Effects of Growing Substrate and Nitrogen Fertilization on the Chemical Composition and Bioactive Properties of *Centaurea raphanina* ssp. *mixta* (DC.) Runemark

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Abstract: The Mediterranean basin is abundant in wild edible species with numerous health beneficial effects due to the presence of various bioactive phytochemicals. In the present work, the effect of nitrogen fertilization rates (0 ppm, (N0), 200 ppm (N1), 400 ppm (N2), and 600 ppm (N3) of total N) and growth substrate composition (soil or peat/perlite (2/1; *v/v*)) on the chemical composition and bioactive properties of *Centaurea raphanina* ssp. *mixta* plants was evaluated. The results of the study showed that both the tested factors affected nutritional value of the edible leaves, with the soil × N1 treatment being the most beneficial for fat, protein, and carbohydrate content and energetic value. On the other hand, the peat/perlite-grown plants that received 200 ppm of N had the highest content in α-, γ-, and total tocopherols, while the control treatment of soil-grown plants was the richest in individual and total sugars. Oxalic, citric, and total organic acids were the highest in the N2 × soil treatment, while malic acid was the highest in control treatment of the same substrate. The main fatty acids were palmitic, α-linolenic, and linoleic acids, with the highest contents being observed in the N0 × soil, N3 × soil, and N3 × peat/perlite treatments, respectively. The major phenolic compounds were pinocembrim neohesperidoside and pinocembrim acetyl neohesperidoside isomer II, with the highest content being observed in the N1 × soil treatment. The highest antihemolytic activity was observed in the N3 × peat/perlite treatment, while the most effective treatments against lipid peroxidation were N0 (in both soil and peat/perlite combinations) and N1 × peat/perlite. Lastly, all the tested extracts (except for N1 × soil) showed promising cytotoxic effects against HeLa (cervical carcinoma), HepG2 (hepatocellular carcinoma), MCF-7 (breast carcinoma), and NCI-H460 (non-small-cell lung cancer), while all the tested extracts exhibited better antifungal activities (lower minimal inhibition concentration (MIC) values) against *Trichoderma viride* than the positive controls. Overall, the present results suggest that the application of cost-effective practices such as the nitrogen application and the selection of growth substrate may regulate the chemical composition and the bioactive properties of *C. raphanina* ssp. *mixta* species and increase its added value under commercial cultivation conditions.

Keywords: phenolic compounds; antioxidant activity; pinocembrin derivatives; antimicrobial properties; γ-tocopherol; nitrogen fertilization

1. Introduction

The Mediterranean basin is thriving in wild edible plants that contain numerous health beneficial compounds and are considered the pillar of agroecosystems and the

agrobiodiversity of the region [1]. Until recently, it was common in rural areas to gather wild edible plants for food purposes, a practice that was handed down from generation to generation along with a general approach of sustainability and respect for the environment and its genetic resources [2–7]. However, despite the transition to diets adapted to the Western and less healthy lifestyles, there is an increasing consumer demand for healthy food products. In addition, the building evidence of the beneficial effects of adherence to the Mediterranean diet has rekindled the interest for wild edible plants [1,8,9]. Under these circumstances, a necessity of sustainable management of plant genetic resources has aroused in order to cover current market needs without putting at risk the valuable genetic resources of the regions [10–14]. Therefore, the commercial cultivation of wild edible species is in the research focus aiming to substitute gathering edible plants from the wild with widely available food products that meet the quality and safety standards and cater to consumer and market needs [1,15,16].

Centaurea raphanina spp. *mixta* (DC.) Runemark belongs to the Asteraceae family and is endemic to Greece [17–19]. Recent reports highlighted the promising bioactive properties of the species, as well as the effect that cultural practices may have on its phytochemical profile and bioactivities [17,19–21]. *Centaurea raphanina* spp. *mixta* is a rich source of various bioactive compounds, including polyphenols and tocopherols with high antioxidant potency and cytotoxic effects [17,19]. Since it is expected to observe variations in the chemical profile of domesticated wild species [22–25], it is very important to define those agronomic conditions that offer the highest yields without compromising the quality of the final product [26–28]. Recent research studies have suggested that cultural practices such as the harvesting and or developmental stage affected the chemical composition of domesticated *Cichorium spinosum* or *Portulaca oleracea* plants [26,29,30], while fertilizer application rates or the composition of the growth substrate also had an impact on quality parameters of various wild species [27,31,32]. Moreover, the fact that most of these wild edible species survive under arduous conditions in natural habitats makes them promising candidates as alternative/complementary crops in sustainable farming systems, especially those of small scale that are very common in the broader Mediterranean region [10]. However, before introducing wild edible species in farming systems, it is essential to define the required quality and safety standards and the means that allow the production of food products that meet these standards. For example, previous reports indicated the high content of cultivated *C. raphanina* spp. *mixta* plants in oxalic acid, which is an antinutritional factor, and high intake (>5 g per day) may result in health complications [8,33,34]. Therefore, agronomic practices that may reduce the oxalic acid content of leaves would increase the quality of the final product in a cost-effective manner.

Considering the current research regarding the effect of various agronomic practices on the quality of *C. raphanina* ssp. *mixta* [2,20,31], the aim of the present study was to further examine the effect of growth substrate and nitrogen application rate on the chemical composition and bioactive properties of the species. The application of such cost-effective cultural practices would allow the improvement of quality parameters in commercially cultivated wild species and further increase their beneficial effects to human health.

2. Materials and Methods

2.1. Plant Material and Experimental Conditions

Collection of *Centaurea raphanina* spp. *mixta* whole plants took place in the wild in 2017, as previously described by the authors [17]. Selected plants were removed carefully from the soil with a pick mattock, trying not to destroy the large tap root. Immediately after the harvest, all leaves were removed except for the apex in order to reduce water losses due to transpiration, then wrapped in wetted absorbent paper, and put in an insulated food bag before being transferred to the laboratory of vegetable production. Then, plants were immediately transplanted in 10 L pots (one plant per pot) filled with soil or peat (Klassman–Deilmann KTS2)/perlite (2:1; *v/v*), irrigated with tap water, and transferred to the experimental farm of the university of Thessaly, Velestino, Greece. Forty plants

were used for each substrate (80 plants in total). Physicochemical properties of peat were as follows: bulk density 0.12 g/cm^3, water holding capacity 218.5%, pH 6.0, electrical conductivity (EC) 0.35 dS/m, organic matter 47.5%, carbon 27.5%, nitrogen 0.14%, C/N 196.8, P 160 mg/L, and K (cmol/kg) 46.03 [32]. Physicochemical properties of soil were as follows: bulk density 1.07 g/cm^3, water holding capacity 49.9%, pH 7.5, electrical conductivity (EC) 0.14 dS/m, organic matter 2.7%, carbon 1.6%, nitrogen 0.08%, C/N 19.9, P (Olsen) 122 mg/kg, and K (cmol/kg) 0.91 [32]. When regrowth of transplanted plants took place (approximately 10 days after transplanting), irrigation was implemented once or twice a week depending on the environmental conditions. Plants remained in the pots and received only irrigation (no fertilizers) throughout the following growing season (October 2017–June 2018) and completed their growth cycle (inflorescence formation, seed production, and senescence) with no harvests taking place. Pots were regularly irrigated during the summer season and after senescence to induce the regrowth of plants and to prevent growth substrates from drying out. The next growing season started when regrowth of established plants took place (at the end of August 2018). After regrowth, plants were regularly irrigated twice a week, while fertilization started 30 days after regrowth (end of September 2018) through fertigation. The fertilization treatments and the application regime, as well as the growing conditions, were described previously by the authors [21]. Briefly, four treatments were applied in each growth substrate (10 pots per treatment): the control treatment (N0, tap water), 200 ppm (N1), 400 ppm (N2), and 600 ppm (N3) of total N. Plants were harvested when the rosette of leaves was fully expanded at the end of November 2018, and batch samples of fresh leaves from each treatment were put in plastic food bags and stored in freezing conditions immediately after harvest. Then, frozen leaves were lyophilized, ground to powder, and stored in deep-freezing conditions until further analysis [20]. Dry weight was determined after oven-drying samples of fresh leaves at 72 °C until constant weight (approximately after 48 h) [35].

