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Seasonal variation of bioactive properties and phenolic composition of *Cynara cardunculus* var. *altilis*

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ABSTRACT

Cynara cardunculus L. (cardoon) has several health benefits mainly attributed to its abundance in polyphenols. In this study, cardoon heads (capitula) were harvested in Greece during the flowering stage, and the hydroethanolic extracts were assessed in terms of phenolic compounds composition and antioxidant, cytotoxic, anti-inflammatory, and antimicrobial activities. The phenolic profile was evaluated by HPLC-DAD-ESI/MS to better understand the seasonal changes in the individual compound levels and how these changes correlate with bioactive properties. The main phenolic compounds identified were caffeoylquinic and dicaffeoylquinic acid derivatives. Immature heads (Car A: principal growth stage (PGS) 5) had the highest phenolic content (34.3 mg/g) and cytotoxic (GI_{50} of 69–268 μg/mL) and anti-inflammatory (IC_{50} of 183 μg/mL) activities. Sample Car D (PGS 6/7) revealed the highest antioxidant (IC_{50} of 23–227 μg/mL) and antifungal (MIC of 0.26–0.51 mg/mL) potential. Regarding the antibacterial activity, Car E (PGS 7) revealed the best results (MIC of 0.59–1.18 mg/mL). This study suggests that the maturity stage of the plant influences the phenolic composition and bioactivity.

1. Introduction

Cynara cardunculus L. (syn. cardoon) is a diploid ($2n = 34$) cross-pollinated perennial plant that belongs to the Asteraceae (sunflower) family. This species includes three taxa: the cultivated cardoon (*C. cardunculus* var. *altilis* DC.), the wild cardoon (*C. cardunculus* var. *sylvestris*), and their ancestor globe artichoke (*C. cardunculus* var. *scolymus*) (Conceição et al., 2016).

Indigenous to the Mediterranean region, this crop is an integral constituent of the so-called "Mediterranean diet", used since antique times in dishes such as salads and soups. Its inflorescences are also widely used in the manufacturing of protected designation of origin (PDO) cheeses, as a result of the clotting capacity. The oil from cardoon seeds is used in the biodiesel production and as edible vegetable oil, while fresh and dried plants for biomass production (Conceição et al., 2016; Gominho, Curt, Lourenço, Fernández, & Pereira, 2018; Piluzza, Molinu, Re, & Sulas, 2019). Besides that, this crop is widely consumed as herbal medicine, due to the known health-promoting effects. Several studies have demonstrated its anticarcinogenic, antioxidant, antimicrobial, anti-inflammatory, anti-HIV, bile-expelling, hepatoprotective, and hypocholesterolemic potential (Gostin & Waisundara, 2019; Kollia, Markaki, Zoumpoulakis, & Proestos, 2017; Petropoulos, Fernandes, Pereira, 2019; Petropoulos, Fernandes, Tzortzakis, et al., 2019; Scavo et al., 2019). Moreover, recently the commercial and economic potential of cardoon has been recognized due to its nutritional value, bioactive potential, as well as its diverse industrial applications. Parameters such as the high crop yield, the stability of agronomic performance, the adaptation capacity to climate changes and the resistance to different types of abiotic stress, are important contributors to the stimulation of this interest (Conceição et al., 2016; Gominho et al., 2018). The pharmaceutical and nutritional properties associated with this crop could be correlated to compounds with beneficial effects, namely flavonoids (apigenin and luteolin derivatives), phenolic acids (mainly caffeoylquinic and dicaffeoylquinic acid derivatives), and anthocyanins (cyanidin), found in different plant tissues of cardoon, in addition to inulin, fibers and minerals (Petropoulos, Pereira, Ntatsi, et al., 2018).

The existence of phenolic compounds in different plant species has

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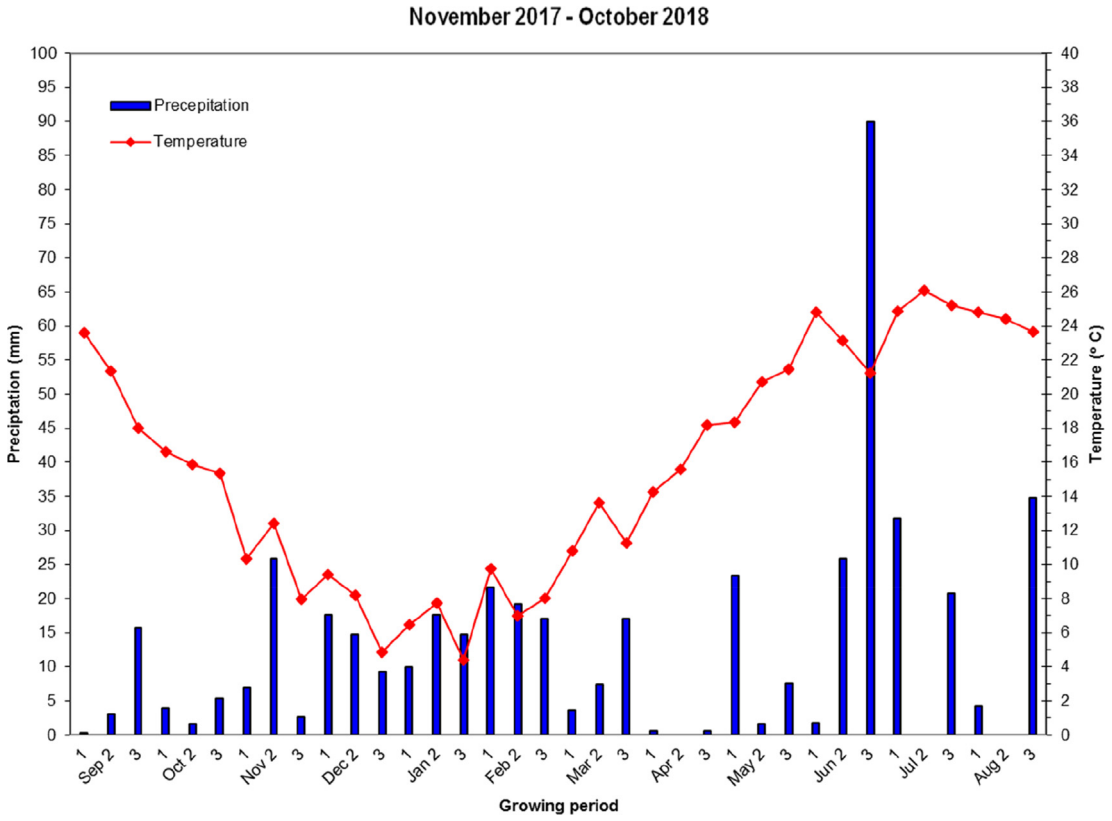

