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Polyphenol Profile, Antioxidant Activity and Yield of *Cynara cardunculus altilis* in Response to Nitrogen Fertilisation

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Abstract: Cardoon leaves are of great pharmaceutical importance due to their high content of polyphenol compounds. Polyphenolic compounds have attracted much interest due to their health-promoting effects. The content of these compounds in *C. cardunculus* depends on several factors, such as genotype, crop management, plant tissues, harvest time, and storage time. In this study, the effects of nitrogen (N) fertilisation (rates and forms) on the biomass yield and polyphenol profile of the leaves were determined. Increasing the amount of N up to 180 kg-ha^{-1} in fertilisation did not significantly increase the air-dried biomass yield of the leaves. On the contrary, it led to lower concentrations of total phenolic compounds (TP), total flavonoids (TF), caffeic acid, cynarin, and luteolin. Improvements in performance were achieved when 120 kg-ha^{-1} N rate was applied and increases in TP, TF content, and radical scavenging activity were observed. The applied N forms (NO_3, NH_4 or urea) had different effects on the concentrations of individual compounds and leaf air-dried biomass. Higher concentrations of cynarin, luteolin, and luteolin-7-*O*-glucoside were found when the N forms NH_4 and urea were applied; higher caffeic acid content was found when urea was applied. The application of NO_3 and urea in fertilisation reduced the level of luteolin-7-*O*-rutinoside, while the application of NO_3 and NH_4 reduced the amount of caffeic acid. The obtained results provide a better understanding of the effects of N rates and forms on cardoon leaves over two growing seasons.

Keywords: cardoon; nitrogen fertilisation; phenolic acids; flavonoids; antioxidant activity; biomass yield

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1. Introduction

The genus *Cynara* belongs to the *Asteraceae* family and includes seven species, which, in their natural state, are found only in the Mediterranean region. The species *Cynara cardunculus* L. consist of the following three botanical varieties: globe artichoke var. *scolymus* L., cultivated cardoon var. *altilis* DC, and their common ancestor, the wild cardoon (var. *sylvestris* Lamk) [1]. Cardoons have long been cultivated in warm climate countries, where the leaves are eaten as a vegetable [2]. In Central Europe, the cultivated cardoon is primarily a valuable herbaceous plant for pharmaceutical raw materials [3,4]. Cardoon is considered a rich source of health-promoting compounds such as polyphenols, inulin, vitamins, and minerals [5–7]. In the last few years, polyphenolic compounds have attracted much interest due to their effects on human health, which include cholagogic, cholepoietic, anticancer, anti-inflammatory, anti-allergenic, and antiviral effects [8,9].

The phytochemicals contained in *C. cardunculus* leaves are attributed to effective action against disorders of the digestive and circulatory systems, protect the body against cancer, and stimulate the immune system [10]. The phenolic acids have a cholagogic effect, enhancing the transport of bile into the duodenum [11]. They increase the amount and flow of bile, which helps to reduce harmful substances that threaten the liver [12]. The increased bile secretion caused by cardoon extract has the effect of lowering serum triglyceride levels [10]. In patients with lipid metabolism disorders, a reduction in low-density lipoprotein (LDL) cholesterol levels after systematic use of cardoon extract was confirmed [13]. The polyphenol compounds of cardoons strengthen and regenerate liver cells [14] and show a liver-shielding effect [15]. The bioactive compounds of cardoons reduce elevated blood pressure, prevent atherosclerosis, and lower triglycerides and cholesterol [16]. The content of these compounds in *C. cardunculus* depends on several factors, such as genotype [17], crop management [18], and plant tissues [19].

The effect of nitrogen fertilisation on the yield and quality of globe artichokes has been studied by other authors under Mediterranean climate conditions [20–26]. Few studies have investigated the impact of N fertilisation in the cultivation of globe artichokes in Central Europe [27,28]. However, published papers on both globe artichokes and cardoons are contradictory. For example, some studies reported that increasing the amount of nitrogen increased the green weight of cardoon leaves but decreased the content of caffeoylquinic acids in the raw material [27,28]. In the Mediterranean Basin, N doses above 100 kg ha^{-1} delayed the harvest of cardoon baskets without an apparent yield-forming effect. Shinohara et al. concluded that the phenolic content was unaffected by the N rate [22], while Lombardo et al. observed an influence of the N rate on the nutrition quality of globe artichokes [24]. Montesano et al. noted a significant "N rate × globe artichoke cultivars" interaction with respect to flavonoid contents [29]. To our knowledge, data available on the polyphenol profile of cardoons in relation to N rates are missing. It is also known that different N forms significantly affect crop yield and quality [30,31].

For the pharmaceutical industry, the green rosettes of cardoon leaves are of great importance due to the large amount of caffeic acid derivatives. The climatic conditions of Central Europe (Poland) favour vegetative growth (leaf formation) and, therefore, create favourable conditions for the cultivation of cardoons as a medicinal plant. Given the poor availability of data on the cultivation of cardoons for the pharmaceutical industry, this study aims to investigate the effects of N rates and forms on biomass yield, the level of polyphenol compounds, and the radical scavenging activity of cardoon leaves over two growing seasons.

2. Materials and Methods

2.1. Site, Climate, and Soil Characteristics

Field experiments were conducted at the Felin research station of the Lublin University of Life Sciences (51.23° N, 22.56° E). The average temperature of the growing season (April–October) for the years 1955–2012 was 13.7 °C, lower by about 2.9 °C in 2018 and by 1.9 °C in 2019 (Table 1). The total precipitation in the years of the study varied compared to the average multi-year total. In detail, in 2018 the amount of precipitation was 33 mm higher, while in 2019 it was 28 mm lower. The individual growing seasons were characterised by high variability. The highest rainfall was recorded in July 2018 (124 mm) and in August 2019 (102 mm). Compared to the average multi-year total, less rainfall was recorded in 2019 during the period of intensive cardoon growth in June and July (38 mm). From April to October 2018, the mean minimum temperature and the mean daily temperature were higher than in 2019 (1.1 and 1.7 °C, respectively) (Table 2). The highest number of sunshine hours (1349) was seen in the cardoon growing period in 2018.

