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# Chemical composition and antioxidant activity of *Cichorium spinosum* L. leaves in relation to developmental stage

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ABSTRACT

In the present study, chemical composition and bioactivity of *Cichorium spinosum* leaves were examined at different growth stages for optimum harvest stage and end-use assessment. Total fresh weight and number of leaves were higher at 4th growth stage; however, at this stage the end-use is not indicated for raw consumption but mostly for pickled or dried products. Regarding chemical composition, the highest content of tocopherols and minerals was observed in the 1st growth stage, whereas sugars content was the highest in 3rd and 4th growth stage. Polyunsaturated fatty acids content was the highest during the first two growth stages, whereas antioxidant activity, phenolic acids and total phenolic compounds content in the 3rd stage. In conclusion, the results of this study indicate that chemical composition of *C. spinosum* is highly depended on development stage, and harvest stage should be considered for alternative uses of end-products with high bioactivity.

## 1. Introduction

*Cichorium spinosum* is a wild edible green, widely appreciated for its high nutritional value and commonly used as a basic ingredient of the so-called Mediterranean diet. Wild *C. spinosum* plants are usually hand-picked for raw consumption at growth stages which depend on growing conditions, consumer needs and accessibility of growing areas, while cultivated plants can be harvested multiple times during the growing season, as soon as they reach marketable size. Moreover, the fact that the species usually grows in coastal rocky areas which limits accessibility and available harvesting time, while modern life style have shifted consumers' needs towards ready to eat food products, both are responsible for the decrease in wild edible greens consumption during the last decades (Tardío, Pardo-de-santayana, & Morales, 2006). However, nowadays there is an increasing trend for consumption of "healthy" or "super foods" which have created a market niche for commercial cultivation of wild edible greens (Petropoulos, Ntatsi, Levizou, Barros, & Ferreira, 2016).

Harvest stage is of the highest importance for marketability of leafy vegetables, since visual quality of the final product defines consumer's acceptance. Apart from appearance, harvest may also affect chemical composition and nutritional value and harvest at the optimum stage could significantly increase quality. There are several reports regarding the effect of harvest stage on nutrients and phytochemicals content of leafy vegetables. Omezzine, Bouaziz, Simmonds, and Haouala (2014) have reported that flavonoids, flavones, flavonols and total phenolics content of *Trigonella foenum-graecum* L. was the highest during the vegetative growth stage. Moreover, Pokkaew et al. (2013) have reported significant fluctuation of phenolic compounds *of Arachis hypogaea* L. during the vegetative stage of plants, while Zeghichi, Kallithraka, and Simopoulos (2003) suggested that the optimum stage of harvest for *C. spinosum* leaves is at 40–50 days after planting.

Although the effect of multiple harvests has been reported in terms of yield for various leafy vegetables (Csizinszky, 1999) and chemical composition of *C. spinosum* in particular (Zeghichi et al., 2003), so far no reports are available regarding the effect of growth

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stage on chemical composition and bioactive compounds content of leafy vegetables, mostly due to their short growing period and the market standards which demand specific plant size. Therefore, the aim of the present study was to assess chemical composition of *C. spinosum* leaves at various growth stages, in order to evaluate the potential of other uses except for raw consumption, such as extracts, decoctions and pickles, which could increase added value of the final product and availability throughout the season.

## 2. Materials and methods

### 2.1. Plant material and growing conditions

Seedlings of *Cichorium spinosum* L. (Asteraceae) were obtained from Viamane S.A. (Timpaki, Greece). Seeds of *Cichorium spinosum* L. (Asteraceae) were sown on September 2nd 2015 in seed trays containing peat, as previously described by Anesti et al. (2016). Young seedlings were transplanted when plants reached the stage of 3–4 true leaves on December 1st, 2015 [90 days after sowing (DAS)] in 2 L pots containing peat (Klassman-Deilmann KTS2, 1.0 L) and perlite (1.0 L), and put in an unheated plastic greenhouse. Plants were fertilized through the irrigation water at regular intervals with the same amount of nitrogen (300 mg $L^{-1}$), starting with 50 mL of nutrient solution per pot and up to 300 mL per pot towards the end of the growth cycle. Growing conditions (mean, max and min temperature, and Relative Humidity) throughout the experiment are presented in Supplementary Material (Fig. S1).

Leaves were harvested at four different stages of plant development and up to flower initiation (133, 175, 195 and 205 DAS) from different plants each time (with no prior harvests being applied), in order to evaluate the chemical composition of the aerial parts at various growth stages. The studied harvest stages are illustrated in Supplementary Material (Fig. S2). On each harvest day, fresh and dry weight of leaves was measured. Dry weight of fresh leaves was evaluated after oven drying of samples at 72 °C to a constant weight (approximately for 48 h).

### 2.2. Chemical composition analyses

For chemical composition, raw samples of leaves were stored at deep freezing conditions (−80 °C) and freeze-dried prior to analysis.