Fig. 1. Meteorological data (mean temperature and precipitation) during the 2017–2018 growing period. Each month is separated in three 10-day intervals (1, 2 and 3).

been extensively studied due to the high variety and potential shown. Their presence and abundance are related to metabolic reactions which are strongly influenced by parameters such as the vegetable tissue analyzed, the physiological (growth) age of the species, the different stages of development (harvesting date), the environmental conditions and interactions, the cultivation practices, and after-harvest conditions (genetic background and geographical location) (Ferreira, Martins, & Barros, 2017). The presence of caffeic acid derivatives, namely chlorogenic acid and cynarine (i.e., 1,5-dicaffeoylquinic acid), in cardoon parts is referred by several authors who suggest the pivotal role of these compounds to the pharmaceutical and nutritional properties associated with this crop (Russo et al., 2017). Therefore, it is of great importance from the production, economical and industrial point of view, to reveal the chemical composition and biological potential of different plant parts through the growing season. Available information suggests that the qualitative and quantitative phenolic composition, and also the biological potential appears to be strongly influenced by parameters such as the environmental conditions, and the genetic background. The full description of the polyphenolic profile and bioactivities of cardoon different parts as affected by factors such as the maturity stage, the genotype or the growing locations, is very important to validate all of its applications and could contribute to the management of agricultural by-products, for the sustainable agricultural practices and the circular economy of the crop (Dias et al., 2018; Pandino, Lombardo, Williamson, & Mauromicale, 2012; Petropoulos, Fernandes, Pereira, et al., 2019; Petropoulos, Fernandes, Tzortzakis, et al., 2019).

Therefore, the present study aimed to evaluate the variations in the phenolic composition and bioactive potential of cardoon heads harvested during the flowering period. According to the findings, the main goal is to provide integrated knowledge towards the improved use of cardoon, regarding the associated potential and the optimal time of the year to harvest its heads and further maximize their added value and their contribution to human health.

2. Materials and methods

2.1. Standards and reagents

The solvents used were of analytical grade and were obtained from Fisher Scientific (Lisbon, Portugal). The sulphorhodamine B (SRB), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), AAPH (2,2′-azobis(2-methylpropionamide) dihydrochloride), ellipticine, tocol, trypan blue, and Tris (tris-(hydroxymethyl) aminomethane) were acquired from Sigma-Aldrich (St. Louis, MO, USA). The phenolic compounds' standards were acquired from Extrasynthèse (Genay, France). The RPMI-1640 and DMEM mediums, foetal bovine serum (FBS), L-glutamine, Hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediaminetetraacetic acid), and penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively) were obtained from Hyclone (Logan, Utah, USA). From Biomerieux (Marcy l'Etoile, France) was obtained the Tryptic Soy Broth (TSB). Blood agar with 7% sheep blood and MacConkey agar plates were purchased from Liofilchem (Italy). Other reagents and solvents of analytical grade were obtained from common sources. Water was treated with a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.2. Plant material

The heads (capitula) of *Cynara cardunculus* var. *altilis* DC. cv. Bianco Avorio (Fratelli Ingegnoli Spa, Milano, Italy) were harvested in central Greece at the experimental field of the University of Thessaly in Velestino (22.756E, 39.396 N), during the growing period of 2017–2018 (principal growth stages (PGS) 5–8, according to the Biologische Bundesanstalt, Bundessortenamt, CHemische Industrie (BBCH) scale) (Archontoulis, Struik, Vos, & Danalatos, 2013). The samples were collected at the beginning of the flower's development, during full maturity, and in seed ripening stages from the main

inflorescence of each plant. For each harvesting date, 15 individual primary heads (n = 15) were collected from different plants. Six different samples of cardoon were analyzed corresponding to six harvesting dates, namely Car A collected at the end of April (PGS 5), Car B collected at the beginning of May (PGS 5/6), Car C collected at the end of May (PGS 6), Car D collected in June (PGS 6/7), and finally the samples Car E and Car F collected at the beginning (PGS 7) and at the end of August (PGS 8), respectively. The climate conditions during the growing period of 2017–2018 are presented in Fig. 1.

The plant material was freeze-dried (Sublimator model EKS, Christian Zirbus Co., Germany), ground to a fine powder (~20 mesh) using a domestic electric blender, and mixed to obtain homogenate batch samples. Then, the samples were put in air-sealed bags and stored under deep freezing conditions (-80 °C) under protection from light until further analysis.

2.3. Extraction procedure

To obtain the extracts, each sample (1.5 g) was stirred (150 rpm) at room temperature for 1 h with EtOH/H_2O (80:20, *v/v*; 30 mL) (Bessada, Barreira, Barros, Ferreira, & Oliveira, 2016). The mixture obtained was filtered through a Whatman No. 4 paper and evaporated under reduced pressure (rotary evaporator Hei-VAP Advantage, Heidolph, Germany) at 40 °C. The aqueous phase was frozen at −20 °C and lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA) before carrying out the remaining analyses.