Table 1. Meteorological conditions during the two growing seasons of the experiment (Meteorological Station in Felin, Poland 51.13° N; 22.37° E).

Growing Season	Month							Average/Sum
	April	May	June	July	August	September	October	
Average Temperature (°C)								
2018	13.4	17.1	18.8	20.7	20.7	15.5	10.0	16.6
2019	9.5	13.4	21.5	19.4	20.3	14.5	11.0	15.6
1955–2012	7.4	13.0	16.2	17.8	17.1	12.6	12.4	13.7
Total Rainfall (mm)								
2018	40	56	65	124	72	68	36	461
2019	49	93	37	38	102	52	29	400
1955–2012	39	58	66	84	69	54	58	428

Table 2. Averages temperatures (°C) during the two growing seasons of the experiment.

Growing Season	Month	Temperature (°C)		Insolation (Sum of Hours)
		Average Maximum	Average Minimum	
2018	April	15.1	13.3	216
	May	18.8	14.4	245
	June	20.4	16.5	206
	July	22.6	19.4	169
	August	23.2	18.1	214
	September	20.3	17.7	165
	October	19.7	16.8	134
	Average/Total	20.0	16.6	209/1349
2019	April	14.1	10.3	183
	May	18.0	13.4	128
	June	22.2	15.5	253
	July	23.1	18.4	198
	August	24.2	19.1	205
	September	18.3	16.6	140
	October	17.7	15.8	138
	Average/Total	19.6	15.5	178/1245

Cultivation was carried out on grey loam soil [32] made of medium silty clay. The characteristics of the soil on which the experiment was conducted are shown in Table 3.

Table 3. Soil characteristics and mineral content in the experimental field.

Characteristics Soil								
Silt (%)	**Loam (%)**	**Sand (%)**	**pH in H_2O**	**Na (mg L^{-1})**	**Cl (mg L^{-1})**	**Salinity (g L^{-1} NaCl)**	**Organic Matter %C**	**C/N**
69	10	21	7.36	57.1	6.13	0.29	1.42	11.3
Macro and Microelement Content (mg L^{-1})								
N-NO_3	**P**	**K**	**Ca**	**Mg**	**Zn**	**Mn**	**Cu**	**Fe**
10.6	204	132	1293	106	8.72	12.80	5.24	42.8

2.2. Plant Material, Experimental Design, and Management Practices

The trials were carried out using cv. Blanco Avorio, provided by the Rijnsburg Seed Company (Rijnsburg, The Netherlands). This cultivar is characterized by a rapid growth rate, abundant foliage, and high bioactive compound contents [33]. In each growing season,

the experiment was set up in a completely randomised block arrangement with three replications. Plants were planted at 0.4 × 0.4 m spacing in plots with areas of 10 m^2 (6.25 plant m^{-2}). Experimental treatments included three levels of nitrogen fertilisation [0 (control), 60, 120, and 180 kg of N ha^{-1}], and three forms of nitrogen: NO_3, NH_4 and $CO(NH_2)_2$—urea [27]. In each growing season, the plants were fertilised with the following amounts of nitrogen (kg ha^{-1}): once 60 (10 May), twice 120 (60 + 60, 10 May and 3 June), and three times 180 (60 + 60 + 60, 10 May, 3 June, 10 July). On the first date, nitrogen fertilisation was carried out before planting (10 May); on the second date at the 7–8 leaf stage (3 June); and on the third date at the 18–20 leaf stage (10 July). For each treatment, the same levels of P and K fertilisation were applied before planting, 44 kg ha^{-1} P_2O_5 and 140 kg ha^{-1} K_2O as a form of mineral: calcium dihydrogen phosphate (46%) and potassium sulphate (50%), respectively. In 2018–2019, seedlings prepared from sowing seeds (11 April) into pots (90 cm^3) in a peat substrate were planted on the same date, 10 May. On the day of planting, the plants were about 12–15 cm tall with 3–5 proper leaves. The cardoons were cultivated according to local practices of ploughing (30 cm deep), harrowing, and fertilising before planting. Common beans were grown in the previous season. Cardoons are grown as annuals because they freeze and die when temperatures drop to around −10 °C. Cardoons are mainly grown from seedlings produced in heated plastic tunnels. In May, after frost, when the plants have 3–5 proper leaves and are about 10 cm high, the plants are planted out in the field. In the field, mineral fertilisation of 90 kg ha^{-1} is applied in spring. Care of the cardoon crop consists of early weeding and loosening of the inter-rows. In dry periods, mineral fertilisation is often combined with drip irrigation. No crop irrigation was used in this experiment. Hand weeding of the inter-rows was carried out twice during the cardoon cultivation period. No protective measures were applied.

2.3. Harvesting of Raw Material and Post-Harvest Treatment

The leaves' biomass was harvested once from 120-day-old plants from each plot. The harvest was conducted in 2018 on 10 August and again on 15 August 2019. Plants had similar developmental characteristics (plant height 40.0–45.0 cm, plants formed a leaf rosette). Based on the fresh biomass after drying, the yield of the air-dried leaf mass was calculated. Each of the three replications was randomly sampled for chemical analyses.

The biomass from each combination of N rate, N form, and repetition was dehydrated in a thermal drier (Ventech, Poland) at an air temperature of 60 °C. After drying, the water content of the leaves was observed to be 12–14% over 5 consecutive determinations. The dry material was ground and passed through a 1 mm sieve and used for further chemical analyses, which were carried out over 30 days.