Free sugars were determined in dried sample powder (1.0 g), spiked with melezitose as internal standard (IS, 5 mg/mL), and extracted with 40 mL of 80% aqueous ethanol at 80 °C for 30 min. The resulting suspension was centrifuged (Centurion K24OR refrigerated centrifuge, West Sussex, UK) at 15,000*g* for 10 min. The supernatant was concentrated at 60 °C under reduced pressure and defatted three times with 10 mL of ethyl ether, successively. After concentration at 40 °C, the solid residues were dissolved in water to a final volume of 5 mL and filtered through 0.2 μm nylon filters from Whatman. The analysis was performed by high performance liquid chromatography with a refraction index detector (HPLC-RI; Knauer, Smartline system 1000, Berlin, Germany), as previously described by Barros et al. (2013). The sugars were identified by comparing their retention times with standard compounds and quantification was conducted by comparison with dose–response curves constructed from authentic standards, using the internal standard (IS, melezitose) method (DataApex, Podohradska, Czech Republic).

Organic acids were determined using ultra-fast liquid chromatography coupled to a photodiode array detector (UFLC-DAD). Samples (∼2 g) were extracted by stirring with 25 mL of *meta*-phosphoric acid (25 °C at 150 rpm) for 45 min and subsequently filtered through Whatman No. 4 paper. Before analysis, the sample was filtered through 0.2 μm nylon filters (Pereira, Barros, Carvalho, & Ferreira, 2013). The analysis was performed using a Shimadzu 20A series UFLC (Shimadzu Corporation, Kyoto, Japan) and detection was carried out in a DAD, using 215 nm and 245 nm (for ascorbic acid) as preferred wavelengths and controlled by LabSolutions multi LC-DAD software (Shimadzu Corporation, Kyoto, Japan). Quantification was performed by area comparison with calibration curves obtained from each standard compound.

Fatty acids were analyzed with a DANI 1000 gas chromatographer (GC, Milan, Italy) coupled to a flame ionization detector (FID), after a transesterification procedure described by Barros et al. (2013). Fatty acids (obtained after Soxhlet extraction) were methylated with 5 mL of methanol:sulphuric acid:toluene 2:1:1 (*v:v:v*), during at least 12 h in a bath at 50 °C and 160 rpm; then 3 mL of deionised water were added, to obtain phase separation; the FAME were recovered with 3 mL of diethyl ether by shaking in vortex, and the upper phase was passed through a microcolumn of sodium sulphate anhydrous, in order to eliminate the water; the sample was recovered in a vial with Teflon, and before injection the sample was filtered with 0.2 μm nylon filter from Whatman. The FAMEs were identified by comparing their retention time with authentic standards and the results were recorded and processed by using Clarity 4.0.1.7 Software (DataApex, Podohradska, Czech Republic).

Tocopherols were determined following a procedure previously described by Barros et al. (2013). BHT solution in hexane (10 mg/mL; 100 μL) and IS solution in hexane (tocol; 50 μg/mL; 400 μL) were added to the sample prior to the extraction procedure. The samples (∼500 mg) were homogenized with methanol (4 mL) by vortex mixing (1 min). Subsequently, hexane (4 mL) was added and again vortex mixed for 1 min. After that, saturated NaCl aqueous solution (2 mL) was added, the mixture was homogenized (1 min), centrifuged (5 min, 4000g) and the clear upper layer was carefully transferred to a vial. The sample was re-extracted twice with hexane. The combined extracts were taken to dryness under a nitrogen stream, redissolved in 2 mL of *n*-hexane, dehydrated with anhydrous sodium sulphate, filtered through 0.2 μm nylon filters from Whatman, transferred into a dark injection vial prior to the analysis. The chromatographic separation was achieved using an HPLC (Knauer, Smartline system 1000, Berlin, Germany) coupled to a fluorescence detector (FP-2020; Jasco, Easton, MD, USA), programmed for excitation at 290 nm and emission at 330 nm. The compounds were identified by chromatographic comparisons with authentic standards and quantification was conducted by comparison with dose–response curves constructed from authentic standards, using the IS (tocol) method (DataApex, Podohradska, Czech Republic).

Oven dried samples of leaves tissues were subjected to dry ashing and extracted with 1 N HCl for mineral content determination. Mg, Fe, Mn, Zn, and Cu content were determined by atomic absorption spectrophotometry (Perkin Elmer 1100B, Waltham, MA, USA) and Ca, Na and K content by flame photometry (Sherwood Model 410, Cambridge, UK).

Nitrate content was assessed colorimetrically by the nitration of salicylic acid, following the procedure previously described by Cataldo, Maroon, Schrader, and Youngs (1975), using a Perkin Elmer Model Lambda 1A spectrophotometer (Perkin Elmer, Waltham, Mass.).

### 2.3. Antioxidant activity assays

For methanolic/water (80:20, *v*/*v*) extraction, one gram of lyophilized material was extracted twice for 1 h in a magnetic stirrer plate (25 °C at 150 rpm), with 30 mL of methanol/water (80:20, *v*/*v*), filtered through a Whatman No. 4 paper and vacuum-dried in a rotary evaporator (rotary evaporator Büchi R-210, Flawil,