2.4. Phenolic composition analysis

The final lyophilized extracts were re-dissolved in EtOH/H_2O (20:80, *v/v*) to prepare solutions at 10 mg/mL. Then, the obtained solutions were filtered through 0.22 μm nylon syringe filters. The polyphenolic composition was determined by HPLC coupled to a diode array detector and electrospray ionization - mass spectrometry (HPLC-DAD-ESI/MS), according to the conditions previously described by Bessada et al. (2016). The tentative identification of the phenolic compounds was based on the comparison of the retention times, and the UV–Vis spectra obtained from commercial standards and the literature information. For the quantification, the area of the peaks was determined and then compared with the calibration curves of the most similar available commercial standards. The results were expressed as mg per g of dry weight of extract.

2.5. Evaluation of the bioactive properties

2.5.1. Antioxidant activity

Two cell-based *in vitro* methodologies were used for the measurement of the antioxidant capacity: the inhibition of lipid peroxidation by the decrease in thiobarbituric acid reactive substances (TBARS), and the oxidative hemolysis inhibition assay (OxHLIA). The positive control used was the commercial antioxidant Trolox.

TBARS assay. The inhibition capacity of the tested samples against the formation of TBARS, such as malondialdehyde generate from the *ex vivo* decomposition of lipid peroxidation products, was evaluated using porcine brain (*Sus scrofa*) cell homogenates (1:2, *w/v*; 0.1 mL), according to the methodology previously described by Souilem et al. (2017). The extracts obtained (see Section 2.3) were re-dissolved in H_2O, obtaining solutions with a final concentration of 5 mg/mL, while further successive dilutions led to obtaining the range of the concentrations tested (2500 to 10 μg/mL). The results obtained were expressed as IC_{50} values (μg/mL), corresponding to the sample concentration that provides 50% of antioxidant activity.

OxHLIA assay. The antihemolytic activity of the extracts was determined following the methodology previously described by Mandim et al. (2019). The erythrocytes used in the assay were isolated from sheep blood samples collected from healthy animals. In a 48-well plate, the erythrocyte solution (2.8% in PBS, *v/v*; 200 μL) was mixed with 400 μL of either PBS solution (control), antioxidant sample dissolved in PBS, or water (for complete hemolysis). After pre-incubating the mixture at 37 °C for 10 min with shaking, AAPH (160 mM in PBS; 200 μL) was added and the optical density was measured at 690 nm. Then, the plate was incubated under the same conditions and the optical density was measured each 10 min at the same wavelength until complete hemolysis occurred. The results were calculated as explained by Mandim et al. (2019) and expressed as IC_{50} values (μg/mL) at Δt 60 and 120 min, which correspond to the extract concentration required to keep 50% of the erythrocyte population intact for 60 and 120 min, respectively.

2.5.2. Anti-inflammatory activity

The anti-inflammatory activity was determined according to the method formerly reported by Mandim et al. (2019). The nitrite concentration in the culture medium was determined to assess the capacity of the extracts to inhibit the lipopolysaccharide (LPS)-induced NO production through a murine macrophage cell line (RAW 264.7). Cardoon heads extracts (see Section 2.3) re-dissolved in water at a final concentration of 8 mg/mL were tested. The positive control used was Dexamethasone, and samples without LPS were used as negative control. The results were expresses as IC_{50} values (μg/mL), which correspond to the extract concentration responsible for 50% of NO production inhibition.

2.5.3. Cytotoxic and hepatotoxic potential

The cardoon head extracts prepared in Section 2.3 were re-dissolved in water at a concentration of 8 mg/mL and subjected to successive dilutions to obtain the concentrations to be tested (6.25–400 μg/mL). Four human tumor cell lines (HeLa – cervical carcinoma; HepG2 – hepatocellular carcinoma; MCF-7 - breast carcinoma; and NCI-H460 – non-small cell lung cancer) (all acquired from Leibniz-Institut DSMZ), and a non-tumor porcine liver primary culture (PLP2) were tested. The cytotoxic and hepatotoxic activities were tested through the sulforhodamine B colorimetric test, as described by Barros et al. (2013). Ellipticine was used as a positive control. The results were expressed as GI_{50} values (μg/mL), which translate the extract concentration responsible for 50% of inhibition of cell proliferation.

2.5.4. Antimicrobial activity

The obtained extracts were re-dissolved in 5% DMSO and diluted according to the protocol described by Petropoulos, Fernandes, Pereira, et al. (2019), Petropoulos, Fernandes, Tzortzakis, et al. (2019). The following Gram-positive bacteria: *Bacillus cereus* (food isolate), *Staphylococcus aureus* (ATCC 6538) and *Listeria monocytogenes* (NCTC 7973); and Gram-negative bacteria: *Escherichia coli* (ATCC 35210), *Enterobacter cloacae* (human isolate), *Salmonella typhimurium* (ATCC 13311) were tested. The antifungal assay was performed with the micromycetes *Aspergillus fumigatus* (ATCC 1022), *A. versicolor* (ATCC 11730), *A. niger* (ATCC 6275), *Penicillium ochrochloron* (ATCC 9112), *P. funiculosum* (ATCC 36839) and *P. aurantiogriseum* (*P. verrucosum* var. *cyclopium* (food isolate)), following the methodology previously described by Petropoulos, Fernandes, Pereira, et al. (2019), Petropoulos, Fernandes, Tzortzakis, et al. (2019). The microorganisms are deposited at Mycological laboratory, Department of Plant Physiology, Institute for biological research "Sinisa Stanković", University of Belgrade, Serbia. Streptomycin, ampicillin, ketoconazole, and bifonazole were used as positive controls. The results were expressed as minimum inhibitory (MIC) and minimum bactericidal (MBC) or fungicidal (MFC) concentrations (mg/mL).

2.6. Statistical analysis

The performed experiments were carried out in triplicate, and the results are expressed as mean ± standard deviation. Means and

Table 1
Phenolic compounds composition of the hydroethanolic extracts of *Cynara cardunculus* var. *altilis* heads. It is presented the retention time (Rt), wavelengths of maximum absorption (λ_{max}) in the UV–Vis region, mass spectral data and tentative identification of phenolic compounds.