2.4. Chemicals and Standards

The pure caffeoylquinic acids and flavonoids: 5-*O*-caffeoylquinic acid (chlorogenic acid), 3,4dihydroxycinnamic acid (caffeic acid), 1,3-di-caffeoylquinic acid (cynarin), luteolin-7-*O*-glucoside, luteolin-7-*O*-rutinoside that were used for determination or calibration were purchased as certified materials from Merck (Darmstadt, Germany). All solvents and reagents used for the preparation of standard solutions and extraction of polyphenols were of analytical-grade purity. They were obtained from Sigma-Aldrich (St. Louis, MO, USA). Methanol, acetonitrile, and other chemicals were purchased from Avantor Performance Materials (Gliwice, Poland).

2.5. Sample Preparation

The powdered cardoon leaves (~3 g) were mixed with methanol (80:20, *v*:*v*, methanol to water) in an RVO 400 SD rotary vacuum evaporator (Ingos, Prague, Czech Republic) at a temperature of 100 ± 8 °C for 3 h. After filtration through Whatman filter paper, gradation 42 (Merck, Warsaw, Poland), the residue was re-extracted with 80% methanol for 2 h at room temperature. Solutions were left in the refrigerator for 24 h. The filtrate was degreased by shaking with light petroleum (30 mL each). Then, purified water solutions

were extracted with diethyl ether (20 mL each). The extracts with diethyl ether were again shaken with this solvent (10 mL each). Ether extracts were joined and dried with anhydrous Na_2SO_4. All samples were stored at T = −22 °C until further analysis. All extractions were performed in duplicate.

2.6. Determination of Total Polyphenols and Flavonoids

The Folin–Ciocalteu assay [34] was used to assess the total polyphenol (TP) content. Sodium carbonate (64 mL, 6% in distilled water) was added to the sample extracts (0.2 mL), and after 1 min, 0.2 mL of the freshly diluted Folin–Ciocalteu reagent was added. The mixture was incubated for 2 h at room temperature, and the absorbance was read at 750 nm using a UV–Vis spectrophotometer (Model UV-1800, Shimadzu Corp., Kyoto, Japan). TP content was standardised against gallic acid and expressed as g of gallic acid equivalents (GAE) kg^{-1} of dry matter (DM).

The aluminium chloride colorimetric method [35] was used for the determination of total flavonoids (TF). The absorbance of the aluminium chloride solution was measured after 45 min against a reference (sample without aluminium chloride) at λ = 425 nm on a Univikon-932 spectrophotometer (Kontron Instruments). The TF content of the raw material was calculated according to the formula, and the results were expressed as g of quercetin equivalents (QE) kg^{-1} DM:

$$X = \frac{A \times K}{M}$$

where:

A—absorbance of the test solution,
K—conversion factor for quercetin acid K = 3.5087,
M—raw material weight.

2.7. HPLC Analysis

Phenolic compounds of air-dried cardoon leaf extracts were separated by high-performance liquid chromatography (HPLC) on a Shimadzu series UFLC instrument (Shimadzu Corp., Tokyo, Japan) coupled to a diode array detector (DAD). The separation was performed on a Phenomenex Synergi Fusion-RP column (4 µm, 250 × 4.6 mm i.d., Phenomenex, Santa Clara, CA, USA) with a sample injection volume of 20 µL. The mobile phase consisted of acetonitrile (eluent A) and 0.1% of formic acid (eluent B). The following gradient programme was applied: 20% A (0 min), 25% A (10 min), 25% A (20 min), 50% A (40 min), 100% A (42–47 min), and 20% A (49–55 min). The flow rate was 1 ml min^{-1}, and the temperature was 30 °C. Detection was performed by scanning in a wavelength range from 190 to 400 nm. The contents of individual phenolic compounds were expressed from the calibration curves of the respective standards according to the recommended IUPAC numbering system [36]. For the qualitative and quantitative profile of polyphenols, the protocol described by Lombardo et al. [24] was adopted. Values were expressed in mg kg^{-1} DM.

2.8. Radical Scavenging Activity Assay

2.8.1. 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic Acid (ABTS) Assay

Methanol extracts were used to determine the antioxidant activity by measuring their ability to scavenge free radicals, i.e., $ABTS^+$ [37]. Absorbance was detected at a wavelength of λ = 734 nm using the spectrophotometer. The values obtained for each sample were compared with the concentration curve of a standard Trolox equivalents (TE) solution and expressed as µM TE 100 g^{-1} DM, respectively.

2.8.2. Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP reagent was prepared according to the procedure described by Gouveia and Castilho, with modifications [38]. Briefly, for each analysis, 30 µL of methanolic solution

was added to 200 µL of distilled water and 1.9 mL of the FRAP solution. The increase in absorbance was recorded at 593 nm in 15 s intervals, for a period of 30 min at 35 °C.

Methanolic solutions of known Fe (II) concentrations were used to prepare the calibration curve. FRAP results were expressed as µM Fe^{2+} 100 g^{-1} DM.

2.8.3. 2,2-diphenyl-1-picrylhydrazyl (DPPH) Assay

The radical-reducing activity of DPPH was determined according to Choi et al., with cation changes [39]. Ethanolic extracts (0.2 mL) were added to 0.8 mL of 0.2 mM ethanolic DPPH solution and kept in the dark for 15 min. Then, the absorbance was recorded at λ = 515 nm. Results are given as µmol of TE 100 g^{-1} DM.

2.9. Statistical Analysis

Data on biomass yield, radical scavenging activity and polyphenol compounds were subjected to a three-factor analysis of variance (N rate × N form × growing season). Analysis of variance (ANOVA) was performed using Statistica PL ver. 13.0 (StatSof Inc., Tulsa, OK, USA). The conformity of the distribution of the determined parameters to the normal distribution was checked using Levene's test for homogeneity of variance and Shapiro–Wilk's test for samples. Results were considered statistically significant at $p \leq 0.05$, and homogeneous groups were identified using the Tukey test. All experiments were performed in triplicate and data in tables and figures are averages of three determinations. Relationships between parameters were assessed by calculating Pearson's correlation coefficients. The functions available in the Statistica package were tested, and the most straightforward function with a sufficiently high coefficient of determination (minimum 0.3) was selected.