2.2. Standards and Reagents

HPLC-grade acetonitrile was obtained from Merck KgaA (Darmstadt, Germany). Formic and acetic acids were purchased from Prolabo (VWR International, Fontenay-sous-Bois, France). Standards of melezitose, fatty acid methyl esters (FAMEs, reference standard mixture 3747885-U), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA); the tocol standard (50 mg/mL) was acquired from Matreya, Pleasant Gap (State College, PA, USA), and the phenolic compound standards were from Extrasynthese (Genay, France). Fetal bovine serum (FBS), L-glutamine, Hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediaminetetraacetic acid), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively), Roswell Park Memorial Institute RPMI-1640, and Dulbecco's Modified Eagle Medium (DMEM) media were from Hyclone (Logan, Utah, USA). Mueller–Hinton agar (MH) and malt agar (MA) were obtained from the Institute of Immunology and Virology, Torlak (Belgrade, Serbia). Dimethyl sulfoxide (DMSO) was purchased from Merck KGaA (Darmstadt, Germany). Phosphate-buffered saline (PBS), acetic acid, ellipticine, sulforhodamine B (SRB), trypan blue, trichloroacetic acid (TCA), and Tris were from Sigma Chemical Co. (St. Louis, MO, USA). Ethanol and all other chemicals and solvents were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA)

2.3. Nutritional Value and Hydrophilic Compounds

The nutritional and energetic values of the lyophilized samples were analyzed for moisture, protein, fat, and ash contents following the Association of Official Analytical Chemists (AOAC) procedures [36]. Briefly, the crude protein content (N × 6.25) was estimated by the macro-Kjeldahl method, the crude fat content was determined by Soxhlet extraction with petroleum ether, and the ash content was determined by incineration in

a muffle furnace at 550 ± 15 °C. Total carbohydrates were calculated by difference, i.e., 100 − (g moisture + g protein + g fat + g ash), and the results were expressed as g per 100 g of fresh weight (fw). The energy value was calculated according to the equation, 4 × (g protein + g carbohydrates) + 9 × (g fat), and the results were given as kcal per 100 g of fw.

The free sugar profile was characterized in a high-performance liquid chromatography (HPLC) system coupled to a refraction index (RI) detector, using the internal standard (IS, melezitose) method as previously described [37]. The identification was made by relating the retention times of the authentic standards to those of the samples, while quantification was made by the IS method, with calibration curves constructed with standards. The free sugar content was expressed in g per 100 g of fw.

The organic acid profile was characterized by ultra-fast liquid chromatography (UFLC) following a procedure earlier described and optimized by the authors [38]. Detection was performed in a photodiode array detector (PDA), using 215 nm and 280 nm as preferable wavelengths. Quantification was performed by comparing the peak area of the samples with calibration curves constructed using commercial standards. Calibration curves constructed using standard compounds were used for quantification. The organic acid content was expressed in mg per 100 g of fw.

2.4. Lipophilic Compounds

The profile in fatty acid methyl esters (FAMEs) was characterized after *trans*-esterification of the lipid fraction obtained by Soxhlet extraction [37] by gas chromatography (GC) with flame ionization detection (FID), using a YOUNG IN Crhomass 6500 GC System apparatus equipped with a split/splitless injector, a flame ionization detector (FID), and a Zebron-Fame column. Identification and quantification were done by comparing the relative retention times of the FAME peaks of the samples with those of the standard. The Clarity DataApex 4.0 Software was used for data processing. The results were expressed in relative percentage of each fatty acid.

The tocopherol profile was characterized following an analytical procedure previously reported by the authors [37]. A high-performance liquid chromatography (HPLC) system coupled to a fluorescence detector (FP) programed for excitation at 290 nm and emission at 330 nm was used. The isoform identification was achieved by chromatographic comparison with authentic standards, and the quantification was based on the fluorescence signal response of each standard, using the internal standard (tocol) method and calibration curves constructed using commercial standards. The results were expressed in mg per 100 g of fw.

2.5. Polyphenolic Profile Characterization

2.5.1. Preparation of Hydroethanolic Extracts

The lyophilized plant material was used to prepare hydroethanolic extracts, which were obtained by stirring the powder (~2.5 g) with 30 mL of ethanol/water (80:20, *v/v*) at 25 °C for 1 h and filtered through Whatman No. 4 paper. The residue was then re-extracted with an additional 30 mL of the hydroalcoholic mixture. The combined extracts were concentrated at 40 °C under reduced pressure and further lyophilized [37].

2.5.2. Analysis of Phenolic Compounds

Phenolic compounds were analyzed in the hydroethanolic extracts, which were re-dissolved in ethanol/water (80:20, *v/v*) to a final concentration of 10 mg/mL, and filtered thought 0.22 μm disposable filter discs. The analysis was performed in a HPLC system coupled with a diode array detector (DAD, using 280 nm and 370 nm as preferred wavelengths) and a linear ion trap (LTQ XL) mass spectrometer (MS) equipped with an electrospray ionization (ESI) source. The separation was made in a Waters Spherisorb S3 ODS-2 C18 column (3 μm, 4.6 mm × 150 mm). The equipment and operating conditions were previously described by Bessada et al. [39], as well as the identification and quantification procedures. The results were expressed as mg per g of plant fw.

2.6. Bioactivities

2.6.1. Antioxidant and Cytotoxic Activity

The lyophilized extracts prepared above were redissolved in water and subjected to dilutions. The lipid peroxidation inhibition in porcine brain cell homogenates was evaluated by the decrease in thiobarbituric acid reactive substances (TBARS) assay; the color intensity of malondialdehyde–thiobarbituric acid (MDA–TBA) was measured at 532 nm [37]. The results were expressed in EC_{50} values (µg/mL, sample concentration providing 50% of antioxidant activity). Trolox was used as a positive control.

The antihemolytic activity of the lyophilized extracts was evaluated by the oxidative hemolysis inhibition assay (OxHLIA) using red blood cells (RBC) isolated from the blood of healthy sheep, as described and optimized by the authors [40]. The Δt values (min) resulting from the half hemolysis time (Ht_{50} values) obtained from the hemolytic curves of each extract sample concentration minus the Ht_{50} value of the PBS control were correlated to the respective extract concentration to obtained IC_{50} values (µg/mL, extract concentration required to protect 50% of the erythrocyte population from the hemolytic action of AAPH for 60 min). Trolox was used as a positive control.

A hepatic cell line, designated as PLP2, was used to evaluate the cytotoxicity. The cytotoxic properties were evaluated in four human tumor cell lines, namely, HeLa (cervical carcinoma), HepG2 (hepatocellular carcinoma), MCF-7 (breast carcinoma), and NCI-H460 (non-small-cell lung cancer), and a sulforhodamine B colorimetric (SRB) assay was followed. The positive control was ellipticine. All results were expressed in GI_{50} values (µg/mL, sample concentration that inhibited 50% of the net cell growth) [41].