Peak	Rt (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification
1	7.00	325	353	191(100), 179(10), 161(5), 135(5)	5-*O*-Caffeoylquinic acid
2	12.08	310	325	163(100), 145(5), 119(30)	*p*-Coumaric acid hexoside
3	13.34	325	607	445(100), 269(47)	Apigenin-*O*-glucuronide-*O*-hexoside
4	16.77	319	677	515(100), 353(9), 191(3)	Tri-*O*-caffeoylquinic acid
5	18.68	345	461	285(100)	Luteolin-7-*O*-glucuronide
6	18.84	345	461	285(100)	Luteolin-*O*-glucuronide
7	20.48	344	515	353(100), 191(5), 173(5), 161(5), 135(5)	3,5-*O*-Dicaffeoylquinic acid
8	21.52	332	577	269(100)	Apigenin-7-*O*-rutinoside
9	22.93	336	445	269(100)	Apigenin-7-*O*-glucuronide
10	28.10	332	517	473(100), 269(50)	Apigenin-*O*-malonylhexoside

standard deviations were determined from the obtained data using Microsoft Excel. All statistical tests were performed using SPSS Statistics software (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). The results were obtained through the analysis of variance (ANOVA), and by Tukey's HSD test ($\alpha = 0.05$).

3. Results and discussion

3.1. Composition in phenolic compounds

The detected phenolic compounds of *C. cardunculus* heads and their quantification (mg/g extract) are presented in Tables 1 and 2, respectively. The phenolic compounds were tentatively identified according to their retention time (R*t*), the wavelength of maximum absorbance (λ_{max}), pseudomolecular ion ($[M-H]^-$), and fragmentation pattern (MS^2). Ten phenolic compounds were found, namely four phenolic acid derivatives (peaks **1**, **2**, **4** and **7**) and six flavone glycosides (**3**, **5**, **6**, **8**, **9** and **10**), the latter being present in higher concentrations. Fig. 2 shows the HPLC phenolic profile of the hydroethanolic extract of the sample Car A. A significant number of previous publications have already given a thorough description of the phenolic profile of cardoon flower and its constituents, and hence the tentative identification of the peaks **1**, **5**, **7**, **8**, and **9** (5-*O*-caffeoylquinic acid, luteolin-7-*O*-glucuronide, 3,5-*O*-dicaffeoylquinic acid, apigenin-7-*O*-rutinoside, and apigenin-7-*O*-glucuronide, respectively) has already been described by the authors in the study performed by Dias et al. (2018) in inflorescences of cardoon samples originated from Portugal. For peaks **4** and **6** (tri-*O*-caffeoylquinic acid and luteolin-*O*-glucuronide), the tentative identification was based on the previously described by Gouveia and Castilho (2012) in artichoke juice and capsules, respectively. Peak **2** was tentatively identified as *p*-coumaric acid hexoside, as previously described in globe artichoke leaf blades by Petropoulos, Pereira, Barros, and Ferreira (2017). Peak **3** was tentatively identified as apigenin-*O*-glucuronide-*O*-hexoside based on the previous description made by Pereira, Barros, Carvalho, Santos-Buelga, and Ferreira (2015) in hydroethanolic extracts and infusions of flowering stems and capitula of artichoke. Finally, peak **10** (apigenin-*O*-malonylhexoside) was tentatively identified based on the description made by Pandino et al. (2012) in cultivated cardoon and globe artichoke samples from Italy.

Quantitatively, cardoon heads in the earliest maturation stage (Car A) presented the highest content in phenolic compounds, mainly due to the presence of apigenin-7-*O*-rutinoside and 5-*O*-caffeoylquinic acid. Overall, the compounds present in higher quantities in all studied samples were 5-*O*-caffeoylquinic acid (0.445–3.55 mg/g extract) and apigenin-7-*O*-rutinoside (0.97–3.51 mg/g extract). It has been verified that the maturity stage shows a significant influence, not only on the content of total phenolic compounds, but also on the polyphenolic profile, while environmental conditions during harvesting may also impose a significant impact on the phenolic compounds composition. According to Lombardo et al. (2010), a significant variation in phenolic compounds composition in inflorescences of globe artichoke was observed depending on the harvesting time (winter or spring harvest), with several polyphenols being detected only in one of the two harvests. In the study of Pandino, Lombardo, Lo Monaco, and Mauromicale (2013), an increase of phenolic compounds content in receptacles was observed from February to April followed by a concomitant decrease in the other plant parts. This finding could suggest a translocation of phenolic compounds to the most biosynthetically active plant parts.

Table 2
Phenolic compounds quantification in the hydroethanolic extracts of *Cynara cardunculus* var. *altilis* heads with different maturation stages.