3. Results and Discussion

3.1. Effect of N Rate and Form on Leaf Biomass Yields

In the experiment, as the amount of N increased (from 0 to 120 kg ha^{-1}), the weight of dry leaf per plant and the yield of air-dried biomass increased (Table 4). Many previous studies on *C. cardunculus* have demonstrated that N fertilisation increases the yield at lower/medium N rates [29]. In the crop without N, the yield of air-dried biomass was 4.27 t ha^{-1}. N fertilisation at 60, 120, and 180 kg ha^{-1} increased the air-dried biomass yield by 50%. In a German study, increasing N rates (from 40 to 240 kg ha^{-1}) increased yields but resulted in lower quality, increased leaf nitrate content, and lower levels of polyphenolic acids and flavonoids [27,28]. In addition, the authors determined an optimal N fertilisation of 120 kg ha^{-1} before the first leaf harvest and 50 kg ha^{-1} before the second harvest. In our study, N fertilisation in each growing season affected the mean leaf weight and air-dried leaf yield ('N rate × growing season' interaction).

The N forms used (NO_3, NH_4, urea) in fertilising the cardoon plants affected yields. When the amide form (urea) and NO_3 were applied, the dry leaf yield of cardoon was higher by an average of 0.72 t ha^{-1} compared to the NH_4 form. Fertilisation with urea increased the average cardoon leaf weight (151 g). Similarly, Matthes and Honermeier found a reduction in cardoon leaf yield and dry matter when the NH_4 form was applied, especially under drought-stress conditions [28]. It has already been stated that, in herbaceous crops, it is crucial to select forms of N that do not harm plant development. High concentrations of NH_4 can cause plant death due to ammonia toxicity and lead to reduced yields [27,28].

Table 4. Effects of treatments (N rate, N form, and growing season) on leaf air-dried biomass, yield of leaf air-dried biomass, (±standard deviation) in cardoon leaves.

Treatment	Source of Variation	Leaf Air-Dried Biomass (g plant^{-1})	Yield of Leaf Air-Dried Biomass (t ha^{-1})
N rate (N) kg ha^{-1}	0	89.50 ^{b} ± 0.16	4.27 ^{b} ± 0.05
	60	165.17 ^{a} ± 0.22	6.40 ^{a} ± 0.06
	120	160.83 ^{a} ± 0.25	6.07 ^{a} ± 0.19
	180	162.50 ^{a} ± 0.28	6.22 ^{a} ± 0.20
	p-value	<0.001	<0.001
N form (F)	NO_3	143.25 ^{b} ± 0.18	5.91 ^{a} ± 0.18
	NH_4	139.25 ^{b} ± 0.27	5.26 ^{b} ± 0.16
	Urea	151.00 ^{a} ± 0.23	6.05 ^{a} ± 0.22
	p-value	0.001	0.001
Growing season (S)	2018	149.00 ^{a} ± 0.24	6.30 ^{a} ± 0.43
	2019	140.25 ^{a} ± 0.19	5.18 ^{b} ± 0.38
	p-value	0.340	0.009
N × F	*p*-value	0.284	0.065
N × S	*p*-value	0.034	0.008
F × S	*p*-value	0.206	0.065
N × F × S	*p*-value	0.002	0.162

Different letters within each column and main factor indicate a significant difference among means.

3.2. Effect of N Rate and Form on Polyphenol Profile

The applied N fertilisation of 120 kg ha^{-1} compared to the crop without N increased the TF content by 0.57 g kg^{-1} DM in the leaves of cardoons and did not affect TP levels (Table 5). The application of a higher rate of 180 kg ha^{-1} in N fertilisation resulted in lower TP, TF levels, and AA values (ABTS, FRAP, DPPH), which is in agreement with the studies of Baier et al. [27] and Montesano et al. [29].

Table 5. Effects of treatments (N rate, N form, and growing season) on total flavonoids and polyphenols content (± standard deviation) in cardoon leaves.

Treatment	Source of Variation	Total Flavonoids (g Quercitin kg^{-1} DM)	Total Polyphenols (g Gallic Acid kg^{-1} DM)
N rate (N) kg ha^{-1}	0	17.93 ^{b} ± 0.15	33.05 ^{a} ± 1.33
	60	16.01 ^{c} ± 0.25	32.52 ^{a} ± 3.14
	120	18.50 ^{a} ± 0.18	33.28 ^{a} ± 1.36
	180	16.39 ^{c} ± 0.27	28.12 ^{b} ± 0.96
	p-value	0.008	<0.001
N form (F)	NO_3	17.33 ^{a} ± 0.25	33.58 ^{a} ± 1.01
	NH_4	17.14 ^{a} ± 0.18	30.60 ^{a} ± 1.05
	Urea	17.15 ^{a} ± 0.29	31.05 ^{a} ± 0.95
	p-value	0.430	0.345
Growing season (S)	2018	17.83 ^{a} ± 0.25	33.23 ^{a} ± 1.98
	2019	16.59 ^{b} ± 0.27	30.26 ^{b} ± 1.05
	p-value	<0.001	0.001
N × F	*p*-value	0.065	0.060
N × S	*p*-value	0.322	0.716
F × S	*p*-value	0.868	0.145
N × F × S	*p*-value	0.003	<0.001

Different letters within each column and main factor indicate a significant difference among means.