2.6.2. Antimicrobial Activity

The following bacteria strains were used for antimicrobial testing: *Staphylococcus aureus* (American Type Culture Collection (ATCC) 11632), *Bacillus cereus* (food isolate), *Escherichia coli* (ATCC 25922), *S. typhimurium* (ATCC 13311), and *Enterobacter cloacae* (ATCC 35030); the following micromycetes were used: *Aspergillus fumigatus* (ATCC 9197), *Aspergillus niger* (ATCC 6275), *Aspergillus versicolor* (ATCC 11730), *Penicillium funiculosum* (ATCC 36839), *Trichoderma viride* (IAM 5061), and *Penicillium verrucosum* var. *cyclopium* (food isolate).

The microdilution method was performed to assess the antimicrobial activity, and the minimum extract concentrations that completely inhibited bacterial growth (MIC values) were determined by a colorimetric microbial viability assay, while minimum bactericidal concentration (MBC values) and minimum fungicidal concentration (MFC) were also calculated. Streptomycin, ampicillin, ketoconazole, and bifonazole were used as positive controls, whereas 5% DMSO was used as a negative control [42].

2.7. Statistical Analysis

The experiment was carried out according to a completely randomized design (RCD) with 10 replications (*n* = 10) for each treatment. All the chemical composition assays were performed in three samples, while the assay for each assay was performed in triplicate. The analysis of the data was carried out with the use of Statgraphics 5.1. plus (Statpoint Technologies, Inc., Virginia, USA). All the data were treated with two-way ANOVA to evaluate the effect of growth substrate and nitrogen fertilization regime, and all means were compared according to Tukey's honest significance test (HSD) test (*p* = 0.05).

3. Results and Discussion

The statistical analysis of the data showed a significant interaction between the tested factors (growth substrate and fertilization regime); therefore, all the means were compared with each other at the same time.

Table 1 presents the results of nutritional value, where growing plants in peat/perlite at the rate of 0 and 400 ppm of nitrogen resulted in the highest moisture content in leaves. On the other hand, growing plants in soil under medium nitrogen fertilization rates (400 ppm of total N) had a beneficial effect for most nutritional value parameters, e.g.,

fat (0.53 g/100 g fw), protein (5.28 g/100 fw), and carbohydrate content (8.9 g/100 g fw) and energetic value (61.4 Kcal/100 g fw), whereas the low fertilization rate (200 ppm of total N) resulted in the highest ash content (3.92 g/100 g fw) in plants grown in the same substrate. These increasing trends in fat, protein, and carbohydrate content and energetic value with increasing nitrogen fertilization rate were also reported in the study of Petropoulos et al. [21], although they also suggested a significant impact of harvesting stage. Moreover, similarly to that study, the present results indicate that plants grown in peat-based substrates are positively affected by increasing nitrogen rates when considering fat and protein content and energetic value, whereas, in plants grown in soil, the highest contents were recorded in medium nitrogen rates (400 ppm of N). Karkanis et al. [31] also indicated the important role of growth substrate composition in proximate composition and nutritional value of *Sanguisorba minor*, and they pointed out that commercial cultivation practices could be practically used as cost-effective means for the improvement of the quality of the final product.

Table 1 also presents the results related to sugar composition with four individual compounds identified, including two monosaccharides (glucose and fructose) and two polysaccharides (sucrose and trehalose), while sucrose was the main detected sugar in most cases (0.18 to 0.40 g/100 g fw, in the case of the control treatment in peat/perlite- and soil-grown plants, respectively). Similar compounds were also identified in previous studies of *C. raphanina* ssp. *mixta* plants, although different free sugar profiles were suggested [17,21,43]. In particular, the composition of free sugars in the leaves of the present study varied depending on the growth substrate and the fertilization rate, although the control treatment of plants grown in soil was the most abundant in all the individual sugars (except for trehalose which was the highest in the case of N1 and N2 × soil, and N0 × peat:perlite treatments) and eventually in total sugars (1.05 g/100 g fw). Petropoulos et al. [21], who studied the effect of successive harvesting and nitrogen fertilization rate on the chemical composition of *C. raphanina* ssp. *mixta* plants, suggested trehalose as the main sugar in the leaves of the first harvest, regardless of the nitrogen rate, whereas, in the second harvest, increased nitrogen rates resulted in increased fructose content, which was also the case in most of the treatments in our study. Moreover, Kołota et al. [44] suggested that increasing nitrogen fertilization rates resulted in a decrease in sugar content in Swiss chard leaf blades, which was also observed in the soil-grown plants of our study. These findings are in the same line with previous reports which highlight the importance of harvesting time and/or developmental stage, as well as of the growing medium for sugar composition in wild or commercially cultivated leafy vegetables [20,29,31,45]. Moreover, nitrogen source may also affect sugar composition in leafy vegetables [27,43,46], which could be related to the fact that the fertilization treatments in our study differed not only in total nitrogen rates but also in the ammonium and nitrate nitrogen ratio.

Table 1. Nutritional value and hydrophilic compounds of *Centaurea raphanina* spp. *mixta* plants in relation to nitrogen fertilization and growth substrate (mean ± SD).

					Treatments			
	N0 Soil	N1 Soil	N2 Soil	N3 Soil	N0 Peat/Perlite	N1 Peat/Perlite	N2 Peat/Perlite	N3 Peat/Perlite
Nutritional value (g/100 g fw)								
Moisture	84.9 ± 0.6 c	82.4 ± 0.7 e	82.4 ± 0.8 e	83.4 ± 0.5 d	86.4 ± 0.1 a	85.8 ± 0.7 b	86.3 ± 0.4 a	85.6 ± 0.2 b
Fat	0.35 ± 0.01 d	0.37 ± 0.01 c	0.53 ± 0.01 a	0.47 ± 0.01 b	0.31 ± 0.01 e	0.32 ± 0.01 e	0.275 ± 0.003 g	0.293 ± 0.001 f
Proteins	4.08 ± 0.01 e	4.82 ± 0.01 c	5.28 ± 0.01 a	4.92 ± 0.04 b	3.07 ± 0.01 h	4.15 ± 0.01 d	3.19 ± 0.01 g	3.89 ± 0.01 f
Ash	2.53 ± 0.01 c	3.92 ± 0.01 a	2.88 ± 0.05 b	2.93 ± 0.05 b	2.37 ± 0.08 d	2.00 ± 0.02 e	2.05 ± 0.04 e	1.99 ± 0.01 f
Carbohydrates	8.1 ± 0.1 d	8.5 ± 0.1 b	8.9 ± 0.1 a	8.3 ± 0.1 c	7.8 ± 0.1 e	7.7 ± 0.1 e	8.1 ± 0.1 d	8.2 ± 0.1 cd
Energy (kcal/100 g fw)	51.9 ± 0.1 d	56.5 ± 0.1 c	61.4 ± 0.1 a	57.0 ± 0.1 b	46.3 ± 0.2 h	50.4 ± 0.1 f	47.8 ± 0.1 g	51.0 ± 0.1 e
Free sugars (g/100 g fw)								
Fructose	0.40 ± 0.02 a	0.238 ± 0.005 d	0.16 ± 0.01 e	0.37 ± 0.01 b	0.18 ± 0.02 e	0.22 ± 0.02 d	0.318 ± 0.001 c	0.212 ± 0.006 d
Glucose	0.292 ± 0.005 a	0.211 ± 0.008 b	0.201 ± 0.005 b	0.159 ± 0.007 c	0.158 ± 0.005 c	0.146 ± 0.003 c	0.198 ± 0.001 b	0.140 ± 0.008 c
Sucrose	0.192 ± 0.006 a	0.049 ± 0.004 f	0.066 ± 0.003 e	0.099 ± 0.001 c	0.079 ± 0.002 d	0.044 ± 0.003 f	0.116 ± 0.002 b	0.128 ± 0.007 b
Trehalose	0.168 ± 0.005 cd	0.199 ± 0.005 a	0.188 ± 0.008 ab	0.168 ± 0.008 cd	0.189 ± 0.009 ab	0.163 ± 0.005 cd	0.152 ± 0.009 d	0.172 ± 0.008 bc
Sum	1.05 ± 0.01 a	0.700 ± 0.005 c	0.62 ± 0.03 d	0.79 ± 0.01 b	0.61 ± 0.03 de	0.58 ± 0.03 e	0.78 ± 0.01 b	0.65 ± 0.03 d
Organic acids (mg/100 g fw)								
Oxalic acid	1131 ± 4 d	1265 ± 3 b	1373 ± 3 a	1254 ± 3 c	910 ± 7 h	998 ± 2 f	1007 ± 5 e	932 ± 4 g
Malic acid	925 ± 5 a	272 ± 4 h	816 ± 1 b	554 ± 4 c	318 ± 3 f	295 ± 5 g	375 ± 4 e	480 ± 3 d
Ascorbic acid	0.090 ± 0.004 d	0.230 ± 0.008 a	0.020 ± 0.005 e	0.18 ± 0.01 b	0.100 ± 0.008 d	0.14 ± 0.02 c	0.13 ± 0.01 c	0.20 ± 0.01 b
Citric acid	878 ± 3 c	814 ± 7 d	1045 ± 4 a	756 ± 3 e	597 ± 5 g	578 ± 4 h	665 ± 2 f	1029 ± 1 b
Fumaric acid	tr	0.020 ± 0.001	tr	tr	tr	tr	tr	tr
Sum	2934 ± 6 b	2352 ± 8 e	3234 ± 7 a	2565 ± 4 c	1825 ± 10 h	1871 ± 1 g	2047 ± 4 f	2442 ± 1 d

