile and the bioactivities of lemon balm extracts, although the extraction protocol also seems to play a pivotal role for the observed activities. Therefore, simple agronomic practices can be adapted to increase the sustainability of lemon balm cropping systems in the Mediterranean, while at the same time they can improve the quality of the marketable product and increase the added value of the crop, especially when combined with the optimum extraction method.

CRedit authorship contribution statement

Izamara de Oliveira: Methodology, Investigation, Writing – original draft. **Antonios Chrysargyris:** Methodology, Investigation, Writing – original draft. **Sandrina A. Heleno:** Methodology, Investigation. **Márcio Carocho:** Methodology, Investigation. **Ricardo C. Calhelha:** Methodology, Investigation. **Maria Inês Dias:** Methodology, Investigation. **Jovana Petrović:** Methodology, Investigation. **Marina Soković:** Methodology. **Spyridon A. Petropoulos:** Methodology, Writing – original draft, Writing – review & editing. **Celestino Santos-Buelga:** Methodology, Writing – review & editing. **Nikolaos Tzortzakis:** Conceptualization, Project administration, Methodology, Writing – review & editing. **Lillian Barros:** Conceptualization, Project administration, Methodology, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

Acknowledgement

The authors are grateful to the Foundation for Science and Technology (FCT, Portugal) for financial support through national funds FCT/MCTES (PIDDAC) to CIMO (UIDB/00690/2020 and UIDP/00690/2020) and SusTEC (LA/P/0007/2021). L. Barros, Ricardo C. Calhelha, S. A. Heleno and M.I. Dias thank the national funding by FCT through the institutional scientific employment program-contract for her contract, while M. Carochi thanks FCT through the individual scientific employment program-contract (CEECIND/00831/2018). I. Oliveira thanks FCT for her PhD grant (BD/06017/2020). To ERDF through the Regional Operational Program North 2020, within the scope of Project GreenHealth—Norte-01-0145-FEDER-000042.Artigo

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodres.2023.113044>.

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