In our study, N fertilisation at 120 kg ha^{-1}, compared to the crop without N fertilisation, increased the content of chlorogenic acid by 28% and cynarin by 30%, and decreased the level of caffeic acid (Table 6). The applied N dose of 120 kg ha^{-1} did not significantly increase the levels of luteolin, luteolin-7-*O*-glucoside, and luteolin-7-*O*-rutinoside (Table 7). At the applied N dose of 60 kg ha^{-1}, plants accumulated less chlorogenic acid, luteolin, and luteolin 7-*O*-glucoside. At a dose of N 180 kg ha^{-1}, plants accumulated less caffeic acid, cynarin, luteolin, and luteolin-7-*O*-glucoside.

Table 6. Effects of main factors (N rate, N form, growing season) on chlorogenic acid, caffeic acid, cynarin (mg kg^{-1} DM ± standard deviation) contents in cardoon leaves.

Main Factor	Source of Variation	Chlorogenic Acid	Caffeic Acid	Cynarin
N rate (N) kg ha^{-1}	0	542.26 ^{b} ± 0.32	71.65 ^{a} ± 0.09	63.35 ^{b} ± 0.02
	60	449.57 ^{c} ± 0.28	75.77 ^{a} ± 0.05	82.33 ^{a} ± 0.05
	120	692.80 ^{a} ± 0.45	67.77 ^{b} ± 0.03	82.10 ^{a} ± 0.06
	180	511.43 ^{b} ± 0.30	52.29 ^{c} ± 0.02	58.60 ^{c} ± 0.18
	p-value	<0.001	<0.001	<0.001
N form (F)	NO_3	572.67 ^{a} ± 0.48	61.91 ^{b} ± 0.02	65.11 ^{b} ± 0.04
	NH_4	515.94 ^{a} ± 0.51	61.54 ^{b} ± 0.05	73.75 ^{a} ± 0.08
	Urea	558.43 ^{a} ± 0.45	77.17 ^{a} ± 0.09	75.93 ^{a} ± 0.12
	p-value	0.170	0.034	0.001
Growing season (S)	2018	650.11 ^{a} ± 0.65	67.77 ^{a} ± 0.05	76.43 ^{a} ± 0.07
	2019	447.92 ^{b} ± 0.24	65.97 ^{a} ± 0.02	66.76 ^{b} ± 0.09
	p-value	0.006	0.054	<0.001
N × F	*p*-value	0.378	0.115	0.474
N × S	*p*-value	0.007	0.698	0.326
F × S	*p*-value	0.268	0.898	0.075
N × F × S	*p*-value	0.567	<0.001	<0.001

Different letters within each column and main factor indicate a significant difference among means.

Table 7. Effects of main factors (N rate, N form, growing season) on luteolin, luteolin-7-*O*-glucoside and luteolin-7-*O*-rutinoside (mg kg^{-1} DM ± standard deviation) contents in cardoon leaves.

Main Factor	Source of Variation	Luteolin	Luteolin-7-*O*-Glucoside	Luteolin-7-*O*-Rutinoside
N rate (N) kg ha^{-1}	0	40.76 ^{a} ± 0.09	13.86 ^{a} ± 0.01	113.43 ^{a} ± 0.35
	60	37.23 ^{b} ± 0.02	12.66 ^{b} ± 0.05	115.15 ^{a} ± 0.35
	120	40.90 ^{a} ± 0.05	13.91 ^{a} ± 0.01	115.30 ^{a} ± 0.36
	180	35.12 ^{b} ± 0.11	11.94 ^{b} ± 0.05	115.20 ^{a} ± 0.41
	p-value	<0.001	0.005	0.105
N form (F)	NO_3	37.38 ^{b} ± 0.06	12.71 ^{b} ± 0.05	113.43 ^{b} ± 0.45
	NH_4	39.15 ^{a} ± 0.02	13.31 ^{a} ± 0.01	116.15 ^{a} ± 0.34
	Urea	38.98 ^{a} ± 0.09	13.25 ^{a} ± 0.03	114.73 ^{b} ± 0.38
	p-value	0.001	0.013	<0.001
Growing season (S)	2018	42.15 ^{a} ± 0.07	14.33 ^{a} ± 0.04	116.52 ^{a} ± 0.15
	2019	34.86 ^{b} ± 0.13	11.85 ^{b} ± 0.01	113.02 ^{b} ± 0.16
	p-value	<0.001	<0.001	<0.001
N × F	*p*-value	0.205	0.216	0.208
N × S	*p*-value	0.367	0.766	0.056
F × S	*p*-value	0.407	0.366	0.344
N × F× S	*p*-value	0.676	<0.001	<0.001

Different letters within each column and main factor indicate a significant difference among means.

In a study by Negro et al., N fertilisation increased the content of caffeoylquinic acids and luteolin derivatives but decreased the content of luteolin and apigenin aglycone [40]. A different effect on the accumulation of individual compounds was found in *Hypericum pruinatum* in relation to the N rate [41]. Moderate fertilisation favours the synthesis of secondary metabolites, and, on the contrary, under conditions of over-fertilisation, the accumulation of polyphenolic compounds tends to decrease [24,27,28]. According to the same authors, excessive N doses increase the degree of vegetative growth, resulting in self-shading of the plants, and, consequently, a reduction in photosynthetic efficiency and the content of caffeoylquinic acids and flavonoids. Stefanelli et al. concluded that, in most cases, the N-deficiency stimulated biosynthesis of secondary metabolites [42].

In this study, the applied N doses in each year of the study significantly ($p \leq 0.01$) influenced the chlorogenic acid levels in the leaves of cardoons ('N rate × growing season' interaction). An interaction effect of all three factors ('N rate × N form × growing season' interaction) on TP, TF, luteolin-7-*O*-glucoside, luteolin-7-*O*-rutinoside, caffeic acid, and cynarin contents was found.