Tr: traces; N0: control (0 ppm N); N1: 200 ppm N; N2: 400 ppm N; N3: 600 ppm N. Means in the same row followed by different letters are significantly different according to Tukey's honest significance test HSD test ($p = 0.05$).

Organic acid composition is presented in Table 1. The main detected compound was oxalic acid, followed by citric and/or malic depending on the treatment, while low amounts of ascorbic acid were detected. Fumaric acid was only detected in the plants that were grown in soil and received the low fertilization rate (200 ppm of total N). Moreover, a varied response to nitrogen application rate and growth substrate was recorded. In particular, the highest amounts of oxalic and citric acid, as well as of total organic acids, were recorded for the soil-grown plants that received 400 ppm of N (1373 mg/100 g fw, 1045 mg/100 g fw, and 3234 mg/100 g fw, respectively), while malic acid was the highest for the control treatment and the same growth substrate (925 mg/100 g fw). When compared to the control, all the treatment where plants received increased nitrogen rates had higher contents of oxalic acid, regardless of the growth substrate, which was also reported in the literature, since oxalic acid is formed by plants during nitrogen assimilation to balance cytoplasm pH [21,31,47]. For the remaining organic acids, no specific trends were observed indicating a complex effect of nitrogen application rate and growth substrate on organic acids biosynthesis. The overall organic acid and oxalic acid contents were higher than those reported in studies carried out by Petropoulos et al. with the same species [17,20,21]. This contradiction could be explained by the differences in the experimental conditions, since several other factors could be involved in the biosynthesis of organic acids, such as the developmental stage, the nutrient solution composition, the harvesting stage, or abiotic stressors, which hinder the direct comparison with the observed recordings [17,20,48,49]. In any case, special attention is needed regarding the oxalic acid content, which is considered an antinutritional factor, and high daily intake could have negative health effects [14,15,50]. Therefore, despite the beneficial health effects that wild edible species may have, commercial cultivation could allow the production of final products with known composition and according to health safety regulations [1].

The analysis of fatty acid composition revealed 18 individual compounds in total, with palmitic (26.60–47.66%), α-linolenic (14.4%0–32.67%), and linoleic (13.8–24.8%) acids being the most abundant ones. A similar profile was reported in previous studies regarding the lipidic fraction of *C. raphanina* spp. *mixta* plants, although with different contents for the individual compounds due to differences in experimental conditions [17,20,21]. Regarding the effect of growth substrate and fertilization regime on fatty acid profile, a varied response was observed, while the major compound content was the highest in soil-grown plants and the control or the high nitrogen rate in the case of palmitic and α-linolenic acids, respectively, or the peat/perlite-grown plants and the high nitrogen rate in the case of linolenic acid (Table 2). The main fatty acid classes were either the monounsaturated fatty acids (only in the N0 $\times$ soil treatment due to the high palmitic acid content) or the polyunsaturated ones (in the rest of the treatments), indicating the significant impact of both growth substrate and fertilization regime, as already reported in previous studies [21,31]. However, the study of Petropoulos et al. [21] also highlighted the importance of harvesting stage on the fatty acid composition of *C. raphanina* spp. *mixta* plants that were cultivated under different nitrogen fertilization rates. On the other hand, the highest ratio of polyunsaturated fatty acids/saturated fatty acids was recorded for the N3 $\times$ peat:perlite treatment due to the high content of α-linolenic acid, while, in all the tested treatments (except for the N0 $\times$ soil), the ratio values were higher than 0.45, indicating an improvement of nutritional value as the result of nitrogen fertilization [17,51]. Similarly, the ratio values of omega-6 (n6)/omega-3(n3) fatty acids were lower than 4.0 in all the tested treatments, suggesting a beneficial effect for the healthy fat composition [17,51].

Table 2. Chemical composition with regard to lipophilic compounds of the studied *Centaurea raphanina* (%) (mean ± SD).