Peak	Tentative identification	Content (mg/g extract)					
		Car A	Car B	Car C	Car D	Car E	Car F
1	5-*O*-Caffeoylquinic acid	2.09 ± 0.01^{c}	2.51 ± 0.01^{b}	3.55 ± 0.02^{a}	0.445 ± 0.001^{e}	n.d.	n.d.
2	*p*-Coumaric acid hexoside	1.40 ± 0.02^{a}	0.82 ± 0.02^{c}	0.40 ± 0.01^{d}	0.225 ± 0.001^{e}	n.d.	n.d.
3	Apigenin-*O*-glucuronide-*O*-hexoside	0.636 ± 0.001^{c}	1.07 ± 0.02^{a}	0.960 ± 0.004^{b}	0.514 ± 0.004^{d}	n.d.	n.d.
4	Tri-*O*-caffeoylquinic acid	1.29 ± 0.02^{a}	0.97 ± 0.01^{b}	0.88 ± 0.03^{c}	0.749 ± 0.004^{d}	n.d.	n.d.
5	Luteolin-7-*O*-glucuronide	0.775 ± 0.003^{a}	0.90 ± 0.02^{c}	0.60 ± 0.01^{d}	0.60 ± 0.01^{d}	n.d.	n.d.
6	Luteolin-*O*-glucuronide	0.564 ± 0.001^{c}	1.03 ± 0.01^{a}	0.517 ± 0.001^{e}	0.563 ± 0.001^{d}	n.d.	n.d.
7	3,5-*O*-Dicaffeoylquinic acid	9.9 ± 0.2^{a}	8.0 ± 0.2^{b}	3.81 ± 0.03^{c}	2.81 ± 0.03^{e}	0.65 ± 0.01^{f}	0.407 ± 0.002 ^{g}
8	Apigenin-7-*O*-rutinoside	3.17 ± 0.01^{b}	3.51 ± 0.02^{a}	1.416 ± 0.002^{e}	0.97 ± 0.04^{f}	2.22 ± 0.03^{c}	1.60 ± 0.01^{d}
9	Apigenin-7-*O*-glucuronide	13.2 ± 0.2^{a}	8.96 ± 0.04^{c}	3.04 ± 0.01^{d}	1.261 ± 0.003^{e}	n.d.	n.d.
10	Apigenin-*O*-malonylhexoside	1.170 ± 0.005^{a}	1.002 ± 0.002^{b}	0.72 ± 0.01^{c}	0.70 ± 0.03^{d}	n.d.	n.d.
	Total phenolic acids	14.7 ± 0.2^{a}	12.3 ± 0.2^{b}	8.64 ± 0.09^{c}	4.23 ± 0.02^{d}	0.65 ± 0.01^{e}	0.407 ± 0.002^{f}
	Total flavonoids	19.5 ± 0.2^{a}	16.48 ± 0.04^{b}	7.26 ± 0.02^{c}	4.608 ± 0.01^{d}	2.22 ± 0.03^{e}	1.60 ± 0.01^{f}
	Total phenolic compounds	34.3 ± 0.4^{a}	28.8 ± 0.2^{b}	15.89 ± 0.07^{c}	8.84 ± 0.02^{d}	2.87 ± 0.04^{e}	2.01 ± 0.01^{f}

Results are presented as mean ± standard deviation. n.d. – not detected. Calibration curves: compounds 1, 4 and 7 – chlorogenic acid ($y = 168823x + 161172$, $R^2 = 0.9999$); compound 2 – *p*-coumaric acid ($y = 301950x + 6966.7$, $R^2 = 0.9999$); compounds 3, 8, 9 and 10 – apigenin-7-*O*-glucoside ($y = 10683x - 45794$, $R^2 = 0.9986$); and compounds 5 and 6 – quercetin-3-*O*-glucoside ($y = 34843x - 160173$, $R^2 = 0.9998$). Different letters correspond to significant differences ($p < 0.05$). Mean statistical differences obtained by Student's *t*-test.

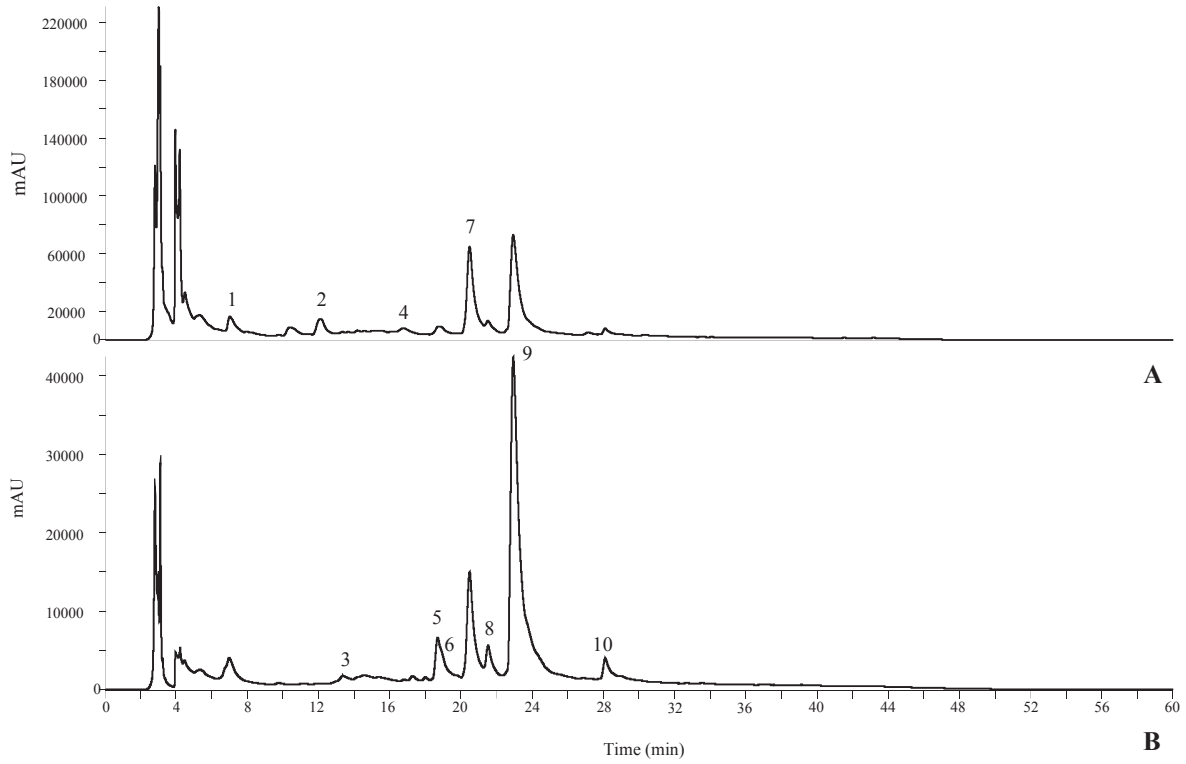

Fig. 2. Phenolic profile of the hydroethanolic extract of the sample Car A of *Cynara cardunculus* var. *altilis* recorded at 280 nm (A) and 370 nm (B). Peak numbers correspond to the compounds identified in Table 1.