In this experiment, the N form did not affect the levels of TP and TF in the leaves of cardoons. Our results were in contrast with the findings of Munene et al., who reported the highest TP content in amaranth species under NH_4 supply [43]. In this study, the choice of N form altered the contents of the polyphenol profile. In the variants with NH_4 and urea, cardoon leaves contained more luteolin, lutein-7-*O*-glucoside, and cynarin compared to NO_3. The variant with NH_4 had higher concentrations of luteolin 7-*O*-rutinoside (116 mg kg^{-1}) than variants with NO_3 and urea, and the variant with urea fertilisation had higher concentrations of caffeic acid (77 mg kg^{-1}) compared to variants with NO_3 and NH_4. Different behaviours of individual compounds in relation to N forms were observed in tea plants [44]. The applied N form had no significant effect on chlorogenic acid levels in leaf biomass.

3.3. Effect of N Rate and Form on Radical Scavenging Activity

The FRAP and DPPH tests showed the highest radical scavenging activity (RSA) abilities of extracts from plants fertilised with 120 kg N ha^{-1} compared to plants grown without N (Table 8). In all assays, the leaves of plants fertilised with 180 kg N ha^{-1} had the lowest RSA. This was likely related to the low TPC found in the same samples. Duan et al. similarly found that blackberries under NO_3-N stimulated DPPH activity [45]. On the contrary, amaranth species supplied with NH_4-N exhibited superior DPPH activity [43]. The highest results in the RSA assays were obtained for the NO_3 form and urea.

The antioxidant activity of the cardoon extracts was correlated with TF content (Figure 1). The correlation coefficients between TF content and the ABTS and FRAP and DPPH test results were 0.67, 0.82, and 0.93, respectively.

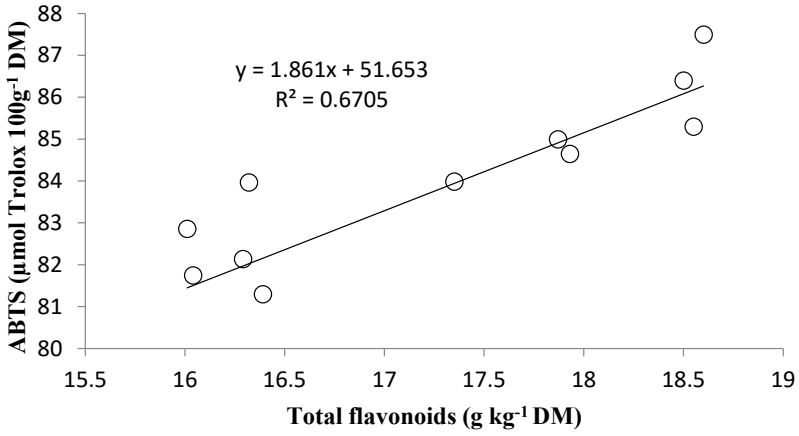

Figure 1. *Cont.*

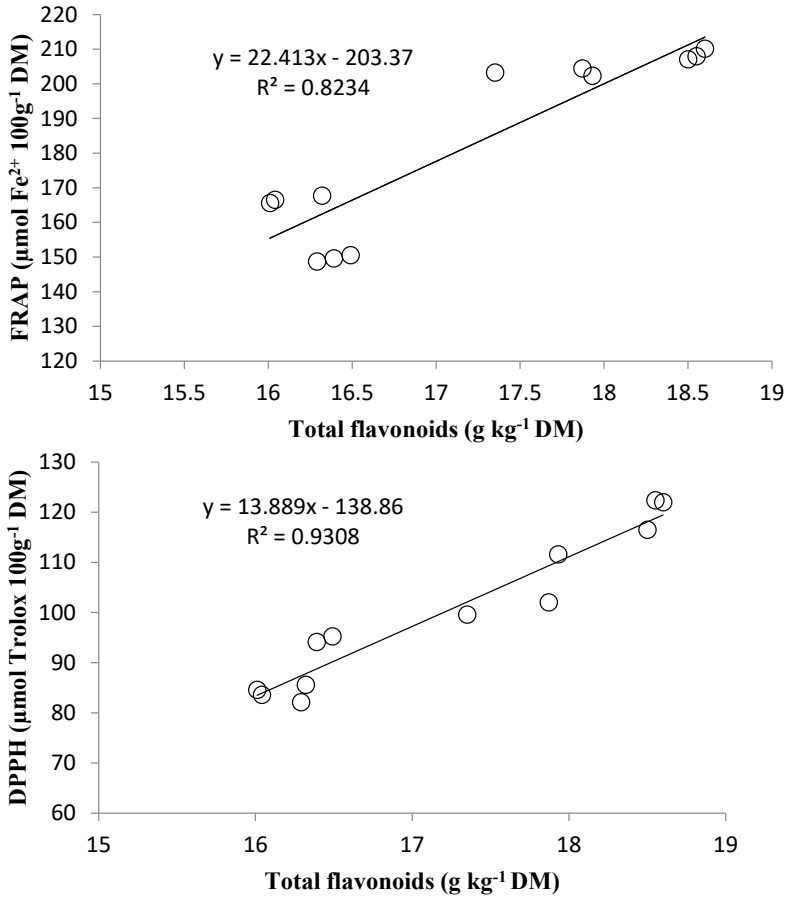

Figure 1. Correlation between the total flavonoids content and ABTS (2,2′-azinobis-3-ethylbenzothia zoline-6-sulphonic acid), FRAP (ferric reducing antioxidant power), and DPPH (2,2-diphenyl-1-picrylhydrazyl) assay.

Table 8. Effects of main factors (N rate, N form growing season) on radical scavenging activity (± standard deviation) in cardoon leaves.