	Treatments							
	N0 Soil	**N1 Soil**	**N2 Soil**	**N3 Soil**	**N0 Peat/Perlite**	**N1 Peat/Perlite**	**N2 Peat/Perlite**	**N3 Peat:/Perlite**
Fatty acids (%)								
C8:0	1.52 ± 0.03 e	0.125 ± 0.001 f	0.21 ± 0.02 b	0.173 ± 0.006 c	0.15 ± 0.01 e	0.26 ± 0.02 a	0.089 ± 0.001 g	0.16 ± 0.01 d
C10:0	0.781 ± 0.008 a	0.227 ± 0.009 d	0.220 ± 0.004 e	0.246 ± 0.008 c	0.28 ± 0.02 b	0.22 ± 0.01 e	0.108 ± 0.005 g	0.173 ± 0.007 f
C11:0	1.90 ± 0.02 a	0.38 ± 0.02 f	0.27 ± 0.03 h	0.73 ± 0.07 d	0.85 ± 0.03 c	1.01 ± 0.09 b	0.30 ± 0.01 g	0.63 ± 0.04 e
C12:0	0.396 ± 0.006 b	0.314 ± 0.001 e	0.328 ± 0.008 d	0.35 ± 0.03 c	0.43 ± 0.01 a	0.27 ± 0.02 f	0.111 ± 0.003 h	0.18 ± 0.01 g
C14:0	4.25 ± 0.04 b	4.47 ± 0.03 a	1.33 ± 0.06 d	1.17 ± 0.06 e	2.84 ± 0.04 c	1.36 ± 0.05 d	0.874 ± 0.009 f	0.58 ± 0.01 g
C14:1	nd	0.58 ± 0.01 a	0.269 ± 0.004 b	0.212 ± 0.008 c	0.185 ± 0.001 d	0.19 ± 0.01 d	0.077 ± 0.003 e	0.083 ± 0.004 e
C15:0	0.91 ± 0.02 a	0.42 ± 0.02 d	0.53 ± 0.02 b	0.47 ± 0.02 c	0.470 ± 0.001 c	0.53 ± 0.01 b	0.411 ± 0.004 d	0.418 ± 0.005 d
C16:0	47.66 ± 0.09 a	26.6 ± 0.7 g	31.33 ± 0.03 c	29.7 ± 0.2 f	32.3 ± 0.4 b	30.8 ± 0.3 d	30.19 ± 0.01 e	30.0 ± 0.3 f
C17:0	0.95 ± 0.04 a	0.54 ± 0.01 de	0.59 ± 0.02 b	0.566 ± 0.007 bc	0.55 ± 0.03 cd	0.534 ± 0.001 e	0.324 ± 0.004 g	0.490 ± 0.004 f
C18:0	6.28 ± 0.08 a	3.78 ± 0.07 e	3.84 ± 0.06 e	4.35 ± 0.06 c	4.44 ± 0.01 c	4.68 ± 0.07 b	4.05 ± 0.01 d	3.20 ± 0.03 f
C18:1 n9 c	1.85 ± 0.05 f	3.91 ± 0.03 b	2.32 ± 0.02 d	2.26 ± 0.02 d	2.09 ± 0.01 e	4.02 ± 0.01 a	3.52 ± 0.01 c	2.26 ± 0.02 d
C18:2 n6 c	13.8 ± 0.4 e	23.5 ± 0.2 c	23.5 ± 0.2 c	22.46 ± 0.02 d	23.7 ± 0.1 c	24.0 ± 0.5 c	26.8 ± 0.2 b	28.0 ± 0.4 a
C18:3 n3	14.4 ± 0.1 f	30.2 ± 0.3 c	30.68 ± 0.05 b	32.67 ± 0.05 a	27.4 ± 0.2 e	27.79 ± 0.03 e	29.3 ± 0.3 d	29.48 ± 0.09 d
C20:0	1.13 ± 0.01 a	0.87 ± 0.03 b	0.74 ± 0.03 c	0.72 ± 0.01 c	0.68 ± 0.03 d	0.732 ± 0.008 c	0.435 ± 0.004 e	0.74 ± 0.02 c
C21:0	0.274 ± 0.001 c	0.28 ± 0.02 c	0.27 ± 0.02 c	0.31 ± 0.01 b	0.394 ± 0.005 a	0.21 ± 0.02 d	0.171 ± 0.009 e	0.27 ± 0.01 c
C22:0	1.71 ± 0.02 a	1.54 ± 0.02 b	1.19 ± 0.08 e	1.43 ± 0.01 c	1.1 ± 0.1 f	1.24 ± 0.01 d	1.24 ± 0.02 d	1.51 ± 0.07 b
C23:0	0.90 ± 0.01 a	0.48 ± 0.02 c	0.52 ± 0.02 b	0.52 ± 0.01 b	0.47 ± 0.01 c	0.506 ± 0.003 b	0.385 ± 0.002 e	0.45 ± 0.01 d
C24:0	1.34 ± 0.03 e	1.77 ± 0.08 b	1.83 ± 0.01 a	1.7 ± 0.2 c	1.70 ± 0.02 c	1.7 ± 0.1 c	1.61 ± 0.01 d	1.38 ± 0.01 e
SFA	70.0 ± 0.2 a	41.8 ± 0.5 f	43.2 ± 0.2 d	42.40 ± 0.07 e	46.6 ± 0.3 b	44.0 ± 0.5 c	40.30 ± 0.04 g	40.2 ± 0.2 g
MUFA	1.85 ± 0.05 g	4.48 ± 0.02 a	2.59 ± 0.02 d	2.47 ± 0.01 e	2.27 ± 0.01 f	4.21 ± 0.02 b	3.60 ± 0.01 c	2.3 ± 0.02 f
PUFA	28.2 ± 0.3 h	53.7 ± 0.5 e	54.2 ± 0.2 d	55.13 ± 0.08 c	51.1 ± 0.3 g	51.8 ± 0.5 f	56.10 ± 0.05 b	57.5 ± 0.3 a
PUFA/SFA	0.40 ± 0.01 h	1.28 ± 0.05 de	1.25 ± 0.02 e	1.30 ± 0.07 cd	1.09 ± 0.3 g	1.18 ± 0.05 f	1.39 ± 0.04 b	1.43 ± 0.03 a
n6/n3	0.96 ± 0.25 a	0.78 ± 0.15 d	0.77 ± 0.15 d	0.69 ± 0.03 e	0.86 ± 0.01 c	0.86 ± 0.04 c	0.91 ± 0.15 b	0.95 ± 0.18 a
Tocopherols (mg/100 g fw)								
α-Tocopherol	0.046 ± 0.003 e	0.056 ± 0.001 d	0.063 ± 0.002 d	0.056 ± 0.003 d	0.036 ± 0.002 f	0.121 ± 0.004 a	0.078 ± 0.001 c	0.110 ± 0.002 b
γ-Tocopherol	0.151 ± 0.006 c	0.17 ± 0.01 b	0.086 ± 0.001 e	0.143 ± 0.001 d	0.069 ± 0.002 e	0.199 ± 0.001 a	0.042 ± 0.002 f	0.069 ± 0.008 e
Sum	0.200 ± 0.007 c	0.23 ± 0.01 b	0.150 ± 0.001 e	0.200 ± 0.001 c	0.100 ± 0.001 g	0.320 ± 0.001 a	0.120 ± 0.001 f	0.18 ± 0.01 d

N0: control (0 ppm N); N1: 200 ppm N; N2: 400 ppm N; N3: 600 ppm N; nd: not determined. Means in the same row followed by different letters are significantly different according to Tukey's HSD test (p = 0.05). Caprylic acid (C8:0); capric acid (C10:0); undecylic acid (C11:0); lauric acid (C12:0); myristic acid (C14:0); pentadecylic acid (C15:0); palmitic acid (C16:0); margaric acid (C17:0); stearic acid (C18:0); oleic acid (C18:1n9); linoleic acid (C18:2n6c); α-linolenic acid (C18:3n3); arachidic acid (C20:0); heneicosylic acid (C21:0); behenic acid (C22:0); tricosylic acid (C23:0); lignoceric acid (C24:0); SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; n6/n3: omega-6/omega-3 fatty acids.

Tocopherol composition is presented in Table 2, with only two vitamin E isoforms (α- and γ-tocopherol) being identified in the studied samples. Interestingly, γ-tocopherol was the most abundant compound in all samples (0.042 to 0.199 mg/100 g fw), especially in peat/perlite-grown plants that received the low nitrogen rate (200 ppm; 0.199 mg/100 g fw). The same treatment was also the richest in α-tocopherol (0.121 mg/100 g fw) and apparently in total tocopherol content (0.32 mg/100 g fw). Similarly to our study, several other reports suggested α- and γ-tocopherols as the main vitamin E isomers in the species [17,20,21], although they indicated α-tocopherol as the main compound instead of γ-tocopherol, and they also recorded significantly higher amounts of individual and total tocopherols compared to the present study. This contradiction should be associated partly with the developmental stage of plants at harvesting time, as well as with the fact that plant material was received from wild plants which were transplanted and cultivated under the tested conditions. According to the literature, α-tocopherols are involved in the antioxidant mechanisms of plants [52]; therefore, it seems that wild *C. raphanina* ssp. *mixta* plants that have already formed large taproots before transplantation are less prone to stressful conditions than seedlings, and the antioxidant mechanisms were not induced under the tested conditions [17]. Moreover, the recent study of Cruz et al. [53] suggested that the increased nitrogen inputs resulted in a decrease in tocopherol content in green and red basil leaves, and that the availability of nitrogen, whether in excess or in deficit, may regulate tocopherol biosynthesis. The connecting link between nitrogen fertilization and tocopherol content seems to be amino-acid and purine biosynthesis which are the main precursors of vitamins [54]. However, apart from the varied response of tocopherol content to fertilization rate, the importance of growth substrate for the chemical composition of leaves also needs to be pointed out, since, according to the literature, it may affect tocopherol composition [31].