Similar trends were observed in our study, where the phenolic compounds content decreased with maturity progress, suggesting a possible translocation of polyphenols to rhizomes which act as reserves for the next vegetative period (Alghazeer et al., 2012). Studies with cardoon leaves suggest that the content in bioactive compounds such as carbohydrates, flavonoids, and other polyphenols increases progressively with the age of the plant (Wahba, Sarhan, Salama, Sharaf-Eldin, & Gad, 2017). Also, different cultivars, genotypes, growing conditions, and plant parts present a considerable variation source regarding the phenolic compounds content. According to Pandino et al. (2012), total phenolic compounds in outer and inner bracts and receptacle varies among various cardoon genotypes and plant parts. The same authors also found that the cultivars and plant parts may affect the content of phenolic compounds. In the study of Pandino, Courts, Lombardo, Mauromicale, and Williamson (2010), apigenin derivatives were detected in the highest concentrations in both wild and cultivated cardoon immature inflorescences. Ramos et al. (2014) studied the polyphenolic composition of different plant tissues of cardoon cultivated in the south of Portugal and found different phenolic profiles depending on the plant tissue under analysis, which were characterized by high levels of hydroxycinnamic acids, mainly dicaffeoylquinic acids in receptacle and bracts, and apigenin and luteolin derivatives in capitulum florets and leaves, respectively. Dias et al. (2018), who analyzed the phenolic profile and bioactivity of inflorescences of different cardoon genotypes, found that the genotype 'F1-25-4′ stands out as the one with the highest content of phenolic compounds, being the best option for protected designation of origin (PDO) cheeses production.

Similar to our results, previous reports on the composition of phenolic compounds suggest that cardoon parts have a higher flavonoid content compared to phenolic acid derivatives (Pandino et al., 2012; Petropoulos et al., 2017). Particularly for head parts, Lombardo et al. (2010) observed significant differences in the phenolic compounds profile of spring- and winter-harvested globe artichoke heads, with results in accordance with the present study, where the majority of the tested samples had higher abundance in flavonoids than in hydroxycinnamoyl derivatives, except the sample Car C. Moreover, the polyphenolic profile of the tested cardoon heads was in general consistent with the literature information (Dias et al., 2018; Petropoulos, Pereira, Tzortzakis, et al., 2018).

3.2. Bioactive properties

The results regarding the cell-based antioxidant activity are presented in Table 3. Amongst the harvesting stages analyzed, sample Car D revealed the lowest IC_{50} values for the TBARS assay, while a varied response of the tested samples was observed for the OxHLIA test. In particular, samples Car A and D were the most effective (lowest IC_{50} values) in hemolysis delay at 60 min (Δt_{60}) and samples Car A, D, and F at 120 min (Δt_{120}). Nevertheless, these IC_{50} values are higher than those of the Trolox, used as a positive control. However, it should be considered that Trolox is a standard antioxidant compound, and the extracts tested are a complex mixture with phytochemicals that may not have antioxidant potential or show antagonistic effects among them. The extract obtained at principal growth stage (PGS) 7 (Car E) revealed the lower effectiveness in the inhibition of reactive substances formation generated by the *ex vivo* decomposition of lipid peroxidation products and did not reveal potential in protecting the erythrocyte population from the haemolytic action. Moreover, all the tested extracts revealed lower capacity in inhibiting the formation of malondialdehyde and other reactive substances that are generated from the *ex vivo* decomposition of lipid peroxidation products.

The antioxidant potential of cardoon plant tissues is widely described in the literature, mainly through the free radical scavenging capacity, measured by the DPPH assay. Several *in vitro* assays with different parts of cardoon plants highlight the antioxidant potential of this crop (Petropoulos et al., 2018Petropoulos, Pereira, Ntatsi, et al., 2018; Petropoulos, Pereira, Tzortzakis, et al., 2018; Piluzza et al., 2019). Furthermore, it has been reported that the antioxidant potential differed significantly among the different plant parts and/or the various genotypes tested, including the genotype evaluated in this study

Table 3
Antioxidant, cytotoxic and anti-inflammatory activities of *Cynara cardunculus* var. *altilis* head extracts.

	Car A	Car B	Car C	Car D	Car E	Car F	Positive control
Antioxidant activity (IC_{50}, μg/mL)							Trolox
TBARS formation inhibition	33 ± 3^{f}	92.0 ± 0.7^{e}	97 ± 3^{d}	23 ± 1^{g}	387 ± 2^{a}	341 ± 1^{b}	9.1 ± 0.3
OxHLIA, Δt = 60 min	122 ± 8^{c}	180 ± 11^{b}	180 ± 9^{b}	110 ± 4^{c}	n.a.	175 ± 13^{b}	21.2 ± 0.7
OxHLIA, Δt = 120 min	272 ± 16^{c}	399 ± 16^{b}	374 ± 3^{b}	277 ± 9^{c}	n.a.	290 ± 4^{c}	41.1 ± 0.8
Cytotoxic activity (GI_{50}, μg/mL)							Ellipticine
MCF-7 (breast adenocarcinoma)	193 ± 5^{c}	> 400	> 400	227 ± 19^{b}	308 ± 34^{a}	> 400	1.21 ± 0.02
NCI-H460 (lung carcinoma)	69 ± 3^{c}	> 400	> 400	149 ± 8^{b}	199 ± 7^{a}	> 400	0.9 ± 0.1
HeLa (cervical adenocarcinoma)	91 ± 5^{c}	> 400	304 ± 20^{c}	314 ± 8^{b}	150 ± 9^{d}	329 ± 4^{a}	1.03 ± 0.09
HepG2 (hepatocellular carcinoma)	75 ± 5^{c}	> 400	264 ± 7^{a}	> 400	73 ± 5^{c}	230 ± 3^{b}	1.10 ± 0.09
PLP2 (porcine liver primary cells)	268 ± 12*	> 400	> 400	> 400	252 ± 9*	> 400	2.3 ± 0.2
Anti-inflammatory activity (IC_{50}, μg/mL)							Dexamethasone
RAW 246.7 (murine macrophage cells)	183 ± 10^{c}	> 400	> 400	> 400	204 ± 8^{b}	291 ± 8^{a}	16 ± 1

Results are expressed as the mean values ± standard deviation. n.a. – no activity. IC_{50} values correspond to the extract concentration needed to inhibit in 50% the formation of thiobarbituric acid reactive substances (TBARS), the oxidative haemolysis (OxHLIA) for Δt of 60 and 120 min, or the nitric oxide (NO) production. GI_{50} values correspond to the extract concentration that causes 50% of cell growth inhibition. Different letters correspond to significant differences ($p < 0.05$). *Mean statistical differences obtained by Student's *t*-test.