Main Factor	Source of Variation	ABTS Assay (µmol Trolox 100 g^{-1} DM)	FRAP Assay (µmol Fe^{2+} 100 g^{-1} DM	DPPH Assay (µmol Trolox 100 g^{-1} DM)
N rate (N) kg ha^{-1}	0	86.65 ^{a} ± 0.64	203.36 ^{b} ± 1.90	101.60 ^{b} ± 0.80
	60	84.86 ^{a} ± 0.46	166.61 ^{c} ± 0.94	84.59 ^{c} ± 0.63
	120	84.40 ^{a} ± 0.40	207.09 ^{a} ± 1.47	106.51 ^{a} ± 0.79
	180	70.30 ^{b} ± 0.39	148.65^{d} ± 0.83	74.15^{d} ± 0.57
	p-value	0.041	0.023	0.001
N form (F)	NO_3	82.50 ^{a} ± 0.42	187.33 ^{a} ± 0.94	96.33 ^{a} ± 0.54
	NH_4	75.03 ^{b} ± 0.36	167.40 ^{b} ± 0.75	82.59 ^{b} ± 0.79
	Urea	87.13 ^{a} ± 0.65	189.56 ^{a} ± 0.89	96.21 ^{a} ± 0.49
	p-value	<0.001	0.040	<0.001
Growing season (S)	2018	88.04 ^{a} ± 0.69	191.41 ^{a} ± 1.24	101.1 ^{a} ± 0.25
	2019	75.05 ^{b} ± 0.45	171.45 ^{b} ± 0.83	82.31 ^{b} ± 0.19
	p-value	<0.001	0.005	<0.001
N × F	*p*-value	0.910	0.343	0.056
N × S	*p*-value	0.407	0.517	0.096
F × S	*p*-value	0.055	0.645	0.065
N × F × S	*p*-value	0.003	<0.001	<0.001

Different letters within each column and main factor indicate a significant difference among means.

The strong positive correlation between the content of TF compounds and the ABTS, FRAP and DPPH results clearly indicates that flavonoid compounds were the antioxidant activity carriers of the cardoon extracts. The results are in line with previous studies.

3.4. Effect of Growing Season on Biomass Yield, Polyphenol Profile and Radical Scavenging Activity

In 2018, the yield of air-dried leaf biomass was 1.12 t ha^{-1} higher than in 2019 (Table 4). This difference may have been due to the different availability of N from the soil caused by the level of rainfall throughout the growing season. In 2019, rainfall totals from April to October were 61 mm lower than in 2018. Water scarcity and the accompanying high air temperatures in 2019, higher than perennial averages by 1.9 °C, probably reduced the availability of nutrients in the soil (Tables 1 and 2). There is little information in the available literature on the effect of meteorological trends on phenolic content in humid temperate climates, where vegetation length and herb yield can be highly variable depending on atmospheric factors [4]. In 2018, the content of TF and TP were, respectively, 7–9%, higher than in 2019. In 2018, the average temperature of the growing season was 2.9 °C higher than the multi-year average, with a high sum of sunshine hours (1349). The high TP and TF content in 2018 was accompanied by high radical scavenging activity (Table 8). The high TP and TF levels were the result of high chlorogenic acid, cynarin, luteolin, luteolin-7-*O*-glucoside and luteolin 7-*O*-rutinoside contents. It is likely that higher N availability and higher temperatures in 2018 compared to 2019 were responsible for the higher accumulation of TP in cardoon leaves. In addition, higher minimum temperatures during the period of intensive plant growth in 2018 during the night in June and July increased the transpiration rate of the cardoon plants, which may have increased the accumulation of chlorogenic acid, cynarin, luteolin, luteolin-7-*O*-glucoside, and luteolin-7-*O*-rutinoside due to lower dilution of the cells. Furthermore, the occurrence of days with high temperatures in 2019 during intensive growth (June–August) may have accelerated plant maturation and consequently reduced TP and TF content. The varying effects of weather conditions on the content of polyphenolic compounds in the cardoons support the hypothesis that the concentration of secondary metabolites in plants depends mainly on the availability of minerals, carbon, temperature, water, and light [46]. Depending on the impact of weather factors, plant metabolism changes and compounds containing mainly carbon in the chemical structure are synthesised first; thus, carbohydrates are formed, followed by phenolic compounds.

Low mineral availability under drought stress is thought to be responsible for the accumulation of phenolic compounds of globe artichokes under Mediterranean climate conditions [24].

3.5. Correlation Analysis of Parameters under Study

The results of the correlation analysis of cardoon leaf bioactive compounds under different N rates are shown in Table 9. Significant correlations (critical value 0.58) were found in 19 cases out of 35 comparisons in the control group; in 16 cases in the group with N fertilisation at 60 kg ha^{-1}; and in 17 cases in the group with N fertilisation at 120 kg ha^{-1}. The lowest values (below the critical value of 0.58) were found in the group with N at 180 kg ha^{-1}. This indicates that the standard correlations between the properties of the bioactive compounds of cardoon leaves were significantly disturbed by the application of a high N rate (180 kg ha^{-1}). The highest correlation coefficients were found between TF content and chlorogenic acid in leaves in the group without N (0.93) and in the group with an N rate of 60 kg ha^{-1} (0.99). Equally high values of correlation coefficients were recorded between TF content and caffeic acid in the group with an N rate of 60 and 120 kg ha^{-1} (0.98 and 0.97, respectively). When analysing TP content in leaves, the highest correlation coefficient (0.97) was found between TP and luteolin in the group without N and in the same group between TP and luteolin-7-*O*-glucoside (0.91). For an N rate of 120 kg ha^{-1}, the highest correlation coefficient (0.92) was found between TP and caffeic acid, and TF and cynarin (0.91). Low correlation values occurred with an N rate of 180 kg ha^{-1}.

Table 9. Coefficients of Pearson's correlation between the chemical composition parameters of cardoon leaves in relation to N rate.