The data related to the phenolic compound identification and quantification are presented in Tables 3 and 4, respectively. Twelve *O*-glycosylated phenolic compounds were identified in all the tested samples including five flavonols, five flavanones, and two flavones (Table 3). These findings are in agreement with previous works on the same species which suggested a similar phenolic compound profile [17,20,31], while an extensive description of the specific compounds has been suggested in other species of the genus [55,56]. The major detected compounds were peaks 9 and 12, namely, pinocembrim neohesperidoside and pinocembrim acetyl neohesperidoside isomer II, a finding which agrees with the results of Petropoulos et al. [17,20,21], who studied the effect of nitrogen application rate and saline conditions, as well as the chemical composition of wild and transplanted *C. raphanina* ssp. *mixta* plants. It is interesting to mention that, in the study of Petropoulos et al. [17], wild plants showed a different profile, with pinocembrim neohesperidoside and pinocembrim acetyl neohesperidoside isomer I being the major compounds, indicating a significant effect of agronomic practices on phenolic compound composition. Regarding the effect of nitrogen application rates and growth substrates tested in our study, the highest content of the major compounds (peaks 9 and 12) was recorded for the soil-grown plants that received 200 ppm of nitrogen, while the same combination of treatments resulted in an increase in compounds 4, 5, 6, 7, and 11. On the other hand, compounds 1, 2, and 3 were the most abundant for the N2 $\times$ peat/perlite treatment and compounds 8 and 10 for the N1 $\times$ peat/perlite treatment. Flavanones were the most abundant class of phenolic compounds, accounting for 83.6% to 94.7% of total phenolic compounds in the N2 $\times$ peat/perlite and N0 $\times$ petta/perlite treatments, respectively. Moreover, the highest content of total flavanones (Tflav), total flavones (Tflavones), and total phenolic compounds (TPC) was recorded for the N1 $\times$ soil treatment, while total flavonol content was the highest for the N2 $\times$ peat/perlite treatment. Similarly to our study, Petropoulos et al. [17,20,21] also suggested flavanones as the major polyphenols in *C. raphanina* ssp. *mixta* plants grown in various agronomic conditions, while they suggested that successive harvesting and abiotic stress (saline conditions) may affect the total phenolic compound content, as well as the individual compound composition, resulting in varied

profiles of polyphenols. Considering the bioactive properties of polyphenols, the use of cost-effective cultivation practices that may regulate their composition could be a helpful means toward an increase in the quality and the beneficial health effects of the species.

Table 3. Retention time (Rt), wavelengths of maximum absorption in the visible region (λmax), mass spectral data, and tentative identification of the phenolic compounds present in the extracts of *Centaurea raphanina* spp. *mixta* plants.

Peak	Rt (min)	λmax (nm)	$[M - H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative Identification
1	14.16	349	493	317 (100)	Myricetin-*O*-hexoside
2	18.1	344	477	301 (100)	Quercetin-3-*O*-glucoside
3	18.63	334	461	285 (100)	Kaempherol-*O*-glucuronide
4	20.4	334	579	285 (100)	Kaempherol-*O*-hexosyl-pentoside
5	22.14	334	563	269 (100)	Apigenin-*O*-hexosyl-pentoside
6	22.9	334	445	269 (100)	Apigenin-*O*-glucuronide
7	25.44	332	665	621 (100), 285 (45)	Kaempherol-*O*-malonyl-pentoside
8	28.28	286/326	549	429 (12), 297 (14), 279 (5), 255 (41)	Pinocembrim arabirosyl glucoside
9	29.47	286/326	563	443 (12), 401 (5), 297 (21), 255 (58)	Pinocembrim neohesperidoside
10	31.39	288/328	591	549 (30), 429 (20), 297 (15), 279 (5), 255 (32)	Pinocembrim acetylarabirosyl glucoside
11	31.79	285/326	605	563 (12), 545 (5), 443 (30), 401 (10), 255 (40)	Pinocembrim acetyl neohesperidoside isomer I
12	32.14	286/328	605	563 (10), 545 (5), 443 (28), 401 (9), 255 (39)	Pinocembrim acetyl neohesperidoside isomer II

The antioxidant activity of leaf extracts was determined using two different assays, namely, TBARS and OxHLIA, and the results are presented in Table 5. The use of cell-based assays was selected since they can provide more useful information regarding target compounds that may have antioxidant effects at a subcellular level, compared to traditional spectrophotometric in vitro assays [57]. A varied response was observed, and the highest activity was recorded for the plants grown in peat/perlite substrate and the high nitrogen rate (600 ppm of N) in the case of OxHLIA assay or the control and the low nitrogen rate (0 and 200 ppm of N) in the case of TBARS assay. Contrasting results were reported in a previous study by Petropoulos et al. [21], where *C. raphanina* ssp. *mixta* plants were grown in the same peat/perlite substrate and received the same nitrogen treatments as in the present study. In particular, in that study, the plant of the second harvest showed the highest activity in the OxHLIA assay, especially the treatment that did not receive nitrogen fertilization, while the highest activity in the TBARS assay was recorded for plants that received 600 ppm of N at the same harvest. Therefore, despite the similar treatments for both studies, the observed differences could be attributed to the plant material used (plants started from seeds vs. transplanted wild plants), as well as to the fact that plants in our study were harvested only once and in different seasons compared to the previous study. According to Petropoulos et al. [30], successive harvesting and harvesting season may affect the antioxidant activity of plant matrices, while the developmental stage or the propagational material (seeds or transplanted wild plants) may also affect the response of plant extracts to various assays that determine antioxidant activity [17,29].

Table 4. Quantification (mg/g of plant fresh weight (fw)) of the phenolic compounds present in the extracts of *Centaurea raphanina* spp. *mixta* plants in relation to nitrogen fertilization and growth substrate (mean ± SD).