(Petropoulos et al., 2018Petropoulos, Pereira, Ntatsi, et al., 2018; Petropoulos, Pereira, Tzortzakis, et al., 2018). The obtained results are in agreement with the literature reports. The antioxidant potential differences in head samples collected at different maturation stages have already been verified in other species. For instance, Chahdoura et al. (2014) reported that the vegetative stage of cactus plant (*Opuntia* spp.) flowers had higher antioxidant potential than full flowering and post flowering stages. Also, the immature peduncles of *Hovenia dulcis* presented higher antioxidant activity comparing to peduncles at advanced maturity stages (Morales et al., 2017). To the best of the authors' knowledge, this is the first study regarding the antioxidant activity of cardoon heads collected in different harvest stages and measuring their (antihaemolytic) activity by the OxHLIA assay.

The cytotoxic effects of cardoon heads extract are shown in Table 3. The results are presented as the concentration of the extracts that causes 50% inhibition in cell proliferation, where lower GI_{50} values represent a higher cytotoxic potential of the tested extracts. The extract obtained from the samples with lower maturity (Car A) revealed the highest cytotoxicity against the tumor cell lines tested. For HepG2 tumour cell line, the extract of sample Car E also revealed high activity, similar to that demonstrated by sample Car A, recording a GI_{50} of 73 μg/mL. The remaining extracts (Car B, C, D, and F) showed no hepatotoxicity to the non-tumour PLP2 cells, with GI_{50} values > 400 μg/mL.

The influence of genotype on the cytotoxic properties of cardoon seeds has been already evaluated by Petropoulos, Fernandes, Pereira, et al. (2019), Petropoulos, Fernandes, Tzortzakis, et al. (2019) using the same cell lines tested herein, finding significant activities against the tumour cell lines, and no activity against the non-tumour cell line (PLP2) (GI_{50} > 400 μg/mL). Mileo, Di Venere, Linsalata, Fraioli, and Miccadei (2012) also demonstrated the toxicity induced by methanolic extracts of cardoon heads against several types of breast cancer (BT549, MCF-7, MDA-MB231, and T47D), while Pagano et al. (2016) reported that artichoke by-products (bracts and leaves) exhibited significant cellular antioxidant activity through the inhibition of hydroperoxide-induced oxidative stress in HepG2 cells, which was attributed to the abundance in caffeoylquinic acids. The possible correlation of the high content in phenolic compounds and the anticancer activities demonstrated by cardoon extracts has also been pointed out by other authors (Petropoulos, Fernandes, Tzortzakis, et al., 2019).

The anti-inflammatory potential was tested through the measurement of the capacity of the extracts to inhibiting the NO production. NO is a proinflammatory mediator with an important role in the inflammatory process and several researches have been carried out to discover new substances that inhibit its production effectively (Aktan, 2004). The IC_{50} values (μg/mL) obtained regarding the anti-inflammatory potential of cardoon inflorescences are presented in Table 3. Only the immature (Car A) and senescent head samples (Car E and F) demonstrated a significant capacity to inhibit the NO production by the murine macrophage cell line (RAW 146.7), particularly Car A which exhibited the lowest IC_{50} value (183 μg/mL). The rest of the samples (Car B, C, and D) did not reveal anti-inflammatory potential (IC_{50} > 400 μg/mL). There are very few studies regarding the anti-inflammatory potential of cardoon. Salem et al. (2017) evaluated the *in vivo* anti-inflammatory activity of artichoke (*C. cardunculus* var. *scolymus*) extracts through the Carr test, suggesting that the anti-inflammatory activity is caused by a synergistic action of the phenolic compounds present in the obtained ethanolic extracts. To the best of authors' knowledge, this is the first study reporting the anti-inflammatory potential of cardoon heads collected at different harvesting times assayed through this cell-based method.

The antimicrobial activities of the studied samples are presented in Table 4. All the extracts revealed bacteriostatic and bactericidal activity, but none of them demonstrated higher or similar activity than the positive controls used, namely streptomycin and ampicillin. In general, the lowest MIC values were obtained for the sample Car E, except for the case of *Bacillus cereus* where Car D showed the highest efficiency (MIC = 0.38 mg/mL) (Table 4). Moreover, the assayed Gram-positive bacteria presented higher susceptibility than the Gram-negative ones. This fact is in agreement with former reports, which described that the membrane characteristics of Gram-positive bacteria led to a higher vulnerability against the plant extracts (Gyawali, Hayek, & Ibrahim, 2014). In general, the MIC and MBC values were lower than those described by Dias et al. (2018) for hydroethanolic extracts of cardoon inflorescences of different genotypes; however, the bacteria strains used in that study were clinical isolates with a strong resistance profile against antibiotics, whereas in the present study they were mainly ATCC strains, with no resistance profile against antibiotics.

The antifungal potential of the cardoon heads against *Aspergillus* spp. and *Penicillium* spp. was also assessed. As presented in Table 4, the tested extracts possessed higher MIC and MFC values than the positive controls, namely ketoconazole and bifonazole, with the extract Car D exhibiting the lowest MIC and MFC values for all the fungi species tested. The remaining extracts showed a variable activity depending on the fungi species. In general, the extracts Car B and Car D were the ones that revealed the lowest potential for all the fungi species, except against *A. niger*, where the extract from sample Car A was the one that revealed the lowest activity, with the highest MIC value (9.32 mg/mL).