Nitrogen Rate (kg ha^{-1})	Parameter	TF	TP	Lut	L7G	L7R	Ch	Caf
0	TF	1						
	TP	0.77	1					
	Lut	0.75	0.97	1				
	L7G	0.70	0.91	0.58	1			
	L7R	0.81	0.82	0.74	0.69	1		
	Ch	0.93	0.73	ns	ns	ns	1	
	Caf	0.86	0.84	ns	ns	ns	0.73	1
	Cyn	0.88	0.80	ns	ns	ns	0.89	0.74
60	TF	1						
	TP	0.56	1					
	Lut	0.73	0.70	1				
	L7G	0.69	0.64	ns	1			
	L7R	0.79	0.89	ns	0.41	1		
	Ch	0.99	0.78	ns	0.58	ns	1	
	Caf	0.98	0.75	ns	ns	ns	0.58	1
	Cyn	0.86	0.74	ns	ns	ns	0.70	0.75
120	TF	1						
	TP	0.87	1					
	Lut	0.80	0.79	1				
	L7G	0.88	0.84	ns	1			
	L7R	0.91	0.63	ns	0.67	1		
	Ch	0.86	0.84	ns	ns	ns	1	
	Caf	0.97	0.92	ns	ns	ns	0.67	1
	Cyn	0.85	0.91	ns	ns	ns	0.70	0.79
180	TF	1						
	TP	0.33	1					
	Lut	0.46	0.45	1				
	L7G	0.34	0.32	ns	1			
	L7R	0.48	0.33	ns	0.34	1		
	Ch	0.48	0.31	ns	ns	ns	1	
	Caf	0.37	0.33	ns	ns	ns	0.32	1
	Cyn	0.42	0.33	ns	ns	ns	0.30	0.30

TF—Total flavonoids, TP—Total polyphenol, Lut—Luteolin, L7G—Luteolin 7-*O*-glucoside, L7R—Luteolin 7-*O*-rutinoside, Ch—Chlorogenic acid, Caf—Caffeic acid, Cyn—Cynarin. ns: not significant.

The results of the correlation analysis of the bioactive compounds of cardoon leaves with different N forms are shown in Table 10. Significant correlations were found in 14 cases in the group with the NO_3 form; in 16 cases in the group with NH_4 form; and in 11 cases with the urea form. This indicates that the N forms used had different effects on the concentrations of bioactive substances. The highest values of correlation coefficients in the NO_3 form were found between TF content and caffeic acid; cynarin and chlorogenic acid (0.93–0.97); and between TP and luteolin (0.98) and luteolin-7-*O*-rutinoside (0.83). The highest values of correlation coefficients in the NH_4 form were found between the TF content and chlorogenic acid, cynarin and caffeic acid (0.89–0.98), and between TP and luteolin (0.95) and luteolin-7-*O*-glucoside (0.88). Average correlation coefficients between TF and chlorogenic acid, cynarin and caffeic acid content (0.61–0.78) were found for the urea form, and between luteolin-7-*O*-rutinoside and luteolin (0.66), and luteolin-7-*O*-glucoside (0.73).

Table 10. Coefficients of Pearson's correlation between the chemical composition parameters of cardoon leaves in relation to N form.

Nitrogen Form	Parameter	TF	TP	Lut	L7G	L7R	Ch	Caf
NO_3	TF	1						
	TP	0.60	1					
	Lut	0.56	0.98	1				
	L7G	ns	0.67	ns	1			
	L7R	ns	0.83	ns	0.34	1		
	Ch	0.97	0.51	ns	ns	ns	1	
	Caf	0.93	0.45	ns	ns	ns	0.67	1
	Cyn	0.96	0.59	ns	ns	ns	ns	0.61
NH^4	TF	1						
	TP	0.41	1					
	Lut	0.47	0.95	1				
	L7G	ns	0.88	ns	1			
	L7R	ns	0.74	ns	ns	1		
	Ch	0.89	0.74	ns	ns	ns	1	
	Caf	0.98	0.66	ns	ns	0.44	0.64	1
	Cyn	0.93	0.75	ns	0.35	0.32	0.65	ns
Urea	TF	1						
	TP	0.33	1					
	Lut	0.48	0.48	1				
	L7G	ns	0.42	ns	1			
	L7R	ns	0.36	0.66	0.73	1		
	Ch	0.61	ns	ns	ns	ns	1	
	Caf	0.78	ns	ns	ns	ns	ns	1
	Cyn	0.77	0.33	ns	ns	ns	ns	ns

TF—Total flavonoids, TP—Total polyphenol, Lut—Luteolin, L7G—Luteolin 7-*O*-glucoside, L7R—Luteolin 7-O-rutinoside, Ch—Chlorogenic acid, Caf—Caffeic acid, Cyn—Cynarin. ns: not significant.

4. Conclusions

The results of this study confirmed that an acceptable yield with high-quality bioactive compounds can be obtained in the cultivation of cardoons for pharmaceutical purposes under a low N rate. The polyphenol compounds identified in cardoon extracts mainly included chlorogenic acid, caffeic acid, cynarin, luteolin, luteolin-7-*O*-glucoside, and luteolin-7-*O*-rutinoside. Under an N rate of 120 kg ha^{-1}, the highest values of TF content and radical scavenging activities (FRAP and DPPH) were observed, which did not affect TP levels. Increasing the N form to 180 kg ha^{-1}, the leaf air-dried biomass did not respond positively, and this led to lower concentrations of TF, TP, caffeic acid, cynarin, and luteolin. The applied N forms had different effects on the concentrations of bioactive compounds. Higher concentrations of caffeic acid, cynarin, luteolin and luteolin-7-*O*-glucoside were found when the NH_4 form and urea were applied. Use of the NO_3 form led to lower levels of bioactive compounds. Positive correlations of the bioactive compounds of cardoon leaves with the amounts of applied N and with the different N forms were found. This study adds to the existing knowledge and suggests that optimal N fertilisation influences the formation of a favourable composition of bioactive compounds without compromising the leaf air-dried biomass. On the other hand, the growing season could also modulate the yield and accumulation of phytochemicals. Here, in 2018, a season with higher precipitation and a higher amount of sunshine resulted in a higher yield of air-dried herbs with higher TP and TF contents than in 2019.

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