Treatments	1	2	3	4	5	6	7	8	9	10	11	12	Tfols	Tflavones	Tflav	TPC
N0 soil	0.088 ± 0.002 f	0.019 ± 0.001 g	0.041 ± 0.003 e	0.027 ± 0.004 d	0.03 ± 0.004 d	0.03 ± 0.01 c	0.018 ± 0.001 d	tr	0.9 ± 0.1 cd	0.04 ± 0.002 f	0.052 ± 0.004 d	0.43 ± 0.02 d	0.192 ± 0.001 g	0.057 ± 0.001 e	1.5 ± 0.1 e	1.7 ± 0.1 e
N1 soil	0.113 ± 0.002 b	0.025 ± 0.001 c	0.057 ± 0.001 b	0.034 ± 0.004 a	0.04 ± 0.001 a	0.035 ± 0.003 a	0.027 ± 0.004 a	0.067 ± 0.001 b	1.4 ± 0.1 a	0.127 ± 0.001 c	0.13 ± 0.01 a	1.4 ± 0.1 a	0.255 ± 0.001 b	0.075 ± 0.001 a	3.0 ± 0.1 a	3.4 ± 0.1 a
N2 soil	0.104 ± 0.003 d	0.021 ± 0.001 f	0.042 ± 0.001 e	0.028 ± 0.003 c	0.04 ± 0.002 a	0.033 ± 0.001 b	0.021 ± 0.001 b	tr	1.06 ± 0.02 c	0.034 ± 0.003 g	0.058 ± 0.002 c	0.43 ± 0.02 d	0.216 ± 0.001 f	0.073 ± 0.002 a	1.6 ± 0.1 e	1.9 ± 0.1 de
N3 soil	0.103 ± 0.001 d	0.022 ± 0.003 e	0.047 ± 0.003 d	0.029 ± 0.001 b	0.035 ± 0.002 b	0.03 ± 0.001 c	0.021 ± 0.001 b	tr	1.2 ± 0.1 bc	0.031 ± 0.002 g	0.062 ± 0.004 c	0.7 ± 0.1 c	0.223 ± 0.001 e	0.065 ± 0.001 c	1.9 ± 0.2 bc	2.2 ± 0.2 bc
N0 peat/perlite	0.095 ± 0.004 e	0.021 ± 0.003 f	0.045 ± 0.001 d	0.027 ± 0.001 d	0.03 ± 0.002 d	0.029 ± 0.002 c	0.019 ± 0.001 c	0.036 ± 0.004 c	1.1 ± 0.1 bc	0.102 ± 0.003 d	0.084 ± 0.01 b	0.39 ± 0.01 d	0.232 ± 0.001 d	0.061 ± 0.001 d	1.8 ± 0.1 cd	1.9 ± 0.1 de
N1 peat/perlite	0.108 ± 0.004 c	0.023 ± 0.002 d	0.047 ± 0.001 d	0.026 ± 0.004 d	0.029 ± 0.001 d	0.028 ± 0.001 d	0.021 ± 0.002 b	0.096 ± 0.002 a	0.85 ± 0.03 d	0.344 ± 0.001 a	nd	0.299 ± 0.003 e	0.226 ± 0.001 e	0.057 ± 0.001 e	1.59 ± 0.03 e	1.87 ± 0.03 de
N2 peat/perlite	0.114 ± 0.001 a	0.027 ± 0.004 a	0.069 ± 0.002 a	0.027 ± 0.001 d	0.036 ± 0.002 b	0.033 ± 0.001 b	0.021 ± 0.001 b	0.06 ± 0.01 b	1.1 ± 0.1 bc	0.196 ± 0.002 b	nd	0.31 ± 0.004 e	0.258 ± 0.001 a	0.069 ± 0.001 b	1.69 ± 0.02 de	2.02 ± 0.02 cd
N3 peat/perlite	0.108 ± 0.001 c	0.026 ± 0.002 b	0.053 ± 0.002 c	0.03 ± 0.001 b	0.032 ± 0.001 c	0.026 ± 0.003 e	0.021 ± 0.002 b	0.001 ± 0.0001 d	1.3 ± 0.2 ab	0.073 ± 0.003 e	nd	0.8 ± 0.1 b	0.238 ± 0.003 c	0.058 ± 0.001 e	2.1 ± 0.3 b	2.4 ± 0.3 b

Tr—traces; nd—not detected; N0: control (0 ppm N); N1: 200 ppm N; N2: 400 ppm N; N3: 600 ppm N. Tfols: total flavonols; Tflav: total flavanones; Tflavone: total flavones. Means in the same column followed by different letters are significantly different according to Tukey's HSD test (p = 0.05). Standard calibration curves used for quantification: apigenin-7-*O*-glucoside ($y = 10{,}683x - 45{,}794$, R^2 = 0.996, limit of detection (LOD) = 0.10 μg/mL and limit of quantification (LOQ) = 0.53 μg/mL, peaks 5 and 6); myrcetin ($y = 23{,}287x - 581{,}708$, R^2 = 0.9988, LOD = 0.23 μg/mL and LOQ = 0.78 μg/mL, peak 1); naringenin ($y = 18{,}433x + 78{,}903$, R^2 = 0.9998, LOD = 0.17 μg/mL and LOQ = 0.81 μg/mL, peaks 8, 9, 10, 11, and 12); and quercetin-3-*O*-glucoside ($y = 34{,}843x - 160{,}173$, R^2 = 0.9998, LOD = 0.21 μg/mL and LOQ = 0.71 μg/mL, peaks 2, 3, 4, and 7).

Table 5. Antioxidant activity, cytotoxicity and antitumor activity of *Centaurea raphanina* spp. *mixta* plants in relation to nitrogen fertilization and growth substrate (mean ± SD).

	N0 Soil	N1 Soil	N2 Soil	N3 Soil	N0 Peat/Perlite	N1 Peat/Perlite	N2 Peat/Perlite	N3 Peat/Perlite
				Treatments				
Antioxidant activity								
OxHLIA (IC$_{50}$; µg/mL); Δt = 60 min	280 ± 8 b	327 ± 9 a	108 ± 8 f	159 ± 11 c	163 ± 8 c	136 ± 6 d	116 ± 7 e	43 ± 3 g
TBARS (EC$_{50}$, µg/mL)	87 ± 2 a	41 ± 1 d	64 ± 4 b	58 ± 2 c	18.4 ± 0.7 f	19.2 ± 0.8 f	57 ± 3 c	38 ± 1 e
Cytotoxicity to non-tumor cell lines (GI$_{50}$ values µg/mL)								
PLP2	350 ± 10	>400	>400	>400	>400	>400	>400	>400
Cytotoxicity to tumor cell lines (GI$_{50}$ values µg/mL)								
HeLa	211 ± 4 g	>400	249 ± 15 d	237 ± 10 e	259 ± 25 c	223 ± 11 f	266 ± 24 b	288 ± 13 a
HepG2	325 ± 22 a	>400	>400	253 ± 12 c	301 ± 3 b	330 ± 12 a	247 ± 14 c	333 ± 13 a
MCF-7	296 ± 20 b	>400	>400	331 ± 16 a	278 ± 11 c	>400	>400	274.8 ± 0.4 d
NCI-H460	316 ± 26 a	>400	>400	283 ± 22 b	266 ± 10 c	255 ± 10 d	249 ± 10 e	>400

EC$_{50}$: extract concentration corresponding to a 50% of antioxidant activity. Trolox EC$_{50}$ values: 23 µg/mL (thiobarbituric acid reactive substances (TBARS) inhibition) and 19.6 µg/mL (oxidative hemolysis inhibition assay (OxHLIA) Δt = 60 min). GI$_{50}$ values correspond to the sample concentration responsible for 50% inhibition of growth in tumor cells or in a primary culture of liver cells-PLP2. GI$_{50}$ values for ellipticine (positive control): 1.2 µg/mL (MCF-7), 1.0 µg/mL (NCI-H460), 0.91 µg/mL (HeLa), 1.1 µg/mL (HepG2), and 2.3 µg/mL (PLP2). N0: control (0 ppm N); N1: 200 ppm N; N2: 400 ppm N; N3: 600 ppm N. Means in the same row followed by different letters are significantly different according to Tukey's HSD test (p = 0.05).

Cytotoxic effects are presented in Table 5, where only the control treatment of soil grown plants showed a mild toxicity against porcine liver primary culture cell lines (350 µg/mL). Moreover, the same treatment was the most efficient against cervical carcinoma cell lines (211 µg/mL), while the N3 soil and N2 peat/perlite treatments were the most effective against HepG2 cell lines (253 and 247 µg/mL, respectively). Lastly, the N3 and N2 treatments of peat/perlite-grown plants had the lowest GI$_{50}$ values against breast carcinoma and non-small-cell lung cancer cell lines (274.8 µg/mL and 249 µg/mL, respectively). Contrasting results were reported in previous studies where *C. raphanina* ssp. *mixta* plants were tested under different agronomic practices [17,19–21]. In particular, wild plants (transplanted or collected in the wild) showed similar mild toxic effects against PLP2 cell lines, whereas plants grown from seeds that were subjected to different nitrogen application rates or saline conditions did not show such effects. Similarly, the same studies also showed a varied response against tumor cell lines, depending on the propagational material and the agronomic practices tested. These findings indicate that cytotoxic effects should be attributed to various compounds whose biosynthesis is up- or downregulated depending on the growing conditions and agronomic practices. Lastly, the extraction protocol may also affect the phytochemical composition of the obtained extracts and result in variable cytotoxic effects against tumor and non-tumor cell lines [55,58,59].