Other reports make reference to the high antimicrobial potential of different cardoon tissues (namely flowers, heads, leaves, and rhizomes). In particular, Falleh et al. (2008) and Scavo et al. (2019) described significant antimicrobial activity of plant tissues against different

Table 4
Antibacterial and antifungal activities of *Cynara cardunculus* var. *altilis* head extracts.

	Car A		Car B		Car C		Car D		Car E		Car F		Streptomycin		Ampicillin	
Antibacterial activity (mg/mL)	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Bacillus cereus	1.75	3.49	1.53	3.06	0.75	1.51	0.38	0.77	0.59	1.18	0.59	1.17	0.10	0.20	0.25	0.40
Staphylococcus aureus	3.49	6.99	3.06	6.12	3.01	6.03	1.54	3.08	0.59	1.18	1.17	2.34	0.04	0.10	0.25	0.45
Listeria monocytogenes	3.49	6.99	3.06	6.12	3.01	6.03	1.54	3.07	1.18	2.35	1.17	2.34	0.20	0.30	0.40	0.50
Enterobacter cloacae	3.49	6.99	3.06	6.12	3.01	6.03	1.54	3.07	0.59	1.18	1.17	2.34	0.20	0.30	0.25	0.50
Escherichia coli	1.75	3.49	1.53	3.06	3.01	6.03	3.075	6.15	1.18	2.35	2.34	4.69	0.20	0.30	0.40	0.50
Salmonella typhimurium	3.49	6.99	3.06	6.12	3.01	6.03	1.54	3.07	0.59	1.18	2.34	4.69	0.20	0.30	0.75	1.20
													Ketoconazole		**Bifonazole**	
Antifungal activity (mg/mL)	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Aspergillus fumigatus	1.16	2.33	4.08	8.16	1.51	3.01	0.51	1.02	1.80	3.60	0.88	1.76	0.25	0.50	0.15	0.20
Aspergillus versicolor	1.16	2.33	1.02	2.04	6.03	> 6.03	0.26	0.51	1.80	3.60	3.52	7.04	0.2	0.5	0.1	0.2
Aspergillus niger	9.32	> 9.32	4.08	8.16	1.51	3.01	0.51	1.02	3.60	7.18	1.76	3.52	0.2	0.5	0.15	0.2
Penicillium funiculosum	0.58	1.16	1.02	2.04	6.03	> 6.03	0.51	1.02	1.80	3.60	0.88	1.76	0.2	0.5	0.2	0.25
Penicillium ochrochloron	0.58	1.16	1.02	2.04	1.51	3.01	0.51	1.02	0.90	1.80	0.88	1.76	1.0	1.5	0.2	0.25
Penicillium verrucosum var. *cyclopium*	0.58	1.16	1.02	2.04	6.03	> 6.03	0.51	1.02	1.80	3.60	0.88	1.76	0.2	0.3	0.1	0.2

MIC – minimal inhibitory concentration; MBC – minimal bactericidal concentration; MFC – minimal fungicidal concentration. Positive controls: streptomycin, ampicillin, ketoconazole, and bifonazole.

bacteria (*Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Salmonella enterica*, and *Staphylococcus aureus*) and fungi (*Aspergillus niger*, *Penicillium oxalicum*, and *Candida albicans*) species.

Overall, the maturation stage of cardoon heads had a significant influence on the *in vitro* bioactivities. Thus, this information can be useful to improve the health benefits of cardoon heads and could be a means to take full advantage of its potential and to increase the added value of the crop. However, further studies are needed to correlate the identified compounds with the observed bioactivities and to reveal the mechanisms of action, as well as to optimize the extraction process for the isolation of these compounds. Finally, the study of the influence of the maturation stage on the remaining plant tissues of cardoon would be extremely useful.

4. Conclusion

The cardoon heads, usually considered as food, could have an added commercial value as a source of bioactive compounds. Caffeoylquinic acids and flavone glycosides (especially apigenin derivatives) were the most abundant classes of phenolic compounds in cardoon heads. The earliest maturation stages (Car A) stood out showing the highest content in polyphenols, to further decrease with the state of maturity. The immature heads also revealed higher cytotoxic and anti-inflammatory potential, with lower GI_{50} and IC_{50} values, respectively. Immature heads (Car A) also exhibited higher antioxidant potential in the cell-based assays, with lower IC_{50} values. All the extracts possessed significant antibacterial activity, with extracts from Car B showing the best results, with the lowest MIC and MBC values. Regarding the antifungal potential, most of the extracts presented inhibitory capacity against the tested fungi species. The sample of advanced maturation stage (Car E) was the one that revealed higher activity, with lower MIC and MFC values. This study allowed for a more complete characterization of the cardoon heads in terms of phenolic compounds composition and the effect of maturation stage on polyphenolic compounds profile and bioactivities. The presented results could be useful, since simple cultivation practices such as harvesting time may allow for a significant enhancement of the bioactive profile of cardoon heads, thus increasing the overall added value of the crop.

CRediT authorship contribution statement

Filipa Mandim: Methodology, Investigation, Writing - original draft. **Spyridon A. Petropoulos:** Methodology, Writing - review & editing. **Kyriakos D. Giannoulis:** Methodology, Investigation. **Maria Inês Dias:** Methodology, Writing - original draft. **Ângela Fernandes:** Methodology, Writing - original draft. **José Pinela:** Methodology. **Marina Kostic:** Methodology. **Marina Soković:** Methodology. **Lillian Barros:** Conceptualization, Methodology, Writing - review & editing. **Celestino Santos-Buelga:** Conceptualization, Methodology, Writing - review & editing. **Isabel C.F.R. Ferreira:** Conceptualization, Methodology, Writing - review & editing.

Declaration of Competing Interest

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

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