The antimicrobial properties of the tested extracts are presented in Table 6. None of the studied extracts exhibited higher antibacterial activity than the positive controls used (streptomycin, ampicillin), a finding that is in agreement with previous reports for the species [17,20,21], which further justifies its mild effectiveness against specific bacteria (Table 6). Moreover, the comparison of the treatments showed significant differences and a varied effectiveness depending on the bacteria and the growth substrate × nitrogen rate treatment. In particular, the soil-grown plants that received 0 or 400 ppm of N were the most effective against *Staphylococcus aureus* and *Listeria monocytogenes*, while the same treatments and the control treatment of peat/perlite-grown plants were the most effective against *Enterobacter cloacae*. Lastly, most of the growth substrate × nitrogen combinations showed similar effectiveness against *Bacillus cereus*, *Escherichia coli*, and *Salmonella typhimurium*. This variability in the antibacterial activity of *C. raphanina* ssp. *mixta* leaf extracts was also reported in other studies, where the effect of nitrogen rate [17] or growing conditions [20,21] was clearly indicated. Moreover, Karkanis et al. [31] highlighted the impact that growth substrate may have on the antibacterial effects of *Sanguisorba minor* extracts, suggesting that changes in phytochemical content and composition could be the main reason for

these findings. The studies of Ćirić et al. [60] and Tekeli et al. [61] identified sesquiterpene lactones as the compounds responsible for the antibacterial activities of the *Centaurea* genus, while Mikropoulou et al. [19] indicated pinocembrin as the major phytochemical with significant bioactive properties.

Table 6. Antibacterial activity (MIC and MBC mg/mL) and antifungal activity (MIC and MFC mg/mL) of *Centaurea raphanina* spp. *mixta* plants in relation to nitrogen fertilization and growth substrate (mean ± SD).

Treatments	Antibacterial Activity	*S. aureus*	*B. cereus*	*L. monocyto-genes*	*E. coli*	*S. typhimurium*	*E. cloacae*
N0 soil	MIC	0.5	0.5	1	0.5	1	1
	MBC	1	1	2	1	2	2
N1 soil	MIC	1	1	2	0.5	2	2
	MBC	2	2	4	1	4	4
N2 soil	MIC	0.5	0.5	1	1	1	1
	MBC	1	1	2	2	2	2
N3 soil	MIC	1	0.5	2	0.5	2	2
	MBC	2	1	4	1	4	4
N0 peat/perlite	MIC	1	0.5	2	1	1	1
	MBC	2	1	4	2	2	2
N1 peat/perlite	MIC	1	0.5	2	0.5	2	2
	MBC	2	1	4	1	4	4
N2 peat/perlite	MIC	1	0.5	2	0.5	1	2
	MBC	2	1	4	1	2	4
N3 peat/perlite	MIC	1	0.5	2	0.5	1	2
	MBC	2	1	4	1	2	4
Streptomycin	MIC	0.1	0.025	0.15	0.1	0.1	0.025
	MBC	0.2	0.05	0.3	0.2	0.2	0.05
Ampicillin	MIC	0.1	0.1	0.15	0.15	0.1	0.1
	MBC	0.15	0.15	0.3	0.2	0.2	0.15

	Antifungal Activity	*Aspergillus fumigatus*	*Aspergillus niger*	*Aspergillus versicolor*	*Penicillium funiculosum*	*Trichoderma viride*	*Penicillium verrucosum* var. *cyclopium*
N0 soil	MIC	0.5	0.5	0.5	0.12	0.12	0.12
	MFC	1	1	1	0.25	0.25	0.25
N2 soil	MIC	0.5	0.5	0.5	0.5	0.25	0.25
	MFC	1	1	1	1	0.5	0.5
N3 soil	MIC	0.5	0.5	0.5	0.5	0.25	0.25
	MFC	1	1	1	1	0.5	0.5
N0 peat/perlite	MIC	0.5	0.5	0.5	0.25	0.12	0.25
	MFC	1	1	1	0.5	0.25	0.5
N1 peat/perlite	MIC	0.25	0.5	0.5	0.5	0.25	0.25
	MFC	0.5	1	1	1	0.5	0.5
N2 peat/perlite	MIC	0.5	0.5	0.5	0.25	0.12	0.12
	MFC	1	1	1	0.5	0.25	0.25
N3 peat/perlite	MIC	0.25	0.5	0.5	0.5	0.12	0.12
	MFC	0.5	1	1	1	0.25	0.25
Streptomycin	MIC	0.15	0.15	0.1	0.2	0.15	0.1
	MFC	0.2	0.2	0.2	0.25	0.2	0.2
Ampicillin	MIC	0.2	0.2	0.2	0.2	1	0.2
	MFC	0.5	0.5	0.5	0.5	1.5	0.3

MIC—minimal inhibition concentration; MBC—minimal bactericidal concentration; MFC—minimal fungicidal concentration; N0: control (0 ppm N); N1: 200 ppm N; N2: 400 ppm N; N3: 600 ppm N.

On the other hand, the results of the antifungal properties were more promising than the antibacterial ones since specific treatments were equally effective to or more effective than the tested controls (bifonazole, ketoconazole; Table 6). In particular, the N0 × soil

treatment exhibited the highest minimal inhibition concentration (MIC) values when compared to the remaining treatments or the positive controls, while the values of minimal fungicidal concentration for the same treatment were equal to bifonazole. Similarly, the MIC values of the N0 × soil, and N0, N1, and N3 × peat/perlite treatments against *Trichoderma viride* were higher than the positive controls and the remaining treatments. For the remaining tested fungi (*Aspergilus fumigatus*, *A. niger*, *A. versicolor*, and *Penicillium verrucosum* var. *cyclopium*), the MIC and MFC values or the treatments were lower than the positive controls indicating mild antifungal activity. When comparing the tested treatments with each other, the N1 and N3 × peat/perlite treatments were the most effective against *A. fumigatus*, while the N0 × soil and N2, N3 × peat:perlite treatments were the most effective combinations against *Penicillium verrucosum* var. *cyclopium*. Lastly, no significant differences were observed among the tested treatments with regard to their efficiency against *A. niger* and *A. versicolor*. The effectiveness of *C. raphanina* ssp. *mixta* extracts against *T. viride* has also been confirmed in the literature in several studies [17,18,20,21], while Panagouleas et al. [18] suggested the effectiveness of wild *C. raphanina* ssp. *mixta* plants against many other fungi due to the high content of extracts in cnicin and flavonoids [18].

4. Conclusions

In conclusion, the present results suggest that the application of cost-effective practices such as the nitrogen application and the selection of growth substrate may regulate the chemical composition and the bioactive properties of *C. raphanina* ssp. *mixta* species and increase its added value under commercial cultivation conditions. For most parameters, the application of up to 400 ppm of total nitrogen in soil- and/or peat/perlite-grown plants was beneficial to specific compounds, whereas the highest nitrogen rate was beneficial for α-linolenic and linoleic acid content and the antioxidant (OxHLIA) and cytotoxic activity against breast carcinoma cell lines. Therefore, this variable response observed for the tested parameters indicates that the proper selection of nitrogen application rates and growing medium may regulate targeted compounds such as tocopherols, polyphenols, organic acids, and free sugars and improve the bioactive properties of the species. Moreover, considering the work already published in the literature in wild and cultivated plants, the research with various ecotypes of *C. raphanina* ssp. *mixta* could allow the selection of elite genotypes with high potential for the commercial exploitation of this valuable species. Another aspect to be considered for future research is the effect of nutrient solution composition and the ratio of ammonium to nitrate nitrogen in the nutrient solution, focusing on reducing the high oxalic acid content of the domesticated species.

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